AUTOIMMUNO-ANTI-TUMOUR IMMUNITY (AATI) – UNDERSTANDING THE IMMUNE RESPONSES AGAINST "SELF" & "ALTERED-SELF"

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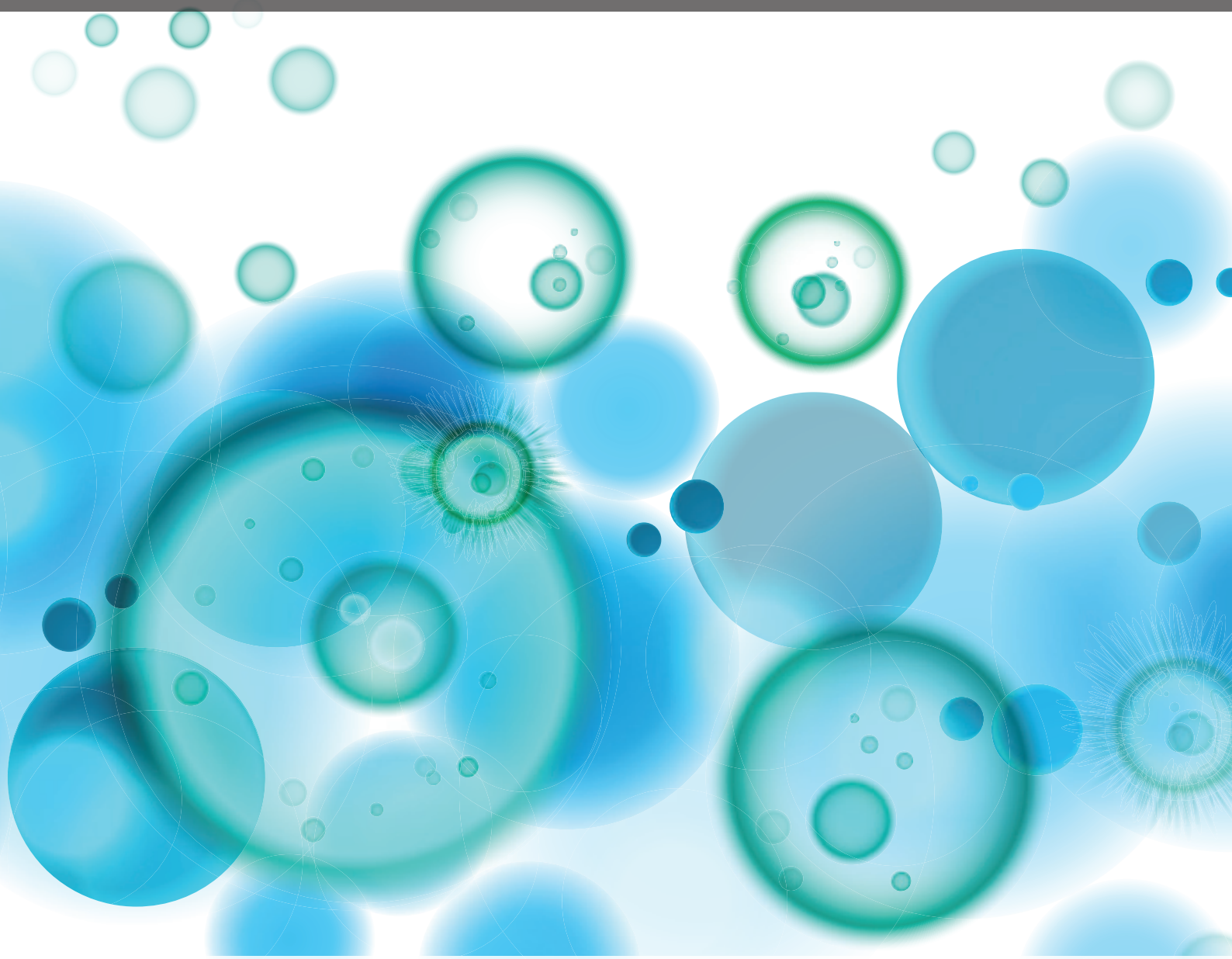

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AUTOIMMUNO-ANTI-TUMOUR IMMUNITY (AATI) – UNDERSTANDING THE IMMUNE RESPONSES AGAINST "SELF" & "ALTERED-SELF"

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The brief description of tumours being "wounds that do not heal" by Dr Harold F. Dworak nearly three decades ago (N Engl J Med 1986) has provided not only a vivid illustration of neoplastic diseases in general but also, in retrospect conceptually, a plausible immunological definition of cancers. Based on our current understanding in the field, it could have even a multi-dimensional meaning attached with. This relates to several important issues which need to be addressed further, i.e. in terms of a close link between chronic inflammation and tumourigenesis widely observed; clinical and experimental evidence of immunity against tumours versus the highly immunosuppressive tumour microenvironment being associated; and their underlying immunological mechanisms, oncogenic basis, as well as the true causal relationship in question.

Recent findings from studies into the pathogenesis of autoimmunity and, more importantly, the mechanisms which protect against it, have offered some new insights for our understanding in this direction. Chronic or persistent autoimmune-like inflammatory conditions are evidently associated with tumor development. The important question is about their true causal relationship. Chronic or persistent inflammation has been shown to contribute directly to tumour development by triggering neoplastic transformation and production of inflammatory mediators which could promote cancer cell survival, proliferation and invasion. On the other hand, tumours are mutated self-tissue cells to which the host immune system is largely tolerized otherwise. Although the mutations may give rise to the expression of tumour-specific antigens (TSA) or tumour-associated antigens (TAA), most of these TSAs/TAAs are found to be poor immunogens. The ongoing inflammatory conditions may therefore reflect a desperate attempt of the host immune system to mount anti-tumour responses, though ineffectively, being a consequence of the continuous yet largely futile triggering by those poorly immunogenic TSAs/TAAs. Furthermore, during autoimmune or overtly persistent immunological responses, many regulatory mechanisms are triggered in the host in attempts to limit the ongoing harmful inflammatory reactions. Such a negative feedback regulation is known to be crucial in preventing normal individuals from immune-mediated diseases. As a result of the negative feedback loop, however, an excessive production of anti-inflammatory or

immunosuppressive molecules followed by the exhaustion of the immune effector cells may instead lower the ability of the host immune system to mount specific anti-tumor responses, allowing the escape of tumour or mutated cells from immunosurveillance. This may also help to explain why the most effective way to enhance host immunity against cancer is by targeting the negative arm of immune regulation.

In this Frontiers Research Topic, we aim to gather current views from experts in these inherent overlapping fields of oncology, autoimmunity and tumour immunology, and to make them available to our potential readership who may be particularly interested in this cutting-edge area. By understanding how the immune system is normally regulated, why dysregulation of which may cause the immunological-oncological related diseases, we also encourage further discussions as to how the so-called "self-reactivity" (autoimmune responses) can be alternatively switched on and redirected, immunologically or molecularly, for effective cancer treatment.

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Table of Contents

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Autoimmuno-anti-tumor immunity – understanding the immune responses against "self" and "altered-self"

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Keywords: autoimmunity, cancer, inflammation, tumorigenesis, immune regulation, immunosuppression, anti-tumor immunity, tumor immunotherapy

The brief description of tumors being "wounds that do not heal" by Dr. Harold F. Dworak nearly three decades ago (*N Engl J Med*, 1986) (1) has provided not only a vivid illustration of neoplastic diseases in general but also, in retrospect conceptually, a plausible immunological definition of cancers. Based on our current understanding in the field, it could have even a multi-dimensional meaning attached with. This relates to several important issues, which need to be addressed further, i.e., in terms of a close link between chronic inflammation and tumorigenesis widely observed; clinical and experimental evidence of immunity against tumors versus the highly immunosuppressive tumor microenvironment being associated; and their underlying immunological mechanisms, oncogenic basis, as well as the true causal relationship in question (2–5).

Recent findings from studies into the pathogenesis of autoimmunity and, more importantly, the mechanisms, which protect against it, have offered some new insights for our understanding in this direction. Chronic or persistent autoimmune-like inflammatory conditions are evidently associated with tumor development. The important question is about their true causal relationship. Chronic or persistent inflammation has been shown to contribute directly to tumor development by triggering neoplastic transformation and production of inflammatory mediators, which could promote cancer cell survival, proliferation, and invasion (2, 3). On the other hand, tumors are mutated self-tissue cells to which the host immune system is largely tolerized otherwise. Although the mutations may give rise to the expression of tumor-specific antigens (TSA) or tumor-associated antigens (TAA), most of these TSAs/TAAs are found to be poor immunogens (6). The ongoing inflammatory conditions may therefore reflect a desperate attempt of the host immune system to mount anti-tumor responses, though ineffectively, being a consequence of the continuous yet largely futile triggering by those poorly immunogenic TSAs/TAAs. Furthermore, during autoimmune or overtly persistent immunological responses, many regulatory mechanisms are triggered in the host in attempts to limit the ongoing harmful inflammatory reactions. Such a negative feedback regulation is known to be crucial in preventing normal individuals from immune-mediated diseases (7). As a result of the negative feedback loop, however, an excessive production of anti-inflammatory or immunosuppressive molecules followed by the exhaustion of the immune effector cells may instead lower the ability of the host immune system to mount specific anti-tumor responses, allowing the escape of tumor or mutated cells from immunosurveillance. This may also help to explain why the most effective way to enhance host immunity against cancer is by targeting the negative arm of immune regulation (8–10).

In this Frontiers Research Topic, we have gathered current views and cutting-edge findings from many experts in these inherent overlapping fields of oncology, autoimmunity, and tumor immunology. It compiles a total of 15 articles in different formats, of concise but informative Mini-review/Reviews, Original Research Articles with novel experimental findings, and some very thought-provoking new Hypothesis/Theory/Opinion/Perspectives. These are now made freely available to our potential readership who may be particularly interested in this cutting-edge area, covering three key issues as outlined below:

- Cancers, Inflammation, and the causal relationship;
- Immune effector and regulatory mechanisms involved in autoimmuno-anti-tumor immunity (AATI);
- Guiding the misguided: AATI alternatively switched on for effective cancer treatment.

For the highly cross-disciplinary nature of this Research Topic, however, the above are reflected in different ways in these articles, crossing throughout the topic. It starts by outlining evidence of the host immune system that may naturally protect against cancers, while it could also cause autoimmunity – being an evolutionally acceptable "side effect" (Chapters 1–2); followed by explaining how autoimmunity could be a "Double-Agent" involved in both tumor-killing and cancer promotion linked to inflammation (Chapters 3–5). It addresses further by dissecting the detailed cellular and molecular mechanisms potentially involved in these processes (Chapters 6–10). These together may help to provide a good basis for the development of novel therapeutic approaches, including stem cell-based immunotherapy, for future cancer treatment (Chapters 11–15). By understanding how the immune system is normally regulated, why dysregulation of which may cause the immunological–oncological related diseases,

we aim and hope that the contents of this Research Topic can also trigger further active discussions among scientists in the fields, as to how the so-called "self-reactivity" (autoimmune responses) can be alternatively switched on and redirected, immunologically or molecularly, for effective cancer treatment.

Finally, I would like to thank all the authors for their valuable contributions to this Research Topic, and to express my great appreciations to many members of the journal editorial team, especially Ms. Rosa Mancebo and Ms. Jessica Kandlbauer, for their professional dedication and kind help throughout the process.

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Does the immune system naturally protect against cancer?

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The importance of the immune system in conferring protection against pathogens like viruses, bacteria, and parasitic worms is well established. In contrast, there is a long-lasting debate on whether cancer prevention is a primary function of the immune system. The concept of immunological surveillance of cancer was developed by Lewis Thomas and Frank Macfarlane Burnet more than 50 years ago. We are still lacking convincing data illustrating immunological eradication of precancerous lesions *in vivo*. Here, I present eight types of evidence in support of the cancer immunosurveillance hypothesis. First, primary immunodeficiency in mice and humans is associated with increased cancer risk. Second, organ transplant recipients, who are treated with immunosuppressive drugs, are more prone to cancer development. Third, acquired immunodeficiency due to infection by human immunodeficiency virus (HIV-1) leads to elevated risk of cancer. Fourth, the quantity and quality of the immune cell infiltrate found in human primary tumors represent an independent prognostic factor for patient survival. Fifth, cancer cells harbor mutations in protein-coding genes that are specifically recognized by the adaptive immune system. Sixth, cancer cells selectively accumulate mutations to evade immune destruction ("immunoediting"). Seventh, lymphocytes bearing the NKG2D receptor are able to recognize and eliminate stressed premalignant cells. Eighth, a promising strategy to treat cancer consists in potentiating the naturally occurring immune response of the patient, through blockade of the immune checkpoint molecules CTLA-4, PD-1, or PD-L1. Thus, there are compelling pieces of evidence that a primary function of the immune system is to confer protection against cancer.

Keywords: cancer immunosurveillance, primary immunodeficiency, cancer risk, organ transplantation, immunosuppressive drugs, HIV, NKG2D, checkpoint blockade

INTRODUCTION

Lewis Thomas and Frank Macfarlane Burnet proposed the concept of immunological surveillance of cancer more than five decades ago (1–4). It was defined by Burnet as follows: "In large long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character" (1). More than 50 years after Burnet proposed his theory, the immunological scientific community remains largely divided with both proponents [e.g., Ref. (5, 6)] and opponents [e.g., Ref. (7, 8)] of the cancer immunosurveillance hypothesis. In fact, an opposite and very influential concept was proposed in 2001 by Frances Balkwill and Alberto Mantovani, who suggested that inflammatory immune cells and cytokines found in tumors may promote rather than suppress tumor growth (9, 10). Although, we are currently lacking convincing data illustrating immunological eradication of precancerous lesions *in vivo*, there are strong indications that a primary function of the immune system is indeed to prevent cancer. Here, I present eight types of evidence in support of the cancer immunosurveillance hypothesis.

PRIMARY IMMUNODEFICIENCY IN HUMANS AND MICE IS ASSOCIATED WITH INCREASED CANCER RISK

As Burnet himself pointed out, an implication of the cancer immunosurveillance hypothesis is that immunodeficiency should be associated with increased likelihood of neoplasia (1). Immunodeficiencies can be divided in two main types: primary (inborn) immunodeficiencies, which are caused by genetic defects and whose incidence is approximately 1:10,000 births; and secondary immunodeficiencies, which are induced by immunosuppressive medication or viral infection and which are much more common. In accordance with Burnet's prediction, severe primary immunodeficiencies have been reported to be associated with increased risk of malignancy (11–14). For instance, patients with defective humoral immunity due to common variable immunodeficiency (CVID) had increased incidence of lymphoma and epithelial tumors of the stomach, breast, bladder, and cervix (12, 15). Selective immunoglobulin A (IgA) deficiency was associated with a high incidence of gastric carcinomas (15). Moreover, patients with X-linked immunodeficiency with hyper-IgM, caused by mutations in the CD40 ligand molecule, had a high incidence of tumors of the pancreas and liver (16). However, it remains unclear to what extent primary immunodeficiency in humans leads to increased cancer

development, due to the relatively low number of patients investigated.

Gene-targeted mice, which selectively lack key components of the immune system have been extensively used to experimentally test the effect of well-defined primary immunodeficiencies on cancer development [reviewed in Ref. (17)]. Mice which lacked both T and B cells, due to a deficiency in the recombination-activating gene 2 (RAG2), were more susceptible to spontaneous and carcinogen-induced carcinomas (18). Mice lacking γδ T cells were highly susceptible to multiple regimens of cutaneous carcinogenesis (19). The cytokines interferon-α/β (IFN-α/β) and IFN-γ were shown to protect mice against spontaneous and carcinogen-induced malignancy (18, 20–22). Moreover, the molecule perforin, which is used by cytotoxic lymphocytes to kill target cells, was reported to be important for surveillance of spontaneous lymphoma (23). Collectively, the human and mouse data reveal a consistent association between primary immunodeficiency and increased incidence of various types of cancer.

ORGAN TRANSPLANT RECIPIENTS ARE MORE PRONE TO CANCER DEVELOPMENT

A breakthrough in organ transplantation was the discovery of immunosuppressive drugs such as cyclosporine A, which prevent organ rejection by the adaptive immune system (24). Immunosuppressive medication is now standard treatment after organ transplantation. Life-long treatment of thousands of transplanted patients with immunosuppressive drugs was defined by Thomas as a "human experiment" to test the cancer immunosurveillance hypothesis (4). Already in 1973, an international registry-based study of renal-transplant recipients from 30 countries revealed that transplantation was associated with increased risk of developing cancer, in particular lymphoma (25). A large cohort investigation of cancer risk after organ transplantation was performed in the Nordic countries, in homogeneous populations with well-documented cancer incidence, on nearly 6000 kidney recipients (26). A two to fivefold excess risk was reported for cancers of the colon, larynx, lung, bladder, prostate, and testis. Strikingly high risks, 10-fold to 30-fold above normally expected levels, were observed for cancers of the lip, skin (non-melanoma), kidney, endocrine glands, cervix, and for non-Hodgkin's lymphoma (26). Another large study of kidney transplantation in 200,000 patients from 42 countries reported that the risk of developing lymphoma was 12-fold higher for transplant recipients than that in a matched non-transplanted population (27). Notably, the majority of posttransplant lymphomas were associated with infection with Epstein–Barr virus (EBV), which primarily infects B cells and is known to cause B cell transformation (28). Thus, most lymphomas arising in transplant patients were likely to be a secondary event resulting from reduced antiviral immunity, rather than a direct effect of reduced antitumor immunity. However, lymphomas not associated with EBV infection have also been reported after transplantation (29). An investigation of 175,000 solid organ transplants in the USA revealed that increased cancer risk occurred not only after kidney transplantation but also after liver, heart, and lung transplantation (30). Risk was increased for 32 different malignancies, some related to known infections (e.g., anal cancer and Kaposi sarcoma) and others unrelated to infections (e.g., lung cancer and melanoma). The most common malignancies with elevated risk were non-Hodgkin lymphoma and cancers of the lungs (30).

Very high rates of non-melanoma skin cancers have been reported for Swedish (20–40%) and Australian (70%) populations 20 years after transplantation (31–33). Cutaneous types of human papillomaviruses have been suggested to be the cause of non-melanoma skin cancers such as squamous cell carcinoma in immunosuppressed patients, but the epidemiological pieces of evidence remain inconsistent (34). Strikingly, non-melanoma skin tumors in the renal-transplant population of Queensland, Australia, were reported to arise predominantly on chronically sun-exposed skin (head, neck, and distal limbs), strongly suggesting a causative role of ultraviolet (UV) light rather than oncogenic viruses (33). Thus, life-long treatment of organ transplant recipients with immunosuppressive drugs leads to increased risk of developing many different types of cancer, some related to known infections and others unrelated.

IMMUNOSUPPRESSION INDUCED BY INFECTION BY HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 LEADS TO ELEVATED RISK FOR CANCER

The HIV-1 virus causes acquired immunodeficiency by selectively infecting and killing $CD4^+$ T cells. Accordingly, HIV-infected patients, receiving or not antiviral treatments, possess reduced levels of $CD4^+$ T cells compared to non-infected individuals. HIV-infected individuals have elevated risk for cancer linked to oncogenic viruses such as Kaposi sarcoma (caused by human herpes virus 8), Hodgkin's and non-Hodgkin's lymphoma (EBV), anal and cervical cancer (human papilloma virus), and liver cancer (hepatitis B and C viruses). Kaposi sarcoma, non-Hodgkin's lymphoma and cervical cancer are particularly frequent and are considered as acquired immunodeficiency syndrome (AIDS)-defining cancers (35). However, several cancers that are not linked to oncogenic viruses, like lung cancer and multiple myeloma, are also more frequent in patients with HIV (35, 36). Lung cancer is the most common non-AIDS-defining cancer and a leading cause of mortality among HIV-infected individuals (37). For the majority of patients with lung cancer, malignant transformation is known to be caused by carcinogens present in cigarette smoke. Higher smoking rates have been reported for HIV-infected populations. After controlling for potential confounders including smoking, a large cohort study of veterans (with 37,000 HIV-infected patients and 75,000 healthy controls) concluded that HIV was an independent risk factor for incident lung cancer (37). Importantly, cancer incidence in HIV-infected individuals was found to be inversely related to $CD4^+$ T cell counts in blood, which supports the association between immunosuppression and increased cancer risk (38). For instance, the risk of lung cancer was doubled by $CD4^+$ T counts in the range of 350–499 cells per microliter blood compared to normal counts $\geq$500, and continued to increase as the $CD4^+$ T cell count fell (38). Thus, acquired immunodeficiency by HIV infection, which selectively depletes $CD4^+$ T cells, leads to increased risk of developing many different types of cancer, some related to known infections, and others unrelated.

QUANTITY AND QUALITY OF THE IMMUNE CELL INFILTRATE IN HUMAN PRIMARY TUMORS REPRESENT AN INDEPENDENT PROGNOSTIC FACTOR FOR PATIENT SURVIVAL

All solid tumors are infiltrated by a variety of immune cells. For many types of human cancers, an association has been reported between the type, density, and location of immune cells within the primary tumor and the clinical outcome [reviewed in Ref. (39)]. The number of intratumoral $CD3^+$ T cells was shown to positively correlate with longer survival of patients with epithelial ovarian and colorectal cancers (40, 41). A high number of stromal $CD4^+$ T cells were found to represent an independent positive prognostic factor in non-small cell lung cancer (42). Tumor-infiltrating $CD8^+$ cytotoxic T cells were shown to predict clinical outcome in colon, lung, and breast cancers (42–45). Concurrent infiltration by both $CD4^+$ and $CD8^+$ T cells was reported to represent a favorable prognostic factor in esophageal squamous cell carcinoma and non-small cell lung cancer, suggesting that both cell types cooperate to fight cancer (46, 47). Among all $CD4^+$ T cell subsets, Th1 cells seem to be particularly advantageous, as reported for colorectal, liver, and breast cancers (39, 40, 48, 49). In patients with gastrointestinal stromal tumors (GIST), the intratumoral density of $CD3^+$ T cells and $NKp46^+$ natural killer (NK) cells were found to represent two independent prognostic factors for progression-free survival (50). Notably, NK and T cells were detected in distinct areas of tumor sections, suggesting that both cell types contributed independently to GIST immunosurveillance (50). Furthermore, a high tumor infiltration by $CD68^+$ macrophages was associated with prolonged survival in prostate, lung, and colon cancers (43, 51–54). Thus, for various types of human cancers, the quantity and the quality of the immune response within the primary tumor appear to represent an independent predictor for patient survival. This correlation between immunological data and clinical outcome strongly suggests that the immune system of the patient had naturally mounted an antitumor immune response before any treatment had started. The efficiency of this response presumably varies from patient to patient, thereby critically influencing survival.

CANCER CELLS HARBOR MUTATIONS IN PROTEIN-CODING GENES THAT ARE SPECIFICALLY RECOGNIZED BY THE ADAPTIVE IMMUNE SYSTEM

Cancer cells originate from normal cells that have accumulated "driver" mutations, which either activate oncogenes by dominant gain of function or inactivate tumor suppressor genes by recessive loss of function. A typical tumor contains two to eight of these driver mutations (55). Cancer cells also accumulate "passenger" mutations, which do not contribute to tumorigenesis. Genome-wide sequencing studies have provided detailed information about somatic mutations in various types of cancers. For common solid tumors such as breast, colon, brain, and pancreas cancers, an average of 30–60 non-synonymous mutations in protein-coding genes was observed (56–59). Most of these mutations (95%) were single-nucleotide substitutions, whereas the remainder was deletions or insertions (55). Metastatic melanoma and non-small cell lung carcinoma, which represent two types of cancers caused by potent mutagens (UV light and cigarette smoke, respectively), had a higher mutation rate with ~150 mutations per tumor (60, 61). Pediatric tumors and leukemias had the fewest mutations with ~10 mutations per tumor on average (55). Thus, it is now established that tumor cells in most cancer types harbor numerous non-synonymous mutations in protein-coding genes.

Driver and passenger mutations, which alter the normal amino acid sequence of proteins, may potentially be recognized by the adaptive immune system. A number of studies have revealed that tumor-specific antigens created by mutations can be recognized either by the T cells or the B cells of the patient. For instance in melanoma, $CD4^+$ T cells were found that recognized a tumor-specific antigen generated by a non-synonymous point mutation in the gene coding for triosephosphate isomerase (62). Another antigen recognized by $CD4^+$ T cells in melanoma had been generated by a chromosomal rearrangement resulting in a fusion of a low density lipid receptor gene with a fucosyltransferase gene (63). In colorectal cancer with microsatellite instability phenotype, $CD4^+$ T cells were identified that recognized a frameshift mutation in the transforming growth factor β receptor II (TGFβRII) (64). In a melanoma patient, the tumor suppressor $p16^{INK4a}$ with a point mutation was specifically recognized by cytotoxic $CD8^+$ T cells (65). In non-small cell lung cancer, several $CD8^+$ T cell epitopes created by point mutations have been reported (66–68). Moreover, in chronic myeloid leukemia, cytotoxic $CD8^+$ T cells specific for a BCR-ABL fusion protein (resulting from the fusion of BCR and ABL genes) were found (69). Tumor-specific IgG antibodies are common in the serum of cancer patients, as revealed by serological identification of antigens by recombinant expression cloning (SEREX) technology (70). This powerful method has allowed the identification of over 2000 tumor antigens recognized by autologous IgG, including the p53 tumor suppressor modified by a point mutation (71). Collectively, these studies demonstrate that the adaptive immune system is able to detect cancer by specifically recognizing the mutated proteins of the malignant cells.

CANCER CELLS SELECTIVELY ACCUMULATE MUTATIONS TO EVADE IMMUNE DESTRUCTION

Recognition of cancer cells by tumor-specific $CD8^+$ T cells is achieved by the presentation of antigenic peptides from mutated proteins on major histocompatibility complex (MHC) class I molecules on the surface of cancer cells. In order to avoid recognition and the resulting elimination by $CD8^+$ T cells, cancer cells often mutate key genes of the MHC class I antigen presentation pathway. Downregulation of surface MHC class I molecules is a common feature of human cancer cells [reviewed in Ref. (72)]. Several mechanisms have been reported, including mutations in the β2-microglobulin gene, which is required for MHC class I molecule expression on the cell surface (73, 74). MHC haplotype loss in various human tumors was shown to be caused by complete or partial loss of chromosome 6, which harbor all MHC class I and class II genes (except for β2-microglobulin) (75). On the basis of its mutation pattern in cancer cells, β2-microglobulin was recently included in a list of 74 tumor suppressor genes (55). A recent study analyzed somatic point mutations in exon sequences from 4742 human cancers across 21 cancer types (76). Based on mutation frequency and pattern, 254 "cancer genes" were identified, including four genes belonging to the MHC class I antigen presentation

pathway (β2-microglobulin, HLA-A, HLA-B, and TAP1), as well as the CD1D gene, which is involved in the presentation of lipid antigens to NK T cells (76). Hence, several mutations frequently observed in cancer cells are likely to result from selective pressure to evade the immune attack, in particular by cytotoxic $CD8^+$ T cells and NK T cells.

Another strategy used by cancer cells to avoid the immune response consists of secreting immunosuppressive cytokines such as transforming growth factor β (TGF-β) and interleukin 10 (IL-10). In contrast to normal cells, which produce very little, malignant cells often secrete large amounts of TGF-β and IL-10 [reviewed in Ref. (77)]. Both cytokines have various effects on non-transformed cells present in the tumor mass, most notably the inhibition of immune cell functions. For several types of cancers, elevated serum levels of TGF-β or IL-10 have been reported to be associated with worse prognosis [reviewed in Ref. (77)]. Surprisingly, TGF-β can function both as a tumor suppressor and a tumor promoter, this duality being known as the TGF-β paradox. In early stage tumors, TGF-β is a potent inducer of growth arrest. In advanced stage malignant cells, TGF-β signaling pathways are severely dysregulated, and TGF-β promotes tumor growth [reviewed in Ref. (78)]. Thus, cancer cells often produce abnormally high levels of immunosuppressive cytokines, which strongly suggests that dampening immunity is a prerequisite for tumor growth.

Experiments with immunodeficient mice have demonstrated that the immune system may exert a strong selective pressure on the cancer cells. By using the chemical carcinogen methylcholanthrene, sarcomas were induced either in wild-type mice or in RAG2-deficient mice, which lack both T and B cells (18). When transplanted into RAG2-deficient mice, all sarcomas grew progressively with equivalent kinetics. In contrast, when the tumor cells were injected into immunocompetent wild-type hosts, all sarcomas from wild-type mice grew progressively, while 8 of 20 (40%) sarcomas from RAG2-deficient mice were rejected (18). These data strongly suggest that in wild-type mice, there was selection of tumor cells that were more capable of surviving in an immunocompetent host. This provides an explanation for the apparent paradox of tumor formation in immunologically intact individuals. Based on these findings, Robert Schreiber and coworkers introduced the term "cancer immunoediting," which was further developed into a general theory, to describe the sculpting actions of the immune response on developing tumors in immunocompetent individuals (18, 79).

LYMPHOCYTES BEARING THE NKG2D RECEPTOR ARE ABLE TO RECOGNIZE AND ELIMINATE STRESSED PREMALIGNANT CELLS

NK cells are innate lymphocytes that can kill malignant or infected cells. All NK cells and some T cells express the NKG2D molecule on the cell surface. NKG2D is an activating receptor, which serves as a major recognition receptor for detection and elimination of transformed cells (80). The ligands for NKG2D are self proteins that are poorly expressed by normal resting cells but upregulated on the surface of stressed cells. NKG2D ligands in humans include MICA, MICB, and six different ULBP proteins (81). In mice, NKG2D ligands include MULT1, five isoforms of RAE-1, and three isoforms of the H60 proteins (82). In humans, cells that express NKG2D ligands may be recognized and killed by either NK cells or γδ T cells in a process called lymphoid stress surveillance (83).

NKG2D ligands were shown to be upregulated in normal cells after treatment with DNA-damaging agents like ionizing radiations and UV light (84). It was concluded that the DNA damage response, which was known to arrest the cell cycle and enhance DNA repair, may also participate in alerting the immune system to the presence of potentially dangerous cells (84). Several studies suggested that expression of NKG2D ligands on transformed cells may be directly induced by oncogenes. For example, the BCR-ABL fusion oncogene was reported to control the expression of MICA in chronic myelogenous leukemia cells at the posttranscriptional level (85). Activation of the Ras oncogene was shown to upregulate the expression of RAE-1α/β in mouse cells, and ULBP1–3 and MICA/B in human cells (86). In a recent study, surface upregulation of NKG2D ligands by human epithelial cells in response to UV irradiation, osmotic shock, or oxidative stress, was shown to depend on the activation of the epidermal growth factor receptor (EGFR) (87). The EGFR pathway is frequently dysregulated in human cancer and it was proposed that activation of EGFR may regulate the immunological visibility of stressed premalignant cells (87). Surprisingly, several isoforms of RAE-1, like RAE-1ε, were found to be expressed not only by cancer cells, but also by some normal proliferating cells such as fibroblasts (88). The E2F transcription factor, which controls cell cycle entry, was shown to regulate RAE-1ε expression. These data suggest that NKG2D-bearing lymphocytes may control the proliferation of both normal and malignant cells (88).

MICA and MICB were found to be expressed by many, but not all, freshly isolated carcinomas of the lung, breast, kidney, ovary, prostate, colon, and liver (89, 90). Moreover, *in vitro* studies revealed that MICA and MICB contributed to the lysis of hepatocellular carcinoma cells by NK cells (90). The importance of NKG2D for cancer immunosurveillance *in vivo* gained support from experiments showing that cancer cells transfected with NKG2D ligands and injected into mice were rapidly rejected by NK cells and by $CD8^+$ T cells (91, 92). Moreover, neutralization of NKG2D with blocking monoclonal antibodies rendered mice more susceptible to carcinogen-induced fibrocarcinoma (93). Gene-targeted mice deficient for NKG2D were shown to be more susceptible to the *in situ* development of prostate adenocarcinoma and B cell lymphoma (94). In humans, an association has been reported between polymorphisms of the NKG2D gene and susceptibility of developing liver and cervix cancers, supporting a protective role of NKG2D against these malignancies (95, 96). Thus, the expression of stress-induced endogenous molecules associated with cell transformation is used by the immune system to recognize and eliminate premalignant cells in mice and humans.

PROMISING NOVEL STRATEGY TO TREAT CANCER CONSISTS IN POTENTIATING THE NATURALLY OCCURRING IMMUNE RESPONSE OF THE PATIENT THROUGH BLOCKADE OF IMMUNE CHECKPOINT MOLECULES

Activation of a naïve T cell requires at least two signals: T cell receptor-mediated recognition of a cognate antigen (signal 1) and engagement of the costimulatory receptor CD28 (signal 2).

Once activated, T cells upregulate on the cell surface two co-inhibitory molecules, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programed death 1 (PD-1). The function of these co-inhibitory molecules is to tightly regulate the immune response by containing excessive T cell activation. For the purpose of cancer immunotherapy, monoclonal antibodies have been generated to potentiate the ongoing antitumor immune response of the patient, through "immune checkpoint blockade" of CTLA-4, PD-1, or PD-1 ligand (PD-L1). The outcome of the initial clinical trials with these new treatments is remarkable (97).

In a phase 3, randomized trial, the CTLA-4 blocking antibody ipilimumab was shown to prolong survival of patients with previously treated metastatic melanoma by ~4 months (98). This was a breakthrough in the treatment of metastatic melanoma because no other therapy had previously been shown to prolong survival in a phase 3 controlled trial. Another phase 3 trial with previously untreated metastatic melanoma patients showed that the overall survival was significantly longer in the group receiving ipilimumab combined with the chemotherapy drug dacarbazine than in the group receiving dacarbazine plus placebo (11 vs. 9 months) (99). Moreover, higher survival rates after 3 years were observed in the ipilimumab–dacarbazine group compared to controls (21 vs. 12%) (99).

Although no phase 3 trial has yet been published based on PD-1 or PD-L1 blockade, phase 1 studies showed promising results. PD-1 checkpoint blockade was tested in a phase 1 trial on patients with several types of advanced cancer. Cumulative response rates (complete or partial responses) were 18% among patients with non-small cell lung cancer (14 of 76 patients), 28% among patients with melanoma (26 of 94 patients), and 27% among patients with renal-cell cancer (9 of 33 patients). Responses were durable, 20 of 31 responses lasting 1 year or more in patients with 1 year or more of follow-up (100). In a phase 1 trial with anti-PD-L1 blocking antibodies, an objective response (complete or partial response) was observed in 9 of 52 patients with melanoma, 2 of 17 with renal-cell cancer, and 5 of 49 with non-small cell lung cancer. Responses lasted for 1 year or more in 8 of 16 patients with at least 1 year of follow-up (101). Finally, combined treatment of advanced melanoma was performed with both anti-CTLA-4 and anti-PD-1 blocking antibodies in a phase 1 trial. The objective response rate for all 53 treated patients in the concurrent-regimen group was as high as 40% (102). Thus, immune checkpoint blockade represents a promising new strategy to treat advanced cancer in humans. The success of this approach, which is based on potentiating the ongoing, naturally occurring antitumor immune response of the patient, provides another piece of evidence that fighting cancer is indeed a primary function of the immune system.

CONCLUDING REMARKS

As summarized in this review, the scientific literature over the past 50 years has provided strong support to the cancer immunosurveillance hypothesis. Thus, it appears that our immune system does not only naturally protect us against infectious non-self (pathogens) but also against malignant self (cancer). Many cell types belonging to both the innate (NK cells and macrophages) and the adaptive (T and B cells) immune systems seem to be involved in cancer control. Our current understanding on how the immune system fights cancer remains very fragmentary. There are pieces of evidence for two main strategies used by the immune system to distinguish cancer cells from normal cells. On one hand, the adaptive immune system recognizes altered (mutated) self proteins in malignant cells. On the other hand, NK cells and γδ T cells recognize stress-induced self molecules (NKG2D ligands) on transformed cells. Yet, cancer cells originate from normal cells and a main challenge for successful antitumor immunity is to restrain the destruction of normal cells (autoimmunity). In fact, a recent study suggested that autoimmune disease may occur as a result of an inaccurate antitumor immune response (103). Scleroderma is an autoimmune connective tissue disease in which patients make antibodies to a limited number of autoantigens, including the RNA polymerase III subunit, encoded by the POLR3A gene. In several patients who had both scleroderma and cancer, genetic alterations of the POLR3A locus were found in the malignant cells, suggesting that POLR3A mutations triggered an adaptive antitumor immune response, which cross-reacted with normal tissue, causing autoimmune disease (103).

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Evolving strategies for cancer and autoimmunity: back to the future

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Although current thinking has focused on genetic variation between individuals and environmental influences as underpinning susceptibility to both autoimmunity and cancer, an alternative view is that human susceptibility to these diseases is a consequence of the way the immune system evolved. It is important to remember that the immunological genes that we inherit and the systems that they control were shaped by the drive for reproductive success rather than for individual survival. It is our view that human susceptibility to autoimmunity and cancer is the evolutionarily acceptable side effect of the immune adaptations that evolved in early placental mammals to accommodate a fundamental change in reproductive strategy. Studies of immune function in mammals show that high affinity antibodies and CD4 memory, along with its regulation, co-evolved with placentation. By dissection of the immunologically active genes and proteins that evolved to regulate this step change in the mammalian immune system, clues have emerged that may reveal ways of de-tuning both effector and regulatory arms of the immune system to abrogate autoimmune responses whilst preserving protection against infection. Paradoxically, it appears that such a detuned and deregulated immune system is much better equipped to mount anti-tumor immune responses against cancers.

Keywords: CD4 T cell, autoimmunity, tolerance mechanisms, cancer, regulation, memory, keyword

INTRODUCTION

In our society today, cancer and autoimmunity are major causes of suffering and death, and a huge financial burden on health services worldwide. The strongest genetic link with autoimmunity is to major histocompatibility (MHC) class II genes, implicating CD4 T cells in autoimmune pathogenesis. Less obviously, CD4 T cells are also implicated in defective immunity to tumors, as CD4 regulatory T cells (Tregs) limit effector responses to tumor antigens.

Our studies have centered on CD4 immunity and its regulation, and have been informed by the striking observation that the key features of the CD4 immune system – high affinity antibody responses, memory, and CD4 regulation – co-evolved with placentation in mammals (1–3). A simple comparison of the numbers of species in different mammalian groups – monotremes, 2; marsupials, ~400; placentals, ~5000 – illustrates the reproductive advantage conferred by placentation. The contribution of the immune system to this advantage is threefold: the bringing of unborn young to immunocompetence at birth; protection after birth by maternal transfer of high affinity IgG; and reduction in exposure of offspring to disease epidemics due to memory responses in the community, the latter two being CD4 T cell dependent functions. Note that the pressure on the immune defenses due to the physical frailty of the placental newborn is enormous, as demonstrated by the fact that even with the immune protections described, mortality is exponentially higher in infants than in adults (4). The high potency of effector immune responses demanded by placentation carries high risk of pathological autoimmunity, which has been substantially addressed by the co-evolution of T regulatory mechanisms. But because Darwinian selection favors reproductive success rather than individual survival, the protection of the developing fetus takes precedence over the risk of personal suffering and even death in post-reproductive adults. And we believe that the less than complete limitation of autoimmunity by T regulation already carries its own risk, also consequent on the stringent necessity of reproductive success. Before placentation, protection of the self from effector responses required tolerance to tissue specific self-antigens; the evolution of placentation required this tolerance to extend to placenta- and fetus-specific antigens as well. Additionally, we think that the intermittent nature of pregnancy fosters the selection of dominant forms of tolerance (see Evolution of the Placenta – A New Organ). Our view is supported by two lines of evidence: – the wide expression of fetus- and placenta-specific antigens by human cancers in both males and females (5), reflecting selection of cancer cells that can gain advantage from the immune regulation protecting the developing embryo and fetally derived placenta; – recent identification of memory Tregs induced specifically by fetally derived antigens (6). Although deletional tolerance no doubt also operates to maintain tolerance to fetally derived antigens, the particular advantage of dominant tolerance to fetal antigens expressed in the placental trophoblast is that it could also confer bystander tolerance to fetal alloantigens at the feto-maternal interface, to which there is no opportunity for maternal thymic tolerance, particularly during first time pregnancies before there is priming to induce Tregs specific for alloantigens (6).

In this article, we start by reviewing the fundamental changes that occurred in mammals over the last 200 million years

[see **Figure 1** Ref. (7)] and that form the context of the sequential genetic changes that enabled new immunological structures and functions to evolve. We then review the evidence that the resulting "modern" mammalian immune system can be detuned to give a minimal essential immune system for health: a system that, without compromising immunity to infection, can both abrogate pathogenic CD4 driven autoimmune responses and augment anti-tumor immunity.

BRIEF HISTORY OF MAMMALS FROM AN IMMUNOLOGICAL PERSPECTIVE

Comparative genomics and the fossil record have been used as a timeline for the evolution of mammals from the common reptilian ancestor [**Figure 1** Ref. (7)]. This timeline highlights the major physiological changes that emerged: the evolution of homeothermy, of hair (insulation), and of lactation (an effective strategy to nourish homeothermic offspring). All mammals, including the egg laying monotremes, have these characteristics, which distinguish them from the cold-blooded common ancestor they share with reptiles and birds.

ORIGINS OF LYMPH NODES

The development of these characteristic mammalian attributes was accompanied by changes in the immune system, both anatomically and functionally. The most striking gross anatomical change is the emergence of intra-lymphatic lymphoid aggregates (the ancestors of lymph nodes) in the common mammalian ancestor. All jawed vertebrates have a spleen and lymphoid aggregates in the mucosal associated lymphoid tissues, but only mammals have lymphoid structures that are intra-lymphatic as opposed to lymphatic-associated. In chickens, lymphoid aggregates are found in the wall but not the lumen of lymphatics (8), which is a similar arrangement to the so called isolated lymphoid follicles (ILFs) found at mucosal sites in all vertebrates, and associated with

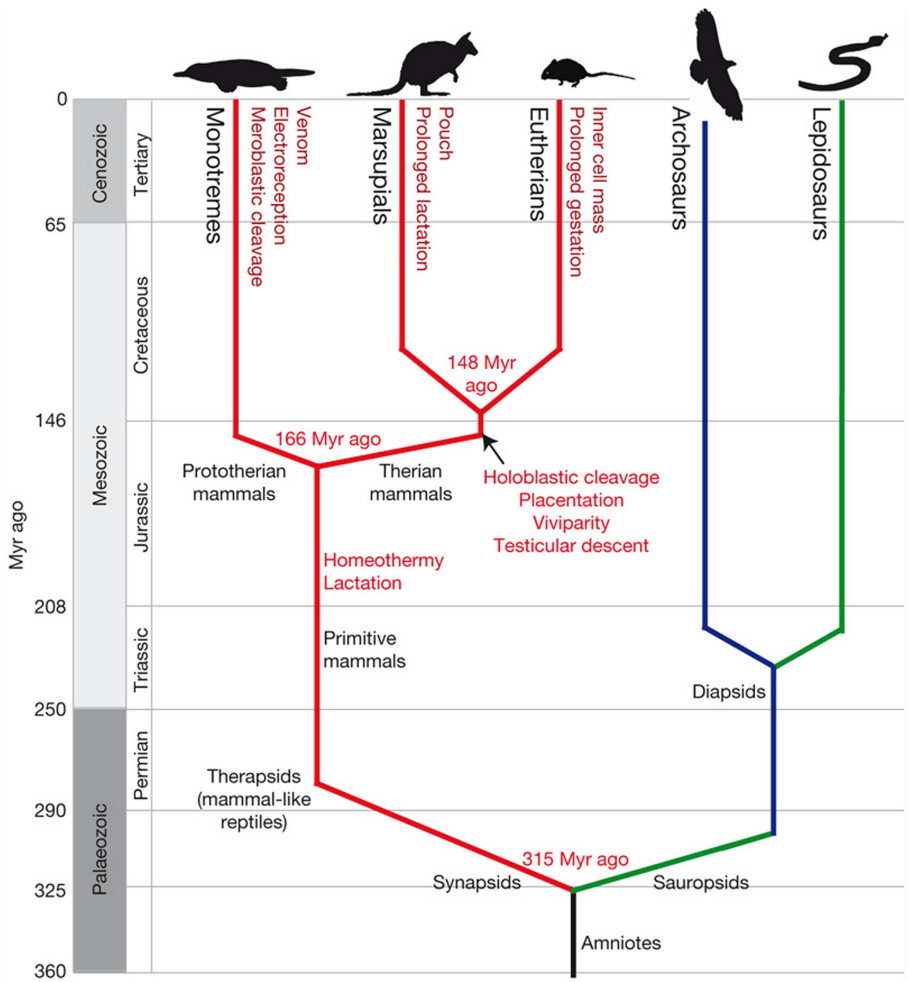

FIGURE 1 | Emergence of traits along the mammalian lineage. Amniotes split into the sauropsids (leading to birds and reptiles) and synapsids (leading to mammal-like reptiles). These small early mammals developed hair, homeothermy, and lactation (red lines). Monotremes diverged from the therian mammal lineage ~166 Myr ago and developed a unique suit of character (dark-red text). Therian mammals with common characters split into marsupials and eutherians around 148 Myr ago (dark-red text). Geological eras and periods with relative times (million years ago) are indicated on the left. Mammal lineages are in red; diapsid reptiles, shown as archosaurs (birds, crocodilians, and dinosaurs), are in blue; and lepidosaurs (snakes, lizards, and relatives) are in green.

but not inside lymphatics (e.g., the lacteals in the gut). In contrast, vascularized intra-lymphatic ILF-like structures are found in both of the extant monotremes (9, 10). Why did these intra-lymphatic structures, which are the precursors of mammalian lymph nodes, evolve? Possible explanations in our view are linked to the evolution of homeothermy in mammals, with the consequent increased demand for nutrients. The mucosal system of mammals has a much more extensive surface area for absorption of food than reptiles, and consequently has increased exposure to bacteria, both commensal and non-commensal, in the gut. One view therefore, is that these intra-lymphatic lymphoid structures evolved in the mesentery of the gut as a "firewall" to block the entry of gut bacteria into the systemic circulation (11). In any case, comparative genomics supports the view that intra-lymphatic lymphoid structures evolved in the common mammalian ancestor, as the L-selectin gene (SELL) first appears in monotremes (www.ensembl.org). This molecule occurs on lymphocytes and directs their exit from blood vessels into lymphatics (as lymph nodes are essentially intra-lymphatic structures), and is not present in reptiles or birds, which however do have genes for the inflammatory selectins present on endothelium, E-(SELE), and P-selectin (SELP). SELE and SELP are chromosomally co-located in mammals with L-selectin, indicating that SELL is likely to have arisen by gene duplication in the common mammalian ancestor.

Microscopically, the ILFs found inside the lymphatics of monotremes contain B cell germinal center (GC)-like structures with follicular CD4 T cells (10), but they do not contribute to affinity maturation of the B cell response or to memory; secondary antibody responses in monotremes are very similar to primary ones (2). In the absence of a link to affinity maturation, it seems probable that these GC-like inclusions mediated the diversification of the B cell repertoire, after the fashion of the GC-like structures seen to develop in the Bursa of Fabricius in chickens, where the B cell repertoire is diversified by activation induced cytosine deaminase (AID)-dependent gene conversion of immunoglobulin variable region genes (12). In placental animals like sheep, primary diversification of the B cell repertoire through gene conversion and somatic hypermutation also occurs in the gut associated lymphoid tissues (13), so it is quite plausible that this is the function first appearing inside intra-lymphatic nodules in monotremes. Orthologs of IgE and IgG also evolved in the common mammalian ancestor; in monotremes (14), unlike placental animals, switched immunoglobulin isotypes are produced in primary immune responses, and it is possible that the sites of AID-dependent class switching, to not only IgE and IgG, but also IgA, are the intra-lymphatic ILFs.

IgA CONTROL OF ANTI-INFLAMMATORY AND RESPONSES TO GUT MICROBIOTA

IgA is the most abundant immunoglobulin, and secreted IgA is the main immunoglobulin at mucosal surfaces. Orthologs of IgA are present in reptiles, birds, and mammals but not amphibians and fish, indicating that this immunoglobulin class evolved in the common amniotic ancestor of land animals. The evolution of the amniotic egg was critical to the capacity of vertebrates to colonize non-aquatic habitats, as it freed their reproduction from the dependency on access to water retained by amphibian species. However, terrestrial habitats brought new challenges in the form of different food sources and different microbiota colonizing the gut. In this context, the anti-inflammatory properties of IgA are likely to have been crucial. Tsuji et al. (15) present several pieces of evidence pointing to the importance of IgA in maintaining a normal gut flora. AID-deficient mice developed abnormal gut flora, which was associated with impaired switching to IgA in ILFs in the mucosa. This IgA-dependent class switching was shown to be T cell independent, but dependent on AID expression in ILF GC-like structures.

ORIGIN OF CD4 IMMUNITY

Modern mammals share a 450-Myr-old common ancestor with cartilaginous fish, and indeed all jawed vertebrates have RAG-dependent adaptive immune systems. However, recent sequencing of the elephant shark genome has revealed no evidence for the CD4 gene, and indeed no evidence of either FoxP3, the gene linked to evolution of CD4 regulation (16), or ROR-gamma (RORc), the transcription factor required for the development of Th17 CD4 T cells (17). This suggests that CD4 T cells were not part of the ancestral RAG-dependent adaptive immune system, but evolved later. Recent data have implicated both CD4 Th17 cells and Tregs in IgA immunity.

FoxP3$^+$ AND Th17 CD4 T CELLS ARE INVOLVED IN IgA PRODUCTION IN THE GUT

The FoxP3 gene is highly conserved in all placental mammals (www.ensembl.org). Given that members of the FoxP gene family are consistently highly conserved across all animal groups, this would not be surprising, except that by comparison, FoxP3 orthologs in marsupials, marsupials, reptiles, monotremes, fish, and amphibians show much reduced conservation. The step gain in conservation of FoxP3 in placental mammals is consistent with a gain of function specific to placentation (18). Similarly, IL17a, the principle IL17 cytokine secreted by Th17 T cells, is exclusive to mammalian genomes, and therefore evolved in the common mammalian ancestor.

The conventional view of FoxP3$^+$ Tregs is that their primary role is to suppress or modulate CD4 dependent immune responses (19), but there is evidence that they also function in the T cell dependent switching to IgA in the gut: Foxp3 expressing Tregs were potent inducers of IgA after transfer in T cell deficient mice (20); Tregs were found to be important mediators of induction of IgA to flagellin, a molecule common to commensal and pathogenic bacteria (21). The latter result raised the very interesting idea that a key function of Tregs is to promote the generation of IgA antibodies to commensal bacteria in the gut, so maintaining homeostasis of gut microbiota and preventing inappropriate inflammatory immune responses. A possible role for the ancestral FoxP3-dependent Tregs could therefore be to moderate immune responses to commensal bacteria by effectively providing help for B cells.

This suggestion is somewhat controversial, as recent studies have implicated Th17 cells rather than Tregs in the induction of IgA in the gut (22, 23). Cao et al. implicated the mammalian specific cytokine IL17a in the production of IgA to flagellin. The available data are however not necessarily contradictory, as in humans at least a significant fraction of Tregs isolated from mucosal surfaces

co-express FoxP3 and RORγt, the key Th17 transcription factor (24), in addition to producing IL17a. A scenario compatible with the evidence is that there was co-evolution of Th17 and FoxP3$^+$ T cells in the common mammalian ancestor, at least in part to promote IgA antibody production in the gut, and in any case to act synergistically to promote integrity at mucosal surfaces.

EVOLUTION OF HIGH AFFINITY ANTIBODIES AND MEMORY IN THE COMMON PLACENTAL ANCESTOR

As discussed earlier (see "Origins of Lymph Nodes" above), despite having intra-lymphatic lymphoid follicles containing GCs, monotremes do not make high affinity antibody responses or demonstrate CD4 memory (2, 9, 10). They do not have true lymph nodes with segregated B and T cell areas, but these structures evolved in the common placental ancestor; all marsupials and placental mammals have lymph nodes, and demonstrate the capacity to make high affinity antibodies and memory (1, 3, 25, 26). These advances are linked to the evolution of new genes, notably the lymphotoxin βreceptor (LTβR) and its ligands, which are not only essential for lymph node development but also for making high affinity antibodies and memory.

LYMPHOID TISSUE INDUCER CELLS: Rorγ DEPENDENT, AND LINKED WITH BOTH LYMPH NODE AND CD4 MEMORY DEVELOPMENT

Reina Mebius was the first to characterize murine lymphoid tissue inducer cells (LTi) (27). Their function in the development of lymphoid tissues was revealed when it was found that mice deficient in the orphan retinoic acid receptor gamma (RORγ) lacked both this CD4$^+$CD3$^-$ LTi population and lymph nodes (28, 29). These studies link LTi unequivocally with the development of lymph nodes, cryptopatches, and ILFs through their expression of the tumor necrosis superfamily members (TNFSF) for the lymphotoxin beta receptor (LTβR), LT$\alpha_1\beta_2$ (30), and TRANCE (31); more recent studies have also shown that LTi are rich sources of the cytokine, interleukin 22 (IL22) (32), which is associated with the promotion of defenses at epithelial sites (33–35). This would permit the LTi a function in the promotion of innate immunity that is distinct from the induction of lymphoid tissues.

Additionally, our work has shown that LTi persist in adult lymphoid tissues in both mouse (36) and man (37), but are distinguished from the neonatal population by their expression of high levels of OX40-ligand (OX40L)(TNFSF4) (36, 37) and in mouse, CD30L(TNFSF8) (36). Our studies have found that CD4 T cell memory function is highly dependent on signaling through both OX40 and CD30 (38), suggesting additional roles for LTi in the mediation of adaptive CD4 dependent immune responses.

IMPORTANCE OF THE IMMUNE CONTRIBUTION TO PLACENTATION

It is difficult to refute the significance of the co-evolution of high affinity antibodies and lymph nodes with placentation. Zinkernagel (39) takes the view that memory and high affinity antibodies are chiefly relevant because they protect offspring via maternal transfer of high affinity antibodies, and the way in which immunological functions have evolved during placentation broadly supports his perception. We know that orthologs of IgG first appeared in the common mammalian ancestor because monotremes have them (14). The neonatal Fc receptor (FCGRT), however, which is the gene that enables the crucial transfer of IgG from mother to offspring as well prolonging the beneficial effects of IgG by increasing its half-life (40), is only present in marsupial and placental genomes. Studies of human infant mortality to most common infections show an exponential decline with the age (4) with exception of the first year of life where transfer of maternal IgG plays a crucial role in infant survival through protective immunity. Indeed in many placental animals failure to transfer maternal IgG is fatal. Comparative genomics therefore supports the idea that the development of the capacity to make high affinity antibodies and transfer them to offspring is an integral component of the evolution of a fundamental change in reproductive strategy.

CO-EVOLUTION OF HIGH AFFINITY ANTIBODIES WITH FoxP3-DEPENDENT REGULATION

In placentals, GCs in B follicles are the locations where T cell dependent B cell selection drives the generation of high affinity antibodies (41), but there must exist mechanisms to edit self-reactive GC B cells that acquire self-reactivity. This can occur because self-proteins can be inadvertently conjugated to foreign proteins (e.g., apoptotic virally infected cells), and therefore GC B cells that acquire self-reactivity by chance have the capacity to get help from GC follicular T helper cells. Indeed, it is very common in viral infections in humans to get transient low affinity autoantibody production, but this does not usually go on to generate high affinity class switched autoantibodies.

Recent studies have shown that Tregs are also present in normal GC (42, 43) and the fact that high affinity IgG autoantibodies to a wide variety of tissue-restricted antigens are found in FoxP3-deficient mice indicates that Tregs must be pivotal in preventing the generation of these autoantibodies. Although it is by no means clear how Tregs in GC prevent autoantibodies being generated, there is better evidence for how self-specific Tregs are selected. Recent work has shown the critical role of the thymic medulla in the selection of thymic derived regulatory but not conventional T cells (44). The gene AIRE, expressed in the thymic medulla, controls the expression of many tissue-restricted antigens (45, 46). Intrathymic deletion of self-specific T cells is substantially AIRE-dependent (47), but the process of selection against tissue-restricted antigens is also a plausible mechanism by which Tregs specific for self-antigen could be selected in thymus and go on to exert their effects in the periphery.

When considering the origin of the requirement for regulation of immune effectors, it is clear that, in the absence of memory and high affinity IgG, the consequences for monotremes of inadvertently making anti-self antibody responses are mild; all their antibodies are low-titer, low affinity, and transiently produced, so one would surmise that the requirement for regulation is limited. Substantial changes in the FoxP3 gene occurred during the evolution of placentation (18), and the relevance of this is further demonstrated by the failure of regulation in placental animals having mutant forms of FoxP3 lacking critical domains (18).

EVOLUTION OF THE PLACENTA – A NEW ORGAN

The major advantage of placentation over oviparous forms of reproduction is that it greatly increases the chances of reproductive

success by prolonging the parental protection of the developing offspring, including increasing the chance of surviving infection courtesy of a more mature immune system, initially supported by high affinity maternal IgG antibodies. A recent detailed study of placental fossils combined with comparative genomic data concluded that all modern placentals (Eutherians) are derived from a common placental ancestor that survived the mass extinction 65 Myr ago that eliminated terrestrial dinosaurs (48). Inferences from this study are that this common Eutherian ancestor had a hemochorial placenta with the fetal and maternal blood circulations in intimate contact. Marsupials represent an intermediate step toward this state; they are born very immature and before their immune system develops, but nevertheless get the benefit of protection from predation by *ex utero* occupation of the maternal pouch, where lactation provides the added advantage of maternally transferred antibodies. Marsupials have a yolk sac (Metatherian) placenta, which is simple and relatively impervious to feto-maternal exchange, thus dodging the issue of maternal recognition of fetal and placental antigens. In Eutherian mammals, however, the placenta is fully adapted to cope with a fetus that develops to maturity. There are many new genes that arose during the evolution of placentation to program the development of the placenta (a fetally derived organ) (49), and in addition there are genes essential for survival of the fetus itself *in utero*. The common ancestor of marsupials and placentals, in which these new genes were evolving, had the capacity to make high affinity antibodies; for the hemochorial placenta with its proximity of maternal and fetal circulations, the selective drive for immune regulation capable of protecting the fetus from rejection by its own mother was clearly decisive.

Two facts need to be remembered concerning the placenta:

- Morphologically it is very diverse in different classes of mammal, reflecting the strong evolutionary pressure for mammals with different lifestyles to adapt reproductively to different external conditions. This is reflected in the evolution of new genes and new gene families in different mammalian classes and is particularly evident on the X chromosome, where many of the placental genes are concentrated and which also evolved from an autosomal chromosome in placentals (5).
- Placentation is an intermittent phenomenon, so maintaining T cell tolerance to the rapidly evolving new proteins that are not present in mammalian females post birth, but to which T cell tolerance will be essential if they are to be reproductively successful is a real challenge. From an evolutionary perspective, it is therefore not difficult to understand why the adaptation to placentation might select for mechanisms of dominant tolerance mediated by Tregs, i.e., where Tregs specific for some of the newly evolving placental and fetal-restricted antigens could suppress maternal effector responses against them, whereas other, maybe less abundant or otherwise less conspicuous, neo-antigens would escape without significant Treg reaction. A further point is that this dominant regulation against thymic expressed placental antigens expressed at the feto-maternal interface could also suppress allorecognition of paternal MHC.

EVOLVING MATERNAL TOLERANCE TO FETUS AND PLACENTA

Because it seems very likely that the problem of maternal tolerance to fetal and placental proteins had already been solved in the common eutherian placental ancestor, we looked for genetic differences between marsupials and eutherian placental mammals in the genes linked to induction and selection of Tregs in mTECs in thymus. The T cell costimulatory molecule CD28 is vital for Treg selection (50), and CD28 ligands, particularly CD80, are expressed on AIRE^{+} mTECs. In all eutherian placental genomes examined, AIRE, the gene associated with selection of antigen-specific Tregs is chromosomally co-located with the CD28 paralog, ICOS-ligand. The ICOS signaling pathway is crucial for the generation of high affinity antibodies in GCs (51); particularly pertinent to the genetic association between ICOSL and AIRE, it is also required for the effective Treg suppressive function (52). Thus the ICOS gene is a pivot between high affinity antibody production and effective Treg function against immune responses to self, raising the very interesting possibility that the expression of this gene facilitated the selection of self-antigen-specific Tregs found in GCs (43), which then edit self-reactive B cells in GCs driven by ICOS signaling interactions.

The comparative genomics of these interactions is instructive. In all marsupial genomes examined, AIRE and ICOSL are not co-located, but are on separate chromosomes, as is also the case in reptiles and birds. Maybe the genetic translocation that brought AIRE and ICOSL together in the common placental ancestor also mediated the co-expression of AIRE with ICOSL on mTECs. We think that this co-expression was likely to have facilitated development of the process of selecting antigen-specific Tregs, and see it as an illustration of how mutually beneficial processes could serve as selective advantages for one another: here the genetic mechanism for improving antibody affinity (ICOS signaling) co-evolved with the genetic mechanism maintaining tolerance to self (selection of antigen-specific Tregs via AIRE) to modify the generation of high affinity antibodies to tissue-restricted antigens – in this case those expressed in the placenta and fetus. This allows antibodies to pathogens to be transferred to offspring, protecting them from infection without causing autoimmunity, which is a genuine risk, as there are many examples of passive maternal transfer of IgG autoantibodies causing disease in the neonate. In FoxP3^{KO}OX40CD30^{KO} mice where not only affinity maturation, but also autoantibody production is impaired, the requirement for Tregs is obviated.

Other studies have linked Tregs specifically with the maintenance of allotolerance at the feto-maternal interface (53–55). However, our data from the FoxP3^{KO}OX40CD30^{KO} mice do not support this interpretation. Our data show that FoxP3^{KO}OX40CD30^{KO} mice are fully able to reject allografts (data not shown) but we found no evidence that female FoxP3^{KO}OX40CD30^{KO} mice rejected allogeneic fetuses. Recent data suggest that immunosuppression in the fetal circulation need not be cell-mediated and implicates the expression of arginase by fetal blood cells (56). Because the fetus is normally sterile, such a global suppression of immune responses in the fetal circulation, and therefore by definition also at the fetal/maternal interface in the placenta, is in our view, a potential mechanism to prevent maternal lymphocytes that enter the fetal circulation inducing

rejection. The selective expression of immunosuppressive arginase in the fetal red blood cells in the fetal circulation also helps to explain why lymphocytes in the maternal compartment are still able to respond. For example, although in pregnancy there is susceptibility to some infections, notably influenza, pregnant women make good antibody responses after vaccination (57). We think, therefore that the requirement for regulation in the form of Tregs is more relevant in the context of the mother, to allow antibody responses in particular to foreign proteins derived from infectious pathogens (maternal IgG then passively protects offspring after birth) to go ahead, but to head off responses to fetal or placental antigens that enter the maternal circulation.

MINIMAL IMMUNE SYSTEM FOR MAMMALIAN HEALTH

Our studies had shown that the TNF-family members, OX40 and CD30-ligand were crucial for the development of high affinity antibodies and memory (38). Although CD30 and its ligand were present in the common amniotic ancestor, true orthologs of OX40-ligand, which plays the dominant role in both affinity maturation and memory, are only present in the mammalian lineage (www.ensembl.org). To ask the question of whether CD4 regulation and memory could have co-evolved in the common placental ancestor we reasoned that mice deficient in FoxP3 (no Tregs) and also deficient in OX40 and CD30 (no high affinity antibodies and memory) would mimic at least to some extent the immune system of the common mammalian ancestral immune system seen in monotremes, and would not exhibit autoimmunity. This was the case (58).

Our mice are interesting in several respects. First, the generation of autoantibodies is abrogated in these mice and they fail to develop the widespread autoimmunity seen in mice and men. The development of FoxP3^{KO} disease is CD4 dependent (19) but the FoxP3^{KO}CD30OX40^{KO} mice do not behave as CD4-deficient as they do generate GCs and switched antibody not dissimilar to that observed in monotreme responses. Furthermore, they control many herpes viruses (CD8 immunity is preserved) as well as bacteria. The behavior of the immune responses in the CD30OX40^{KO} mice is mirrored in a single reported case of OX40-deficiency in humans (59). Although the individual did suffer from Kaposi's sarcoma, she controlled common Herpes virus infections, and was not unduly susceptible to bacterial infections, despite the absence of recall CD4 memory responses.

If one accepts Zinkernagel's view that the development of memory and high affinity antibodies is a strategy optimized for reproductive success rather than individual survival, then the Foxp3^{KO}OX40CD30^{KO} immune system in our view represents the "minimal immune system for health," the necessary and sufficient platform that had evolved in the common mammalian ancestor before the evolution of high affinity antibodies and memory induced a compromise in the form of susceptibility to autoimmunity.

HUMAN AUTOIMMUNITY AND BLOCKADE OF OX40 AND CD30 SIGNALING PATHWAYS

In human genome wide association studies (GWAS) class II polymorphisms are the strongest genetic link, highlighting the role of CD4 T cells in immunopathology, either due to failure to select antigen-specific Tregs in thymus that subsequently protect, or because CD4 effector T cells drive pathology. The significance of the Foxp3^{KO}OX40CD30^{KO} mice with a global defect in Tregs is that it suggests that blocking OX40 alone, or in combination with CD30, would be very effective treatment for wide number of CD4 driven autoimmune diseases without rendering patients susceptible to infection. This is further supported by evidence that shows that FoxP3^{KO} disease can also be blocked by co-injection of antibodies that block OX40 and CD30 signaling pathways (58).

CANCERS MIMIC THE IMMUNE EVASION STRATEGY OF PREGNANCY

The link between placentation and cancer is hardly new (60). From our perspective, a substantial role for dominant FoxP3-dependent regulation was to facilitate the growth of the fetus and placenta, both expressing many neo-antigens, while at the same time being permissive particularly for the generation of antibodies to pathogens. Recent whole exome sequencing of human cancers has also revealed their enormous heterogeneity (61). Although in theory this should render tumors immunogenic to the CD8 immune system in particular, it is clear that dominant Treg tolerance is a major stumbling block to the development of effective anti-tumor immune responses if Tregs specific for self-antigens can suppress immune responses to tumor-specific antigens.

Two gene examples illustrate the point. The first is the gene alpha-fetoprotein (AFP), the fetal albumin adapted to *in utero* survival, an autosomal gene. The second is the gene PLAC1 expressed in the trophoblast of the placenta of all placental animals, and exclusive to placental animals. Both of these genes are part of the genetic adaption to placental reproduction, but they are also widely expressed in human cancers. This fact suggests that the suppressive effect of fetal and placental antigens on immune responses might have led to the success of cancers that express them. As stated earlier, medullary epithelium in thymus (mTEC) is crucial for the selection of Tregs but not conventional T cells (44). Both PLAC1 and AFP are over-expressed in mTECs [compared to cortical epithelium (cTECs)] (our own data and also www.immgen.org), so it is quite plausible that Tregs specific for these proteins could be selected in thymus. Support for this type of dominant tolerance preventing immune responses to cancer is also provided by the following study (62).

In this study, T cell receptors (TCRs) from tumor infiltrating Tregs found in a murine model of prostate cancer were cloned. TCR transgenic mice positively selected Tregs in thymus in both male and female mice, indicating that they were not tumor-specific Tregs, and as they were found in female mice, were not selected in prostate! Their mTEC thymic derivation was further supported by the observation that selection was dependent on AIRE, the gene that controls expression of many tissue-restricted antigens in the thymus (45).

DE-TUNING THE IMMUNE SYSTEM TO UNBLOCK CD8 ANTI-CANCER IMMUNE RESPONSES

Strategies that suppress Treg function [CTLA4 blockade (63, 64) and PD-1 (65)] have been effective in releasing CD8 anti-tumor

immune responses, particularly when used in combination (66). Because Tregs suppress CD4 driven autoimmunity, autoimmunity is a major cause of morbidity and mortality in these treatments. Like $Foxp3^{KO}$ mice, $CTLA4^{KO}$ mice die of CD4 driven autoimmunity (67) so in reality CTLA4 blockade can only be partial in human patients.

However, our studies in $FoxP3^{KO}OX40CD30^{KO}$ mice suggest that that CD4 mediated immunity can be obviated in $FoxP3^{KO}$ without seriously compromising autoimmunity. To test whether these mice were capable of mounting anti-tumor immune responses we used the well established murine melanoma line B16 (68). This tumor grows rapidly in syngeneic B16 mice but tumor growth is virtually abrogated in $FoxP3^{KO}OX40CD30^{KO}$ mice (our unpublished observations). To us this observation has potential important implications for the treatment of human cancers as it offers the option of permitting effective CD8 anti-tumor responses while preventing the unpleasant CD4 driven autoimmune side effects.

SUMMARY

In this perspective we outline a strategy for attenuating CD4 driven immunopathology by blockade of the TNF super family members, OX40L (in particular), and CD30L (synergistic with OX40L). Studies of immune function in mice deficient in OX40 and CD30 reveal that although CD4 immunity is reduced, deficient mice are able to deal with the common viral and bacterial infections that can be associated with conventional immunosuppressive strategies. We suggest that antibodies that block these pathways may have therapeutic benefit in human autoimmune diseases mediated by CD4 T cells without compromising resistance to infection.

Recent work has shown that blockade of regulatory T cell function with CTLA4-blocking antibodies has revealed impressive "repressed" CD8 immune responses to neo-antigens expressed by human cancers, particular melanoma, but also some other solid tumors. However, this has been at the expense of CD4 driven autoimmunity that can have considerable morbidity and even mortality. Our work indicates that in the absence of OX40 and CD30, FoxP3-dependent Tregs are dispensable, and mice deficient in OX40, CD30, and FoxP3 mount excellent CD8-dependent anti-tumor immune responses.

As stated above, it is our view that human susceptibility to autoimmunity and cancer are the evolutionarily acceptable side effects of the immune adaptations that evolved in early placental mammals to accommodate a fundamental change in reproductive strategy, and by reversing this process, a detuned and deregulated immune system is much better equipped to mount anti-tumor immune responses against cancers but is also resistant to chronic CD4 driven autoimmune disease.

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Corrigendum: Evolving strategies for cancer and autoimmunity: back to the future

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Autoimmunity as a double agent in tumor killing and cancer promotion

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Cancer immunotherapy through manipulation of the immune system holds great potential for the treatment of human cancers. However, recent trials targeting the negative immune regulators cytotoxic T-lymphocyte antigen 4, programed death 1 (PD-1), and PD-1 receptor ligand (PD-L1) demonstrated that clinically significant antitumor responses were often associated with the induction of autoimmune toxicity. This finding suggests that the same immune mechanisms that elicit autoimmunity may also contribute to the destruction of tumors. Given the fact that the immunological identity of tumors might be largely an immunoprivileged self, autoimmunity may not represent a wholly undesirable outcome in the context of cancer immunotherapy. Rather, targeted killing of cancer cells and autoimmune damage to healthy tissues may be intricately linked through molecular mechanisms, in particular inflammatory cytokine signaling. On the other hand, since chronic inflammation is a well-recognized condition that promotes tumor development, it appears that autoimmunity can be a "double agent" in mediating either pro-tumor or antitumor effects. This review surveys the tumor-promoting and tumoricidal activities of several prominent cytokines: IFN-γ, TNF-α, TGF-β, IL-17, IL-23, IL-4, and IL-13, produced by three major subsets of T helper cells that interact with innate immune cells. Many of these cytokines exert divergent and seemingly contradictory effects on cancer development in different human and animal models, suggesting a high degree of context dependence in their functions. We hypothesize that these inflammatory cytokines could mediate a feedback loop of autoimmunity, antitumor immunity, and tumorigenesis. Understanding the diverse and paradoxical roles of cytokines from autoimmune responses in the setting of cancer will advance the long-term goal of improving cancer immunotherapy, while minimizing the hazards of immune-mediated tissue damage and the possibility of *de novo* tumorigenesis, through proper monitoring and preventive measures.

Keywords: autoimmunity, antitumor, tumorigenesis, inflammation, cytokine

INTRODUCTION: PARADOXICAL RELATIONSHIPS BETWEEN AUTOIMMUNITY AND TUMOR

The process of inducing the immune system to selectively destroy tumor tissues *in vivo* faces numerous conceptual as well as practical hurdles. Foremost among these is the self-derived immunological identity of tumors (1, 2). The prevalence of self-antigen expression on cancer cells implies that many tumors will be protected from cytotoxic immune responses via intrinsic host mechanisms of self-tolerance. It is therefore evident that any biologic therapy capable of provoking a therapeutically useful antitumor immune response will carry some risk of off-target autoimmune toxicity. The resulting destruction of normal host tissues, besides contributing to morbidity and mortality in its own right, can potentially lead to *de novo* tumorigenesis by initiating chronic inflammation, which is a feature of premalignant states in numerous organs including breast, bladder, prostate, cervix, ovary, stomach, and lungs (3). The molecular sequence that links chronic inflammation to cancer development involves intricate and context-dependent interactions among differentiated tissue cells, immune cells, organ-specific stem cells, and other cell types present at the incipient tumor site (4). In light of these disparate outcomes, autoimmunity may be regarded as a "double agent" implicated in both immune-mediated tumor elimination and the cellular, genetic, and epigenetic changes that underlie carcinogenesis. Given the complexity and interconnections of the associated signaling networks, navigating this new therapeutic realm demands a formidable balancing act: any cancer treatment that seeks to modify immune system function must induce a degree of self-reactivity that leads to immune-mediated tumor killing while containing the destructive and cancer-promoting aspects of that self-reactivity within tolerable limits.

After decades of hard struggle in cancer immunotherapy, exciting opportunities have emerged, especially in monoclonal-antibody-based therapies designed to elicit antitumor immunity either through inhibition of negative immune system regulators or activation of costimulatory receptors (5). Remarkable benefits in patient survival have been demonstrated in clinical trials of novel monoclonal antibodies blocking immune checkpoints,

Table 1 | Anti- and pro-tumor activities of selected cytokines.

Cytokine	Antitumor activities	Pro-tumor activities
IFN-γ	Required for Th1 differentiation, effective cytotoxic antitumor immune responses, and transplanted tumor rejection induced by bacterial endotoxin (10); mediates immune surveillance against spontaneous tumor development (11); enhances tumor antigenicity by upregulating expression of antigen presentation machinery (12); has immunosuppressive functions that can restrain chronic inflammation in certain contexts (13, 14)	Mediates chronic inflammation in gastric epithelium (15, 16); protects tumor cells from CTL lysis by altering their surface MHC expression (17, 18)
TNF-α	Mediates transplanted tumor rejection induced by bacterial endotoxin (19); local administration damages tumor vasculature, and has been used in isolated limb perfusion to treat melanomas and soft tissue sarcomas (20)	Mediates chronic inflammation, oxidative stress, and genomic damage, promoting malignant transformation in various organs (21–23); activates growth and survival-promoting pathways that drive angiogenesis, proliferation, invasion, and metastasis in established tumors (24, 25)
TGF-β	Regulates the cell cycle and inhibits proliferation in stromal and epithelial tissues (26, 27); restrains chronic inflammation by opposing Th1 differentiation and downregulating IFN-γ production by NK cells and DCs (28–30)	Induces tolerant, cancer-promoting phenotypes in tumor-associated macrophages (M2) and neutrophils (31); promotes differentiation and recruitment of immunosuppressive Treg cells in the tumor microenvironment (32, 33); promotes angiogenesis, invasion, and metastasis of cancer cells (34); may suppress antitumor immunity by inhibiting Th1 signaling (35)
IL-17/IL-23	May promote IFN-γ secretion and Th1 differentiation (36, 37); possible mediator of innate and adaptive antitumor immunity through interactions with the Th1 signaling axis (38–41), or direct cytotoxicity of Th17 cells (42)	Mediates chronic inflammation in the liver (43–46); drives differentiation and expansion of Th17 cells in the tumor microenvironment, which has been shown to favor disease progression and suppress antitumor immunity (47, 48); activates proliferative and survival-promoting pathways in cancer cells (49–51)
IL-4/IL-13	Poorly characterized	Drives differentiation of tolerogenic CD4+ Th2 cells in the tumor microenvironment (52); induces cancer-promoting phenotypes in tumor-associated macrophages (M2) and DCs (31, 53); IL-4 mediates survival and proliferation of cancer stem cells (54–56); IL-13Rα2 signaling promotes tumor invasion and metastasis (57–59)

including cytotoxic T-lymphocyte antigen 4 (CTLA4) (6), programed death 1 (PD-1) (7), and PD-1 receptor ligand (PD-L1) (8). In practice, however, many biologic therapies have fallen short of expectations in clinical trials, failing to deliver enhancements in disease-free or overall patient survival (9). A partial explanation for this disappointment is the context-dependent nature of immune signaling pathways themselves. In many cases, a given signal can exert diverse, and often opposing, effects on the progression of cancer depending on a variety of factors including the tissues involved, expression level of the signal molecule(s), tumor stage and antigenic profile, and host genetic background. Thus, there is a remarkable degree of overlap between the signaling mechanisms that mediate the desired outcome of tumor destruction, and those which fuel the detrimental processes of cancer development, tumor progression, and autoimmunity. Many cytokines with therapeutic potential have demonstrated these paradoxical effects, revealing both tumoricidal and tumor-promoting activities under different experimental conditions. The task of eliciting potent cytotoxic immune responses, while managing the concomitant dangers of autoimmunity, therefore requires detailed mechanistic knowledge of immune system signaling. This review will summarize the current understanding of the pro- and antitumor activities of several major cytokines (**Table 1**): interferon-gamma (IFN-γ); tumor necrosis factor-alpha (TNF-α); transforming growth factor-beta (TGF-β); the Th17 cytokines IL-17 and IL-23; and the Th2 cytokines IL-4 and IL-13. Concluding remarks will address a hypothetical loop of autoimmunity-mediated antitumor immunity, leading to further induction of autoimmune responses, inflammatory cytokine signaling, and tumor promotion, including potential *de novo* tumorigenesis in solid organs such as the gastrointestinal tract with demonstrated susceptibility to inflammatory carcinogenesis.

ANTI- AND PRO-TUMOR ROLES OF AUTOIMMUNITY COULD BE MEDIATED THROUGH INFLAMMATORY CYTOKINES OF Th CELLS

The pathological correlation between inflammation and cancer has been known to clinical science for 150 years (60). However, it is only in recent times that our understanding of the immune system has become sophisticated enough to yield practical applications in the realm of cancer biology. For example, ample evidence has been gathered for the roles of inflammatory signals derived from innate immune response in promoting tumor growth and progression [for recent reviews, please see Ref. (4, 61, 62)]. There is emerging

evidence for a tumor-promoting role by inflammatory effectors from the adaptive immune system, for example, in the TRAMP model of prostate cancer initiated with transgenic expression of an oncogene (the SV40 large T antigen) (63, 64), or implanted model of melanoma (65). Given the long-lasting nature of memory responses that is characteristic of adaptive immunity and the potency of autoimmune memory T cells [for review see Ref. (66)], one might speculate that inflammatory signals originated from the adaptive immune system, compared to their innate-derived counterparts, might sustain a longer effect, regardless of their pro- or antitumor nature. However, we should also emphasize that in a complex *in vivo* setting of immune responses, it is likely that the intimate interaction between innate and adaptive immune cells determines an immunological outcome (67–69). Nevertheless, definitive evidence remains to be gathered to show whether an inflammatory signal(s), at a physiologically relevant setting, can initiate *de novo* tumorigenesis.

Numerous parameters of immune system function have been correlated with clinical outcomes in cancer patients, including the cellular composition of tumor inflammatory infiltrate (70), expression of pro- and anti-apoptotic genes in circulating PBMCs (71), and cytokine profiles measured in peripheral blood (72). It has long been recognized that the same inflammatory cytokine may play a prominent role in either tumor killing or cancer promotion. Although the original stimuli of inflammatory cytokine production are unknown in most cancer settings, given the largely "self" constituents of tumors, we reasoned that autoimmune T cells, especially the three main T helper subsets, Th1, Th2, and Th17, could be a major source of these cytokines, or play a major role in driving other immune cells to produce them.

Early experimental models offered hope that the induction of selective antitumor immune responses, even those mediated by CTL recognition of self-antigens, was possible without clinically significant autoimmunity. One study reported the eradication of established p53-overexpressing tumor cells in mice, achieved through adoptive transfer of a clone of cytotoxic T cells, which recognized wild-type p53. No autoimmune damage was observed in normal tissues, despite the p53+/+ genotype of the treated mice (73). *In vitro* assays also suggested that epitope-specific CTL clones had the capacity to initiate cytotoxic immune responses against non-mutated, tumor-associated p53, while simultaneously avoiding reactivity with the same antigen when endogenously expressed (74). Other experiments used transgenic models to search for evidence of autoimmune pathology in an organ-specific fashion. In one study, transgenic mice were engineered to express low levels of Friend murine leukemia virus envelope protein (FMuLV) from an immunoglobulin promoter; adoptive transfer of FMuLV-specific T cells mediated complete destruction of leukemia tumor cells, without concurrent autoimmunity. Lymphoid tissues in the treated mice were unharmed, despite the fact that they expressed the targeted cancer-associated antigen in a "self" context (75). Another study employed transgenic mice with tissue-specific expression of influenza virus hemagglutinin (HA) in pancreatic islet β cells. Administration of an anti-HA vaccine to these animals produced CTL-mediated rejection of renal carcinoma cells, which had also been genetically engineered to express HA. Prior to vaccination, the immune systems of the transgenic mice exhibited HA self-tolerance, and supported growth of implanted tumors expressing this antigen. Remarkably, in addition to tumor rejection, immunohistologic analysis of treated mice showed intact structure and function in the pancreatic islets (76).

Despite these favorable preclinical results, most types of cancer immunotherapy tested in human patients have revealed serious and persistent risks of treatment-associated autoimmunity. Reported manifestations include vitiligo, uveitis, psoriasis, and colitis, with potential consequences ranging from cosmetic complaints to permanent disability and death (77). However, it has been suggested that autoimmunity, in addition to mediating these adverse events, makes an essential contribution to antitumor immunity. Indeed, it may be the case that effective cancer immunotherapy requires a significant degree of self-reactivity, since antitumor immune responses must surmount both the pre-existing tolerance to self-derived tumor antigens and immunosuppressive signals from the tumor microenvironment (1, 77). For some cancer immunotherapies, such as blockade of the inhibitory T cell signaling molecule CTLA4, autoimmune toxicity shows a positive correlation with therapeutic response (78). Meanwhile, antitumor vaccines, probably the category of cancer immunotherapy least associated with severe autoimmunity in human studies (77), have generally failed to show therapeutic efficacy in large-scale Phase 3 trials (79). Thus, it appears that the future of cancer immunotherapy will not feature the elimination of autoimmunity, which is unlikely to be feasible or wholly desirable. Rather, the cytokines that mediate autoinflammation can be harnessed as effective agents in tumor destruction, if their toxic and tumor-promoting potentials are understood and judiciously managed.

IFN-γ

Interferons (IFNs) are a family of cytokines that bind to cell-surface receptors and mediate numerous functions related to pathogen defenses, immune function, cell survival and differentiation, and angiogenesis (12, 80). Type I interferons are broadly expressed (80), while IFN-γ, the sole member of the Type II interferon family, is produced mainly by T lymphocytes, NKT cells, and natural killer (NK) cells as a crucial component of the inflammatory response (10). IFN-γ is integral to the development of Th1 adaptive immunity, by maintaining IL-12 signaling in $CD4^+$ T lymphocytes while simultaneously suppressing Th2 differentiation (10). Murine models support a range of anti-carcinogenic properties for IFN-γ (11). Antibody-mediated suppression of IFN-γ signaling has revealed that this cytokine is required for LPS-stimulated rejection of transplantable tumors in mice. IFN-γ receptor knockout mice (IFN-γ$R1^{-/-}$) exhibit susceptibility to both spontaneous and chemically induced tumors, in addition to poor resistance to a variety of pathogens (10, 11). IFN-γ is a powerful inducer of Class I and Class II antigen presentation machinery, suggesting its primary importance in the production of specific antitumor immune responses (12). *In vitro* assays have also revealed pro-apoptotic, antiangiogenic, and antiproliferative effects (10–12, 81). Indeed, reduced IFN-γ production has been observed in a variety of human malignancies, including melanoma, gastric cancer, lung cancer, glioblastoma, nasopharyngeal carcinoma, colorectal cancer, and head and neck cancer (72).

Certain human cancers, notably melanoma, are known to develop resistance to IFN-γ through various mutations in downstream molecules in the IFN signal transduction pathway, such as JAK and STAT1 (12, 81).

Despite this promising body of experimental evidence, anti-cancer therapy with exogenous IFN-γ has generated mixed results. A 2003 clinical trial in malignant melanoma patients showed no positive responses to intratumoral injections of IFN-γ (82). A Phase 3 trial for ovarian cancer and peritoneal carcinoma was prematurely ended in 2006, due to decreased survival and more frequent adverse events in patients treated with subcutaneous IFN-γ, as compared to conventional platinum-based chemotherapy (82). To understand why this cytokine has not demonstrated the broad therapeutic efficacy that prior data would suggest, it is necessary to examine the dual nature of the inflammatory process. While inflammation can promote the cell-mediated destruction of tumor cells, its chronic forms may lead to pathological changes that promote cancer development in a variety of tissues. These changes include accumulation of reactive oxygen species, epithelial hyperplasia, extracellular matrix generation, and angiogenesis (3, 83, 84). Besides mediating anticancer immunity, IFN-γ is a major player in chronic inflammation, as illustrated by its contributions to Th1-driven autoimmune disease. In clinical trials, anti-IFN-γ antibodies have been useful in treating a variety of inflammatory disorders, including rheumatoid arthritis, Crohn's disease, corneal transplant rejection, and skin diseases such as vitiligo and alopecia areata (82, 85, 86).

The interplay between IFN-γ signaling, aberrant chronic inflammation, and neoplastic disease has been explored in the pathogenesis of gastric cancer, one of the classic models of human malignancy precipitated by chronic inflammation (87–90). With regard to gastric carcinogenesis, the role of Th subsets would also perhaps be best surveyed while keeping in mind the robust evidence of innate immunity in gastric cancer development (91–93). In the natural course of this disease, infection with the bacterium *Helicobacter pylori* during childhood precipitates a chronic inflammatory response which, in a small portion of patients (<5%), progresses to malignancy decades in the future (84, 94). Studies of mice infected with the related pathogen *Helicobacter felis* showed that a Th1-biased inflammatory response is involved in gastric cancer development (94). Knockout of the transcription factor T-bet, which controls commitment to the Th1 lineage (95), curtailed gastric tumorigenesis induced in mice infected with *H. felis* (15). A recent experiment employed a murine model that directly implicates IFN-γ in this course of preneoplastic change. The transgenic mice, which were engineered to overexpress IFN-γ from a stomach-specific, H/K ATPase β promoter, exhibited a prominent inflammatory infiltrate along with accelerated histological changes characteristic of a premalignant phenotype: metaplasia, loss of parietal and chief cells, gastric gland atrophy, and dysplasia beginning as early as 3 months of age (16).

IFN-γ can thus be seen as an essential mediator of both immune-mediated tumor rejection and the destructive chronic inflammation that precedes malignant transformation. There is, however, another vital dimension to this cytokine's effects, that of a master regulator which restrains inflammation in a variety of contexts. In two key murine model systems of Th1-driven autoimmunity, collagen-induced arthritis (CIA) and experimental autoimmune encephalitis (EAE), it has been established that abrogation of IFN-γ signaling through monoclonal antibody therapy or genetic knockout produces a seemingly paradoxical increase in disease susceptibility and severity (13, 14). The mechanisms for this apparent downregulation of the inflammatory response are not yet clear, but suppression of pro-inflammatory Th17 cytokines, induction of T cell apoptosis, and enhanced Treg cell differentiation appear to play prominent roles (13, 14, 96). Another mechanism of IFN-γ mediated immunosuppression is suggested by its ability to alter tumor cell MHC presentation in a manner that *decreases* tumor antigenicity and protects tumor cells from CTL killing. One *in vitro* experiment demonstrated that IFN-γ could protect ovarian carcinoma cell lines from CTL-mediated lysis by upregulating HLA-E on cancer cells, which engaged the inhibitory CD94/NKG2A receptor on $CD8^+$ effector T cells (18). A similar cancer-promoting role for IFN-γ was revealed in a mouse model of melanoma, where tumor protection from CTL killing was associated with IFN-γ-stimulated upregulation of non-cognate MHC-I molecules (17).

Given the evidence above, IFN-γ-mediated downregulation of the inflammatory response can be regarded as an obstacle to effective cancer immunotherapy. However, it remains an important physiological mechanism for preserving tolerance to self-antigens and protecting tissues from the damaging effects of autoimmunity (80, 97). In keeping with this fact, experimental data also suggest that IFN-γ mediated suppression of inflammation may be protective in certain contexts. One recent experiment employed a transgenic mouse line engineered to overexpress IFN-γ in a stomach-specific manner, using the same H/K ATPase β promoter as Syu et al. (16). In this case, however, no spontaneous gastritis or metaplasia occurred (98). Moreover, IFN-γ was reported to be protective against gastric dysplasia produced by either *H. felis* infection or stomach-specific overexpression of the cytokine IL-1β (a well-established mediator of gastric carcinogenesis). IFN-γ was also shown to inhibit proliferation of gastric epithelial cells, enhance autophagy in a manner recognized as protective against tumor development (99), and diminish expression of pro-inflammatory Th1 and Th17 cytokines (98). As noted by Syu et al. (16), the seemingly contradictory results of these two studies can most likely be explained by differing degrees of overexpression. These findings underscore the challenges of discerning which aspect of IFN-γ will prevail in a particular patient, tumor type, and stage of malignancy. Attempts to broaden the therapeutic use of this cytokine must take into account these intricate and context-dependent multidirectional effects.

TNF-α

Activated macrophages and T lymphocytes produce TNF-α in response to pro-inflammatory stimuli. This cytokine stimulates inflammation through multiple mechanisms, including recruitment of neutrophils and monocytes and induction of cell adhesion molecule expression on the endothelial surface (72, 100). TNF-α is involved in the classical pathway of macrophage activation (M1), which plays a central role in immune defenses against tumors and intracellular parasites (31). Although usually undetectable in the tissues and circulation of healthy people, TNF-α exerts

important effects on immune homeostasis during states of disease. It is known, for instance, that systemic TNF-α mediates the wasting observed in humans and animals afflicted with chronic illness (24, 100, 101). As with IFN-γ, aberrant TNF-α signaling is associated with a variety of autoimmune disorders. Five TNF inhibitors have been approved for clinical use in the United States for the treatment of inflammatory illnesses such as Crohn's disease, ankylosing spondylitis, and rheumatoid arthritis (100, 102).

The observation that the human body's response to infection is capable of producing regression of tumors has been recorded by clinicians since at least the eighteenth century (103). It was not until 1975, however, that TNF-α was identified as one of the principal mediators of this anticancer immune response. The name "tumor necrosis factor" reflects early experiments, which demonstrated this cytokine's capacity to induce hemorrhagic necrosis of subcutaneously transplanted sarcomas, leukemias, and mastocytomas in mice whose immune systems were primed by exposure to bacterial endotoxin (19). However, 38 years of data subsequent to this discovery have not fulfilled the early promise of TNF-α as a safe, potent, or selective tumoricidal agent. Unlike mice with abrogated IFN-γ signaling, TNF-α-knockout animals do not develop spontaneous tumors, and peritoneal tumors transplanted into them do not show accelerated growth (104). These knockout animals have also shown an unexpected resistance to chemically induced skin tumors (105). Indeed, there seem to be numerous contexts in which TNF-α signaling helps to initiate carcinogenesis and sustain tumor growth. In obese patients, TNF-α is suggested to be a key mediator of cancer-promoting inflammation in various organs, including the pancreas (22), colon (21), and liver (23). Picogram amounts of TNF-α are constitutively secreted by many tumor types, and appear to stimulate cancer growth, although the underlying signaling mechanisms are not completely understood (24, 106). The pro-carcinogenic functions of TNF-α appear to be mediated predominantly through downstream activation of the proliferative and survival-promoting pathways NF-κB and AP-1 (20, 21, 100, 104). Elevated serum concentrations of TNF-α have been described as a clinical feature of eight independent cancer types (72). A direct link between the pro-inflammatory effects of TNF-α and carcinogenesis can be seen by returning to the example of gastric cancer. The presence of a specific, inflammation-associated single nucleotide polymorphism in the *TNF-A* gene has been found to increase the odds ratio of non-cardia gastric cancer (83). The importance of this association has been further established by examining the genome of the initiating pathogen: *H. pylori* harbors the Tipα gene family, whose members are known to induce high levels of TNF-α expression in the gastric epithelium (24, 106). Experimental transfection of a transformed BALB/3T3 cell line with *H. pylori* genes revealed that tumor progression was dependent on Tipα-induced production of TNF-α (106).

TNF-α has also been implicated as a disease promoter in hepatocellular carcinoma (HCC), from chronic inflammation to tumor progression including invasion, metastasis, and angiogenesis in established HCC tumors (107). *In vitro* studies of numerous cell types, including murine hepatocytes, have demonstrated that TNF-α-mediated inflammation leads to an increase in markers of oxidative stress, which in turn can lead to genomic damage (108–111). Moreover, the development of obesity-induced hepatosteatosis and steatohepatitis in mice is dependent on signaling by TNF-α, along with IL-6 (21). In the Mdr2-knockout mouse, an experimental model of inflammation-induced HCC, the spontaneous development of hepatic malignancy is dependent on TNF signaling, and can be attenuated through downstream inhibition of NF-κB (23). Overall, experiments on HCC and many other tumor types have established that TNF-α is intimately involved in all aspects of cancer development, including transformation, proliferation, angiogenesis, invasion, and metastasis (25).

Owing to a high frequency of inflammation-related adverse events, the therapeutic applications of exogenous TNF-α in cancer have been quite limited (24). Localized TNF-α infusion has demonstrated antitumor efficacy against melanomas and soft tissue sarcomas in human patients. In this context, high concentrations of TNF-α were shown to induce hyperpermeability and structural disruption in tumor vasculature, thus promoting tumor necrosis and enhancing the efficacy of traditional cytostatic drugs. However, this intervention was aimed at limb sparing and had no impact on patient survival (20). Given the broad range of cancer-promoting activities of TNF-α, blockade of its signaling remains a tempting therapeutic approach. Unfortunately, the fundamental role of TNF-α in pathogen defense represents a formidable obstacle to implementation (24). The feasibility of modifying TNF-α signaling without triggering destructive autoimmunity on one hand, or vulnerability to opportunistic infections on the other, remains to be seen.

TGF-β

Transforming growth factor-beta mediates a vast array of functions related to wound healing, immune responses, cell proliferation and differentiation, and carcinogenesis, via receptors expressed on nearly all human cells. It plays a crucial role in T cell tolerance (112). Its classical signal transducers are transcription factors known as Smads, which combine with each other as well as additional cofactors to form a variety of DNA-binding complexes. These intricate assemblies of transcriptional regulators allow TGF-β to implement a versatile, yet precisely controlled, range of downstream effects. Additional "Smad-independent" signaling pathways are known to further augment this functional repertoire (27, 29, 34, 35). This cytokine has been extensively characterized as a negative regulator of immune responses, with anti-inflammatory, antiproliferative, and immunosuppressive activities (27, 29, 34, 35). Thus, TGF-β helps restrain the destructive effects of uncontrolled inflammation and proliferation that might otherwise occur in the context of infection or tissue damage. Mouse models reveal that knockout of TGF-β or its receptor produces a phenotype characterized by lethal autoimmune disease (113, 114). One recent study featured a murine model with the TGF-β receptor gene deleted in stromal fibroblasts. The transgenic animals experienced an excessive, aberrant inflammatory response in adjacent epithelial tissue, characterized by molecular markers of DNA damage, oxidative stress, cell cycle dysregulation, and death from invasive squamous cell carcinoma by the age of 7 weeks (26). Other models have examined the specific effects of TGF-β silencing in the innate immune and T cell compartments. One experiment blockaded TGF-β signaling in mouse NK cells through transgenic expression of a dominant negative receptor. NK cells

were more numerous in lymphatic tissues of the transgenic mice, and showed enhanced secretion of IFN-γ. Consistent with the Th1-promoting effects of IFN-γ signaling, transgenic mice also demonstrated an enhanced Th1 inflammatory response, which protected against infection with cutaneous Leishmaniasis (28). These effects suggest the importance of TGF-β in maintaining NK cell homeostasis, as well as downregulating Th1 differentiation. Another study reported a dendritic cell-specific deletion of the TGF-β receptor gene, which resulted in multiorgan autoimmunity, a pro-inflammatory DC phenotype characterized by IFN-γ overproduction, and reduced Foxp3 expression in Treg cells (30). Accumulating evidence suggests that TGF-β is directly involved in many aspects of T cell homeostasis, including differentiation of Treg cells and $CD8^+$ effectors, maintenance of peripheral tolerance, and preservation of naïve T cell populations (112). The precise molecular mechanisms behind these effects are largely unresolved, and represent an area of active investigation.

In the early stages of carcinogenesis, TGF-β is known to suppress tumor growth through induction of cell cycle inhibitors and promotion of apoptosis. However, in many advanced cancers, paracrine and autocrine TGF-β signaling drives tumor progression and metastasis. TGF-β is produced in large quantities by a variety of human cancers, to the extent that it is arguably the most ubiquitous immunosuppressive mediator in cancer progression (34). Elevated systemic levels of TGF-β have been reported in breast cancer, lung cancer, pancreatic cancer, glioblastoma multiforme, colorectal carcinoma, HCC, renal cell carcinoma, and gastric carcinoma (72). TGF-β is a crucial inducer of pro-tumor phenotypes in both tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs), leading to cancer cell proliferation and the curtailing of antitumor immune responses (31). The capacity of TGF-β to promote the differentiation of Treg cells appears to be highly deleterious in this context, since Treg cells are key inducers of immune tolerance in the tumor microenvironment. The association between TGF-β signaling and Treg cell recruitment has been experimentally demonstrated in a lung cancer cell line (115), as well as in animal models of pancreatic cancer (32) and HCC (33). One key observation from these studies is that a tolerogenic immune microenvironment is not exclusively the result of tumor-secreted cytokines. Rather, it requires elaboration of TGF-β and other anti-inflammatory signals from immune cell populations such as dendritic cells and TAMs.

Several other mechanisms are believed to underlie the pro-carcinogenic role of TGF-β, including enhanced extracellular matrix formation, cytoskeletal rearrangements to facilitate epithelial-to-mesenchymal transition (EMT), angiogenesis, and cell cycle dysregulation (27, 29, 34, 35). In a clinical context, TGF-β serves as a marker of metastasis and poor prognosis for many malignancies (35, 72). The transition of TGF-β from a protective role early in tumor development to a tumor-promoting one in more advanced disease appears to be a watershed moment in many cancers, reflecting global derangement of signal transduction through genetic and epigenetic mechanisms. Indeed, *in vitro* experiments have linked specific forms of oncogenic transformation to alterations in TGF-β responsiveness. One study found that engineered overexpression of HER2 in mesenchymal human breast cancer cells caused a loss of sensitivity to the antiproliferative effects of TGF-β (116). Another reported that loss of TGF-β growth inhibition correlated with the loss of c-myc downregulation in ovarian carcinoma cells (117).

The prevalence of TGF-β overexpression across a broad range of human malignancies has made this cytokine a tantalizing therapeutic target. Four classes of TGF-β inhibiting molecules have already been tested in clinical trials, with responses that generally fell short of hopes (118). At least one class of TGF-β inhibitor has also shown the capacity to elicit biochemical resistance in mouse models (119). This observation, coupled with the integral roles of TGF-β in wound healing and tissue homeostasis, suggests that long-term inhibition of TGF-β signaling may be a dangerous prospect. Rather, it has been suggested that TGF-β inhibition will find its first clinical applications as part of a combined drug regimen, administered to cancer patients over relatively brief spans of time to minimize resistance and adverse events (118).

IL-17 AND IL-23

The Th17 subset of $CD4^+$ T cells has added a new dimension of complexity to the Th1/Th2 paradigm since its initial discovery in 2005. Extensive studies in murine models have implicated this T cell population in a number of pro-inflammatory functions, including the pathogenesis of autoimmune diseases of the brain (EAE) and joints (CIA), mediated by the characteristic cytokine IL-17 (36, 120–122). Knockout mice have also revealed an important role for IL-17 signaling in the defense of mucosal surfaces against a variety of bacterial and fungal pathogens, including *Staphylococcus aureus, Klebsiella pneumoniae*, *Citrobacter rodentium*, and *Candida albicans* (36, 120). Th17 cells also engage in physiologically important interactions with key cytokines of the Th1 immune system. One attribute, noted in the initial characterization of this helper T cell subset, is the inhibitory effect of IFN-γ on Th17 differentiation and production of IL-17 (123, 124). Meanwhile, IL-23, a cytokine that is essential to sustain the survival and proliferation of Th17 populations, appears to participate in divergent regulatory pathways with the Th1-associated cytokine IL-12, whose release is promoted by IFN-γ (125–127). The dichotomous signaling of IL-12/IL-23 is especially intriguing, as these two cytokines share a common subunit: IL-12 is formed through the covalent linkage of p40 and p35 subunits, whereas IL-23 is a combination of p40 and p19 (128). TGF-β signaling also appears to make a major contribution to Th17 differentiation, although the precise nature and mechanism of this association remains a subject of intense controversy (122, 129).

Th17 signaling through the canonical IL-23/IL-17 pathway has been shown to contribute to cancer development in a variety of experimental contexts. During the preneoplastic stage, excessive Th17-mediated signaling is a probable contributor to the chronic inflammation that can precipitate cancer in a variety of tissues. In the case of Hepatitis B-induced inflammation, evidence from human and animal experiments suggests that Th17, not Th1, signaling is the primary mechanism underlying liver immunopathology and eventual malignant transformation to HCC (43–46). Th17 cells have been discovered in human tumors in many different organs, and a growing body of evidence suggests that their presence, like that of immunosuppressive Treg cells, may be a general feature of the tumor microenvironment (37, 121). One

comprehensive examination of gene expression profiles in human cancers revealed overexpression of IL-23 in the vast majority of human carcinomas, when compared to expression profiles of adjacent, non-cancerous tissue (130). A recent quantitative PCR analysis revealed statistically significant IL-23 upregulation across a panel of non-small cell lung carcinoma (NSCLC) patient samples, in comparison to matched normal tissue controls (49). In mouse models, knockout of IL-23 conferred resistance to both chemically induced and transplanted epithelial tumors, whereas knockout of IL-12 produced the opposite effects (130). Moreover, there is mounting evidence that tumor cells produce chemokines in order to selectively recruit Th17 lymphocytes (121). In the 4T1 mouse model of breast cancer, Qian et al. reported that signaling via mammary tumor-derived prostaglandin E_2 (PGE_2) caused overexpression of IL-23 (but not IL-12) in the tumor microenvironment (48). This overexpression, in turn, was associated with expansion of Th17 cell populations in the tumor tissue, spleens, and peripheral blood of experimental animals (48). This discovery is likely to have significant implications in a variety of human cancers, since PGE_2 is the most abundant prostanoid in epithelial cell tumors (127). In the same study, exposure of murine dendritic cells to tumor-conditioned medium enhanced their expression of the IL-23 subunit p19, and also reduced expression of p40 and the IL-12 subunit p35, in a dose-dependent manner (48). These changes collectively indicate a shift from Th1 to Th17 immune phenotype, which seems to be favorable for cancer persistence. As in the case of experimental models of TGF-β signaling, cells of the innate immune system were key participants in the generation of an immune microenvironment promoting carcinogenesis.

The pathways through which Th17 signaling exerts its tumor-promoting effects are not yet clear, but a variety of relevant observations have emerged. One probable mechanism is the stimulation of angiogenesis and cell proliferation: the p19 subunit of IL-23 is transcriptionally upregulated by growth-promoting signals from NF-κB and AP-1 (48). Administration of recombinant IL-23 has been shown to enhance proliferation in an IL-23 receptor-positive lung adenocarcinoma cell line (49, 51). One study reported IL-23-mediated enhancement of cancer growth and proliferation in cultures of human oral squamous cell carcinoma; in this case, IL-23 exposure was accompanied by enhanced nuclear translocation of NF-κB (50). Mouse models have also furnished valuable mechanistic clues. In one experiment, IL-17 knockout mice showed reduced growth of transplanted B16 melanoma and MB49 bladder carcinoma, whereas acceleration of tumor growth occurred with knockout of IFN-γ. In mice with both knockouts, tumor growth was reduced relative to WT controls (65). In the IFN-γ knockout mice, elevated concentrations of IL-17 were measured in tumor tissue compared to tumors in wild-type controls. Moreover, IL-17 was found to enhance signaling by the pro-survival, pro-angiogenic transcription factor Stat3 in both tumor and stromal cells (65). Another study reported similar results using receptor knockouts: IL-17 receptor-deficient mice showed diminished tumor growth of transplanted melanoma and lymphoma cell lines, while IFN-γ receptor knockout led to growth enhancement. The double-knockout genotype was also protective in comparison to WT controls (47). In addition, IL-17 receptor deficiency was correlated with increased tumor infiltration of $CD8^+$ effector T cells, and decreased numbers of myeloid-derived suppressor cells (MDSCs), both favorable indications for the generation of antitumor immunity (47). Interestingly, these pro- and antitumor effects were successfully reproduced in wild-type mice through administration of either recombinant IL-17 (which accelerated tumor growth) or antibody-mediated IL-17 blockade (which suppressed it), hinting at the viability of anticancer therapies targeting this pathway (47). It is also notable that elimination of IL-17 signaling in both studies produced an antitumor effect that was potent enough to compensate for the concurrent loss of IFN-γ signaling, despite the role of IFN-γ as a potent mediator of Th1-driven antitumor immunity.

The complex and heterogeneous functions of the Th17 signaling axis, and their relationships to cancer progression, are only beginning to be elucidated. Despite the results mentioned above, numerous studies support the existence of antitumor Th17 effects, including those which may be mediated through signals other than IL-17 and IL-23 (121). For instance, subsets of Th17 cells are capable of producing IFN-γ, indicating possible cross-talk with the Th1 pathway of differentiation, as well as the ability to stimulate cytotoxic and tumoricidal immune responses (36, 37). In human pancreatic ductal adenocarcinomas, one recent study found correlation of protective benefit with activity of Th17 cells specific to α-enolase, a pancreatic tumor-associated antigen (42). Another recent study reported that a mouse model deficient in RORγt (a transcription factor required for Th17 differentiation) exhibited accelerated growth of transplanted melanoma tumors, along with a diminished percentage of Th1 $CD4^+$ cells at the tumor site; this phenotype was rescued by adoptive transfer of Th17 cells, a portion of which began to produce measurable quantities of IFN-γ (41). One study of IL-17 knockout mice demonstrated increased susceptibility to lung melanoma. Subsequent treatment by Th17 adoptive transfer served to prevent tumor development by inducing a specific $CD8^+$ antitumor response (40). It has also been found that systemic administration of high-dose IL-23 led to reduced tumor growth and prolonged survival in a mouse fibrosarcoma, due to Th1-mediated activation of cytotoxic T cells, helper T cells, and NK cells (38). Increased growth and lung metastasis of murine colon carcinoma has also been reported in IL-17 deficient animals, with corresponding reductions in IFN-γ^+ NK cells and IFN-γ^+ tumor-specific T cells (39).

Although characterized less than a decade ago, the Th17 lymphocyte population has already become the focus of a vast and diverse body of scientific literature. However, this expanded knowledge contains apparent contradictions, which will challenge the field of cancer immunology for years to come. As with the pathways and cytokines previously discussed, the tumor-related effects of IL-17 and IL-23 exhibit a high degree of context dependence. Some of these discordant results may therefore be attributable to the source of tumor cells, the tissue involved, the stage of cancer growth, the genetic background of the organism, and other features of the experimental model employed in a particular study. Meanwhile, our understanding of Th17 interactions with other elements of the immune system, including NK cells, antigen-presenting cells and other helper T cell subsets, remains incomplete. Experiments designed to address the cross-talk between Th17 cytokines and other branches of the immune system should

help resolve some of the inconsistencies in their reported effects. While a variety of IL-17/IL-23 antagonists are currently being developed for the treatment of autoimmune diseases (131), safe and effective modification of Th17 signaling in cancer therapy will require a more thorough understanding of the forces which underlie Th17 differentiation, recruitment, and interaction with malignant cells.

IL-4 AND IL-13

The Th2 subset of $CD4^+$ T cells plays an important physiological role in implementing immune defenses against helminths and other extracellular parasites. Th2-mediated responses include generation of high-affinity IgE antibodies, mucus overproduction, and heightened smooth muscle contractility, all of which function in the clearance of invasive multicellular organisms. However, aberrant and excessive Th2 activation also provides the foundations for allergic disease (132). The major cytokine responsible for differentiation of naïve $CD4^+$ T cells into the Th2 phenotype is IL-4, while Th2 effector functions are mediated through a combination of IL-4, IL-13, and IL-5 (133). All three of these cytokines exert varied effects on cancer development, which remain an area of ongoing investigation. Th2 cytokines appear to be involved in shifting the immune response to forms favorable to tumor growth, particularly in the context of innate immunity. IL-4, IL-13, and IL-5 promote the differentiation of macrophages into an "M2" or alternatively activated form, which displays poor antigen-presenting capacity and local anti-inflammatory effects (31, 83, 134). M2 macrophages play a variety of physiological roles in tissue homeostasis, including wound healing, extracellular matrix remodeling, and scavenging of debris (132, 134). This M2 phenotype contrasts with the classically activated (M1) macrophage, which is specialized for the production of pro-inflammatory cytokines (e.g., IFN-γ), cytotoxic immune responses, and efficient destruction of phagocytosed microbes (132, 134). In the context of cancer, M2 polarization of TAMs is associated with suppression of antitumor immune responses, disease progression, and poor prognosis (31, 83). Moreover, it has been established that tumors are capable of producing Th2 cytokines in order to bias innate and adaptive immune responses toward this more favorable phenotype (134). A study of pancreatic cancer patients demonstrated that tumor-produced cytokines (TNF and IL-1β) triggered activation of a Th2 phenotype in cancer-associated fibroblasts, dendritic cells, and naïve $CD4^+$ T cells. Moreover, the ratio of Th2:Th1 $CD4^+$ T lymphocytes present at the tumor site was negatively correlated with patient survival (52). In a humanized mouse model implanted with human breast carcinoma, Th2 cytokine expression was detected in both cancer cells and tumor-promoting $CD4^+$ T cells within the tumor microenvironment. Dendritic cells isolated from these tumors also potently induced Th2 cytokine secretion from naïve $CD4^+$ T cells *in vitro*, suggesting that tumor growth is facilitated by a complex network of Th2 paracrine signals (53).

The Th2 cytokine IL-4 is capable of signaling through two distinct cell-surface receptor complexes. The Type I receptor, found on cells of hematopoietic stem cell origin, is composed of IL-4Rα and the common gamma chain γc. The Type II receptor, expressed on cells of non-hematopoietic origin, contains IL-4Rα and IL-13Rα1, and also binds the cytokine IL-13 (135). IL-13Rα2 is a second type of IL-13 binding receptor, whose physiological role remains uncertain. The receptor bears structural similarities to IL-13Rα1, but is expressed in two forms: as soluble IL-13Rα2, and as a transmembrane protein, which interacts with a number of signal transduction pathways (54). IL-4 and IL-13 exert both overlapping and distinct physiological effects by binding these receptors, whose structure and function have been extensively studied as potential therapeutic targets in asthma and other allergic diseases (135, 136). Emerging evidence indicates that these receptors can influence cancer development through pathways other than macrophage polarization, although the molecular details of this process are only starting to become clear.

The Type II IL-4/IL-13 receptor has been found to be overexpressed in a variety of epithelial tumors, and treatment of cancer cell lines with IL-4 is associated with pro-proliferative and anti-apoptotic effects (54, 137). The effects of IL-4 signaling have been studied extensively in the development of colon cancer: one study found pro-proliferative effects of IL-4Rα signaling in mouse colon tumors, as well as human and mouse colon adenocarcinoma cell lines examined *in vitro* (55). Of particular interest is the relationship between intestinal malignancies and multipotent stem cells, which have been identified in recent experiments as an integral driving force in the growth of both premalignant adenomas and established tumors (138, 139). It now appears that IL-4 signaling is vital to the functioning of at least some of these tumorigenic stem cells. Research on colon cancer has identified a subset of tumor cells with a $CD133^+$ stem-like phenotype, which was found to be necessary and sufficient for the establishment of transplanted human colon tumors in immunodeficient mice (56). In keeping with the cancer stem cell (CSC) hypothesis (4), these cells possess self-renewing capacity, an especially high resistance to death-promoting signals, and the ability to effect regeneration of the overall tumor mass (56, 140). One study revealed that resistance to drug-induced apoptosis in $CD133^+$ colon cancer cells was mediated through increased production of IL-4 (56). Preliminary experiments suggest that this IL-4-mediated, pro-survival pathway may be a promising therapeutic target. In the same study, blockade with either IL-4 neutralizing antibody or a mutant, inhibitory form of IL-4 (IL-4DM) reduced the viability of $CD133^+$ and $CD133^-$ tumor cell cultures, while increasing the efficacy of cytotoxic treatment with standard chemotherapeutic agents: oxaliplatin, 5-fluorouracil, and the death receptor ligand TRAIL. IL-4 antagonism also enhanced the effectiveness and duration of chemotherapy response in mice bearing transplanted tumors, suggesting a role for combined therapy in the treatment of human disease (56). Both the *in vitro* and *in vivo* effects of IL-4 blockade were mediated by decreases in the anti-apoptotic molecules cFlip, Bcl-xL, and Ped (56, 140).

Another remarkable finding has been the role of IL-13Rα2 in models of cancer development, indicating signaling functions far beyond the previously suggested role of a decoy receptor (141). This receptor is known to be overexpressed in several human cancers. An immunohistochemical analysis of human tissues found IL-13Rα2 overexpression in 71% of pancreatic ductal adenocarcinoma samples in comparison to normal pancreas controls (142). Indeed, experiments in an orthotopic mouse model of pancreatic cancer suggest that IL-13Rα2 is an important mediator

of the pro-tumor effects of IL-13, including activation of AP-1 growth signals, production of immunomodulatory cytokines such as TGF-β, and promotion of metastasis (58, 142). Similar IL-13Rα2-dependent effects have been demonstrated in other cancer models, including ovarian carcinoma (59), colorectal cancer (57), head and neck squamous cell carcinoma (143), and malignant glioma (144). As with IL-4, IL-13 signaling has been suggested as a possible target of anticancer therapy. To this end, recombinant cytotoxic proteins have been developed, which consisted of IL-4 or IL-13 joined to a mutant form of *Pseudomonas* exotoxin. These agents have been found to restrain tumor growth in numerous animal models (142–144). However, in a Phase 3 clinical trial, IL-13 *Pseudomonas* exotoxin failed to improve median survival time in patients with glioblastoma multiforme when compared to conventional chemotherapy (145). While this agent may still find use as an adjuvant therapy (146), the outcome suggests that the development of Th2-targeted treatments with robust antitumor efficacy will require further exploration.

Despite considerable progress in the field, many Th2-mediated influences on tumor development remain poorly characterized. For instance, populations of eosinophils and mast cells, crucial mediators of Th2-driven allergic responses, also contribute to the inflammatory infiltrate in numerous human tumors. However, experimental data exploring their effects are lacking, and conflicting results have been published regarding their impact on clinical prognosis (83). This ambiguity underscores the fearsomely complex, and incompletely understood, nature of signaling among discrete immune system components in the context of cancer development. Owing to these knowledge gaps, the possibility that an anti-Th2 intervention could impede antitumor immune responses *in vivo* cannot be prematurely dismissed. Systemic therapies to antagonize Type I or Type II receptors will also confront a high risk of adverse events, owing to the global effects of Th2 cytokines on immune homeostasis and other physiological functions. Nevertheless, a fascinating body of experimental and clinical data suggests that the pro-carcinogenic effects of IL-4 and IL-13 will remain a source of therapeutic interest for years to come.

POTENTIAL FEEDBACK BETWEEN AUTOINFLAMMATION AND TUMOR: IMPLICATIONS FOR CANCER IMMUNOTHERAPY

Virtually all of the immune signaling pathways relevant to tumor biology play major physiological roles in the maintenance of self-tolerance and tissue homeostasis. Moreover, most well-characterized tumor-associated antigens are self-antigens, meaning that they are also expressed by normal cells in the course of growth and differentiation. Conversely, antigenic proteins expressed exclusively in cancerous tissue, such as viral products or mutated oncogenes, have been characterized in only a small number of tumor types. Overall, it seems that tumors, despite their aberrant characteristics, may remain antigenically "self" entities first and foremost (1, 2). In the clinical context, this suggests that useful antitumor immune responses elicited in patients may be functionally inseparable from those directed against healthy tissues. The seemingly inextricable association between off-target autoimmune damage to healthy tissues and antitumor immunity induced by effective cancer immunotherapy during recent clinical trials can be seen as supporting this hypothesis. Evidence from molecular studies in this regard and a large body of research on inflammation and cancer suggest a feedback loop of autoimmunity, antitumor immunity, inflammation, and *de novo* tumorigenesis that reinforces the remarkable entanglement between autoimmunity and cancer (**Figure 1**). In particular, the persistence of antigens in most autoimmune conditions likely leads to the formation of a type of autoimmune memory cells called effector memory T (T_{EM}) cells [for review see Ref. (66)], which could further perpetuate this feedback loop. Advances in not only experimental and clinical research, but also in computational biology tools for large datasets, will be needed to understand the complexity of molecular and cellular interactions in such chronic human disease settings (66, 147).

Along this line, one may argue that a combination of immunomodulation with conventional cancer therapies such as chemotherapy could be used to augment tumor-specific immune responses. Despite the severe immunosuppression produced by many standard chemotherapeutic agents, current data suggest that patients with chemotherapy-induced leukopenia retain a functional T cell compartment that is capable of mediating clinically significant antitumor immunity (148). Within solid tumors, it has been demonstrated that chemotherapy can deplete immune suppressor cells (MDSCs and Treg cells) in the tumor microenvironment, increase tumor antigenicity, and upregulate the expression of costimulatory molecules for CTL activation (79). All of these mechanisms point toward possible synergistic effects that could enhance the clinical efficacy of existing cancer immunotherapies, including agents that have shown meager benefits when administered alone. Indeed, synergy between cancer vaccines and chemotherapy has already been demonstrated in studies of advanced small-cell lung cancer and follicular B-cell lymphoma (149).

Immunological research continues to produce crucial mechanistic insights into the tumor-related effects of major cytokines. For instance, a recent murine experimental model has demonstrated that IFN-γ and TNF-α produced by Th1 cells are capable of inducing prolonged senescence in pancreatic tumors, by inducing expression of the transcription factors JUNB and INK4A (150). The identification of specific signaling pathways for tumor cell growth inhibition and apoptosis heralds a new and improved generation of cytokine-based therapies. More broadly, this knowledge may eventually enable a combinatorial approach to cancer immunotherapy, in which multiple treatments can be jointly administered to yield superior therapeutic outcomes. The current field of cancer immunotherapy is divided between treatments that encourage global activation of cytotoxic immune responses, such as exogenous cytokines and antibodies targeting T cell-inhibitory signals (e.g., anti-PD-1, anti-PD-L1, anti-CTLA4), and treatments based on tumor antigens, which aim to stimulate destruction of cancerous tissues by engaging a specific population of tumor-reactive CTLs (e.g., cancer vaccines and autologous T cell transfers). With further advancement in the clinical and investigative realms, it may become possible for these two approaches to complement each other within the same patient. A cancer vaccine that produces a meager antitumor response *in vivo* could have enhanced efficacy when administered alongside a treatment that

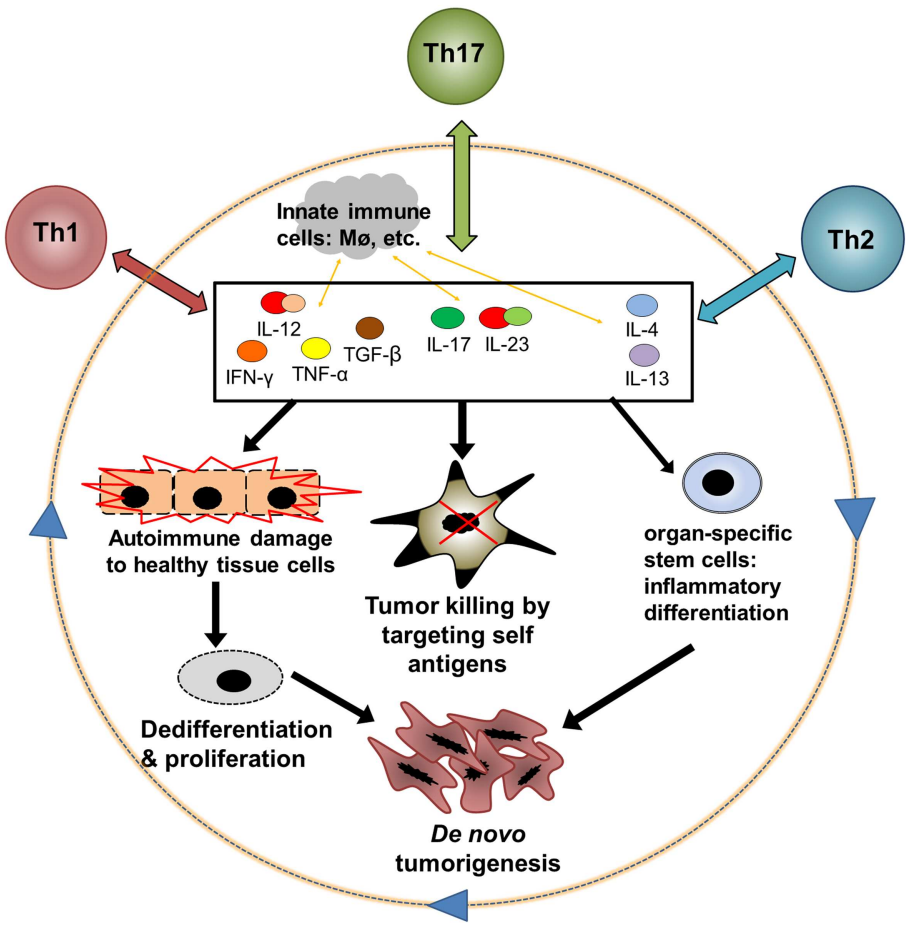

FIGURE 1 | A hypothetic feedback loop between autoimmunity, antitumor immunity, and inflammatory tumorigenesis. Three major subsets of autoantigen reactive T helper cells, Th1, Th2, and Th17, could mediate both antitumor and pro-tumor effects through interaction with innate immune cells and CD8 T cell effectors. (1) Th1, Th2, and Th17 cells release cytokines after encountering autoantigens. These cytokines may exert direct and indirect effects on cell cycle regulation, apoptosis, and differentiation in both tumors and healthy tissues. (2) Cytokines can promote tumor killing by various mechanisms including stimulation of CD8 effectors, innate immune cell activation, and direct toxicity. (3) Inflammation in healthy tissues, particularly when chronic, could contribute to loss of antiproliferative signals and aberrant differentiation in normal cells, leading to a premalignant phenotype. Continued proliferation of these abnormal cells, with accumulation of DNA damage, leads to the emergence of cancer. (4) Chronic cytokine signaling may induce abnormal differentiation of organ-specific stem cells or other precursor cells and drive a cascade of cancer development. The feedback between autoinflammation and cancer may particularly affect solid organs such as the gastrointestinal tract that are demonstrated to be susceptible to inflammatory carcinogenesis.

elicits global T cell activation, such as CTLA4 blockade. Moreover, the systemic autoimmune toxicity produced by these broad-acting treatments might be mitigated if a lower dose was combined with a cancer vaccine or other antigen-focused immune stimulus. Adding to the possibilities, multiple immunomodulatory agents from either treatment category could theoretically be combined (e.g., anti-PD-1 in combination with one or more exogenous cytokines) to provide unprecedented control over the targeting, intensity, and duration of the induced immune response. The potential advantages of a combined regimen are already supported by data from numerous preclinical models (5). The true capabilities of cancer immunotherapy may only be realized once multiple treatments can be synthesized into a therapeutic strategy tailored to the pathological and molecular characteristics of every patient's disease. Although the harmful clinical sequelae of autoimmunity may never be banished entirely, this integrative approach has the potential to harness its tumoricidal functions better than any single agent administered in isolation.

In summary, clinical data suggest that both the anticipated benefits of cancer immunotherapy and its associated adverse events share autoimmunity as a common originating process. Thus autoimmunity, regarded until recently as a "side effect" of cancer immunotherapy, may be more properly considered a correlate of antitumor immunity, or even more appropriately as an antitumor effector in its own right. Despite the lopsided benefit versus risk ratio in cancer immunotherapies that succeed in providing

substantial survival benefit, it should be noted that autoimmune damage to healthy tissues is a justifiably dreaded cause of morbidity and mortality in patients receiving these treatments. This hazard may be even more dire if one considers that an immunosuppressive microenvironment effectively designates tumors as an immunoprivileged self, which is more resistant to immune targeting than its healthy counterpart (1, 2). Furthermore, the calculation of benefit versus risk must account for the possibility that inflammatory signals arising from therapeutically induced autoimmunity may ultimately contribute to *de novo* tumorigenesis in the clinical setting. Therefore, achieving optimal benefit of cancer immunotherapies awaits advances in tumor-specific targeting, either by site or by unique antigens, coupled with proper monitoring and prevention of potentially catastrophic autoimmune damage or long-term risks of *de novo* tumorigenesis.

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Significance of interleukin-33 and its related cytokines in patients with breast cancers

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Interleukin-33 (IL-33) is a recently identified cytokine, an important member of the interleukin-1 family. IL-33 binds to its receptor ST2 to induce type 2 cytokines and exert both pro-inflammatory and protective functions in host defense and disease. Murine breast carcinoma models suggest disruption of ST2 signaling may enhance the anti-tumor immune response, suggesting IL-33 impedes anti-tumor immunity. However, the role of IL-33 in patients with breast cancers (BC) is not elucidated. We detected the expression of IL-33 in tumor tissue, and IL-33 and its related cytokines in serum from BC patients. Using Luminex and immunohistochemistry methods, we found that serum levels of IL-33 were nearly twofold higher in patients with BC, compared to patients with benign breast diseases. In cancer tissues, expression of IL-33 was higher than matched normal breast tissues from the same patients, and was also associated with a well-differentiated phenotype, HER2 overexpression, more lymph nodes involvement, and a family history of malignant carcinoma. These results suggest that IL-33 may play an important role in the progress of BC and may be a useful biomarker for predicting the progress and metastasis of BC.

Keywords: interleukin-33, ST2/ST2L, breast cancer, cytokines, immunosuppression

INTRODUCTION

Breast cancer (BC) is the second leading cause of cancer-related deaths amongst women in the United States, and morbidity and mortality of this disease increases each year (1). Although standard multi-modality treatment has improved the overall outcome and quality of life for patients with BC, identification of new prognostic markers, therapeutic targets, and new therapeutic approaches are needed. Recent insights into the cancer development mechanisms have revealed that immune system functionally regulates development and progression of epithelial malignancies and tumor-infiltrating leukocytes may be causal players in cancer development (2).

The interleukin-1 (IL-1) family is a growing group of cytokines, consisting of at least 11 members, and the balance between pro- and anti-inflammatory cytokines is crucial in the pathogenesis of many human diseases (3). Interleukin-33 (IL-33) is an important member of the IL-1 family, and in humans is expressed predominantly in skin, lung, adipocytes, and synovial fibroblasts (4). IL-33 is an endogenous ligand for the ST2/T1 receptor, and depending on the cellular and cytokine context, participates in many immune diseases with dual, pro-inflammatory, or protective roles. IL-33 induces T cells to produce IL-4, IL-5, and IL-13, and potently induces pro-inflammatory cytokines and chemokines through a Th2-dependent pathway, and also promotes Th1-type responses (5). IL-33 is involved in the pathogenesis of immune diseases, such as rheumatoid arthritis and atopic dermatitis, and may reflect the degree of inflammation in patients with immune diseases (6, 7). Deletion of IL-33/ST2 function enhances cytotoxicity of NK cells and increases levels of TNF-α, IFN-γ, and IL-17, and systemic pro-inflammatory cytokines, leading to attenuated tumor growth (8).

Recent studies demonstrated that high serum levels of soluble ST2 (sST2) are a worse prognostic factor in hepatocellular carcinoma (9), and that serum IL-33 is a diagnostic and prognostic marker in non-small cell lung cancer, independent of the therapeutic intervention (10). In studies on mouse mammary carcinoma, the IL-33/ST2 pathway promotes BC progression and metastasis through increased intratumoral accumulation of immunosuppressive cells and by diminishing innate anti-tumor immunity (11). Conversely, Gao et al. reported that transgenic expression of IL-33 may activate CD8(+) T cells and NK cells, and inhibit tumor growth and metastasis in B16 melanoma and Lewis lung carcinoma metastatic models (12). Thus, the data on the role of IL-33 in cancer progression was limited, and in particular, the function mediated by IL-33 in human BC is under-investigated. In this study, we aim to determine the serum level and to detect the expression of IL-33 in human tissues in patients with breast carcinoma, using Luminex-based measurements and immunohistochemistry, to further explore the role of IL-33 in anti-tumor immunity in BC.

MATERIALS AND METHODS

PATIENTS

This study was approved by the Ethics Committee of Shantou University Medical College and conducted according to the principles in the Declaration of Helsinki (13).

Blood samples were drawn from 64 patients with BC and 10 patients with benign breast diseases (BBD) as controls, who

visited the Shantou University Medical College Cancer Hospital Breast Center between April 2013 and July 2013. The mean age was 52 ± 11 years (25–80 years old) for patients with BC, and 41 ± 11 years (28–68 years old) for patients with BBD. All patients were pathologically diagnosed using specimens obtained either by core-needle biopsy or by surgery. The clinicopathological characters of the BC patients are summarized in **Table 1**.

The central regions of tumors were collected from 29 BC patients, as well as microscopic normal tissues from either tumor-adjacent normal tissue (<1 cm) or normal tissue ≥5 cm away from the tumor margins, for which paraffin-embedded samples were available. The mean age was 53 ± 13 years (25–80 years old) for all enrolled patient. The clinicopathological characteristics of the 29 BC patients are summarized in **Table 3**.

Table 1 | Relationship of serum IL-33 levels with clinicopathological parameters of breast cancer patients.

Variables	IL-33 level (pg/ml)			
	N	Mean	SD	P value
Age				
≤50 years	30	35.87	11.00	0.481
>50 years	34	38.21	14.78	
Menopausal status[a]				
No	33	36.19	11.33	0.992
Yes	30	36.52	12.26	
Size				
≤2 cm	26	36.50	11.52	0.760
>2 cm	38	37.53	14.21	
AJCC stage				
I + II	44	37.93	12.11	0.465
III	20	35.32	15.23	
Histological grade[b]				
1 + 2	30	36.51	13.58	0.915
3	23	36.14	10.64	
ER expression[c]				
Negative	26	32.79	10.84	0.033
Positive	37	39.92	13.97	
PR expression[c]				
Negative	32	36.76	16.28	0.896
Positive	31	37.20	9.17	
HER2 expression[c]				
No	46	37.82	14.28	0.409
Yes	17	34.71	9.48	
Ki-67 expression[c]				
Low	15	43.74	19.40	0.021
High	48	34.86	9.86	
Lymph node metastasis				
≤3	56	37.59	13.52	0.445
>3	8	33.78	9.67	
Family history				
No	58	36.00	11.69	0.034
Yes	6	47.81	21.29	

[a] *One case missing this information because the patient was male.*

[b] *Two cases with invasive lobular carcinomas, one case with an intraductal papillary carcinoma, and in eight cases, only biopsy samples were available without information on histological grade.*

[c] *One case was discharged after biopsy, without information on ER, PR, HER2, and Ki-67 expression.*

ASSESSMENT OF SERUM CYTOKINES

The levels of IL-33, IL-12, IL-13, IL-17, IFN-γ, and TNF-α in serum were measured based on a Luminex assay, using Milliplex™ MAP (Millipore, MA, USA) multiplex magnetic bead-based antibody detection kits according to the manufacturer's protocols (14).

Blood from patients was collected and centrifuged for 10 min at room temperature. Serum was removed carefully and stored at −80°C until use. Twenty-five microliters of neat samples were added into each well of 96-well plate, and then 25 μl mixed beads were added to the samples. The plate was incubated with agitation on a plate shaker overnight at 4°C. Twenty-five microliters of anti-cytokine antibody was then added and incubated for 1 h at room temperature. Lastly, 25 μl streptavidin–phycoerythrin was added into each well containing the detection antibodies for 30 min at room temperature. Cytokines were quantified using a BioPlex 200 platform (BioRad, CA, USA).

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Surgical specimens of cancer tissues, adjacent tissues to tumors, and normal tissues collected from patients with BC were formalin-fixed, paraffin-embedded, and cut into four-micron-thick sections. Sections were deparaffinized by immersion in xylene, and rehydrated in a series of graded alcohols. Epitope retrieval and inactivation of endogenous peroxidase activity was achieved as described (15). Samples were incubated overnight at 4°C with anti-IL-33 (R&D Systems, Minneapolis, MN, USA), then visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB). Sections without primary antibody were used as negative controls. Counterstaining was carried out with hematoxylin, and sections were visualized and photographed under a bright-field microscope (Olympus, Tokyo, Japan). Immunohistochemical staining was mainly cytoplasmic, and the percentage of positive cells for IL-33 was calculated for analysis by counting at least 200 cells in five or more high power fields.

Estrogen receptor (ER) and progesterone receptor (PR) were interpreted as negative or positive, if equal to and less than 1% or more than 1% of tumor showed nuclear positivity, respectively. HER2 was interpreted as negative or over-expressed, if there was 0–1+ or 3+ membranous staining, respectively. If there was 2+ membranous staining, FISH was conducted to determine whether HER2 was over-expressed (16). A Ki-67 cut-off point of 15% was defined according to the experience of different pathologists as well as national and international recommendations at present (17).

STATISTICAL ANALYSIS

Serum levels and tissue expression are expressed as the mean ± standard deviation (SD). Differences between groups were analyzed using the Student t-test or the non-parametric Mann–Whitney test. All statistical differences were considered significant at the level of $p < 0.05$. All data were analyzed with SPSS 19.0 software for Windows.

RESULTS

SERUM LEVELS OF IL-33 AND RELATED CYTOKINES IN BBD AND BC

Among six cytokines, the concentrations of IL-33 were nearly twofold higher in the BC group (34.49 ± 1.65 pg/ml) compared with BBD group (17.71 ± 2.60 pg/ml) ($p = 0.0008$), with a non-normal distribution (**Figure 1A**). Conversely, serum levels of IL-13, a Th2-associated cytokine, in the BC group (14.79 ± 0.45) were 40% lower than in the BBD group (24.92 ± 8.68 pg/ml) with borderline difference ($p = 0.0608$) (**Figure 1B**), and the concentrations of IL-12, a Th1-type cytokine, were 35% lower in the BC group (6.143 ± 0.25 pg/ml) than in the BBD group (9.39 ± 2.11 pg/ml) with statistical significance ($p = 0.0178$) (**Figure 1C**). The concentrations of other cytokines, IL-17, TNF-α, and IFN-γ, did not show any significant difference between the BBD and BC groups ($p > 0.05$, **Figures 1D–F**).

CLINICOPATHOLOGICAL ANALYSIS OF SERUM LEVELS OF IL-33 IN BREAST CARCINOMA PATIENTS

In patients with ER-positive breast tumors, the serum levels of IL-33 were 39.92 ± 13.97 pg/ml, and were significantly higher than in patients with ER-negative tumors, which were 32.79 ± 10.84 pg/ml, $p = 0.033$ (**Table 1**). In patients who showed lower Ki-67 expression, the serum levels of IL-33 were higher than the high Ki-67-expressing group (43.74 ± 19.40 pg/ml vs. 34.86 ± 9.86 pg/ml, $p = 0.021$). The serum concentrations of IL-33 were significantly associated with family history of malignant tumors ($p = 0.034$). No correlation was observed between serum IL-33 levels and patient age, menopausal status, tumor size, AJCC stage, histological grade, lymph node status, PR, and HER2 expression.

We also analyzed the association of clinicopathological parameters with IL-33-related cytokines. The serum levels of INF-γ were associated with tumor size ($p = 0.039$), with INF-γ being higher in patients with tumors <2 cm in size (22.57 ± 2.63 pg/ml) than patients with tumors >2 cm (15.22 ± 5.13 pg/ml). In pre-menopausal patients, the serum levels of IL-17 were significantly higher ($p = 0.048$) than that in post-menopausal group (15.51 ± 6.29 vs. 13.13 ± 2.16 pg/ml), and serum levels of IL-17 were also significantly associated with AJCC stage ($p = 0.049$) and HER2 expression ($p = 0.012$). No statistical significance was

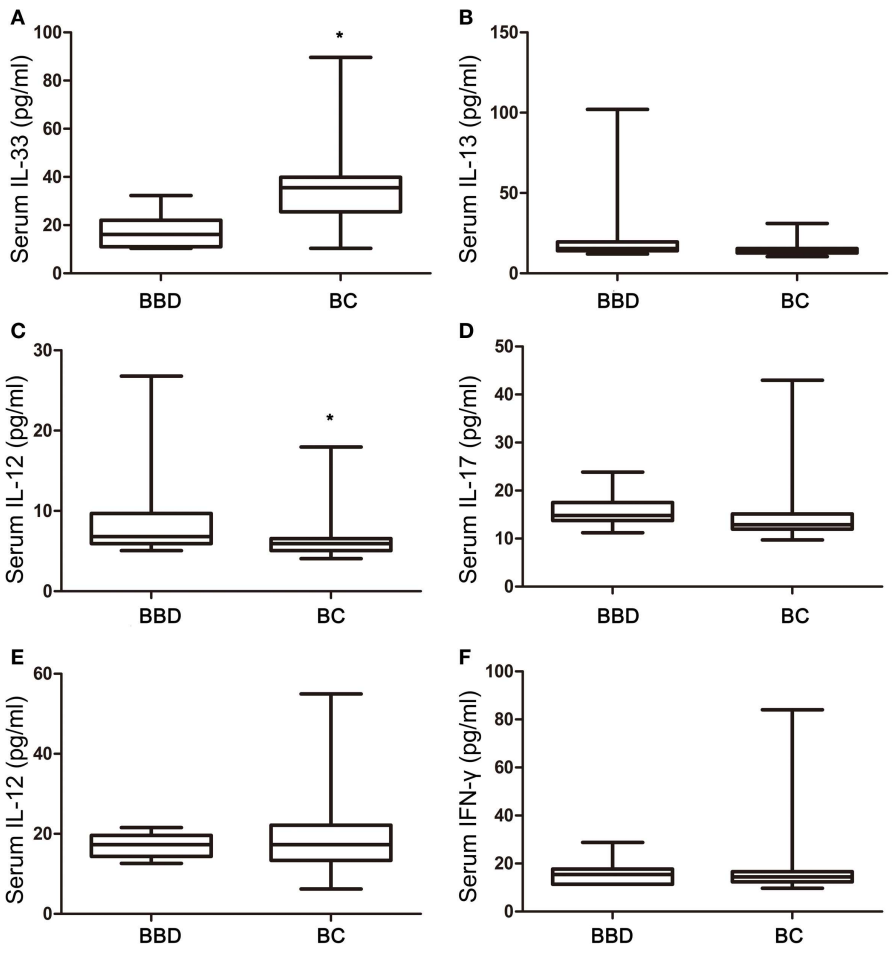

FIGURE 1 | Interleukin-33 and associated cytokine levels in benign breast diseases (BBD) and breast cancers (BC). **(A)** Serum IL-33 expression in BC was significant higher than that in BBD ($p = 0.0008$); **(B)** no significant difference between serum IL-13 expression in BC and BBD ($p = 0.0608$); **(C)** serum IL-12 expression in BBD was significant higher than that in BC ($p = 0.0178$); **(D)** no significant difference between serum IL-17 expression in BC and BBD ($p = 0.0526$); **(E)** no significant difference between serum TNF-α expression in BC and BBD ($p = 0.9748$); **(F)** no significant difference between serum IFN-γ expression in BC and BBD ($p = 0.7693$); $*p < 0.05$ by unpaired two tailed Student's t-test.

found between the serum levels of TNF-α, IL-12, or IL-13 with clinicopathological parameters of BC patients (specific data not shown).

IL-33 IS HIGHER IN CANCER TISSUES, COMPARED WITH ADJACENT AND NORMAL TISSUES

Figure 2 shows a representative immunohistochemical staining of IL-33 in tissues from patients with BC. In 29 patients with BC, mean expression of IL-33 in carcinoma was 72.6% of cells in the tumor, which was significantly higher than in normal breast tissues from the same patients ($p < 0.0001$) as shown in **Table 2**. Interestingly, the mean expression of IL-33 in adjacent tissues to tumor was 64.1%, which was also significantly higher than in normal breast tissues from the same patients ($p = 0.0002$). However, the mean expression level of IL-33 was not statistically different between in cancer and adjacent tissues ($p = 0.3561$).

CLINICOPATHOLOGICAL ANALYSIS OF CYTOPLASMIC IL-33 EXPRESSION IN BREAST CANCER TISSUES

Interleukin-33 was mainly detected in cytoplasm of BC cells as shown in **Figure 2**. With quantitative analysis, mean expressions of IL-33 were not significantly associated with the age at diagnosis, menopausal status, tumor size, AJCC stage, ER, PR, and Ki-67 expression (**Table 3**). In comparison with high grade tumors, low grade tumors showed significant higher IL-33 expression ($p = 0.027$). Interestingly, high IL-33 expression was more frequently observed in tumors with HER2 overexpression ($p = 0.017$). In patients with more than three lymph nodes involved, the expression of IL-33 was significantly higher than the patients with ≤3 metastases ($p = 0.002$). High IL-33 expression was also associated with family history of malignant tumor ($p = 0.002$).

DISCUSSION

Interleukin-33, the newest member of IL-1 family, is a recently identified cytokine with diverse and context-dependent functions, and has been shown to bind to ST2. IL-33 has also been characterized as a potent inducer of T helper (Th) 2 immune responses, and is an important mediator for mucosal healing and epithelial

Table 2 | The percentage of IL-33-positive tissue in breast tumors, tumor-adjacent tissues, and normal tissues from breast cancer patients.

Samples	IL-33 (%)			
	N	Mean	SD	P value[a]
Breast carcinoma tissue	29	72.6	34.5	<0.0001
Adjacent tissues to tumors	29	64.1	34.7	0.0002
Normal breast tissues from BC patients	25[b]	26.8	33.0	

BC, breast cancer.

[a] Statistically significantly different was found between cancer tissues vs. normal breast tissues, and between adjacent tissues vs. normal breast tissues from BC patients, using unpaired Student's t-test. No statistical significance was found between cancer tissues and adjacent tissues.

[b] In four cases, no normal breast tissues were available.

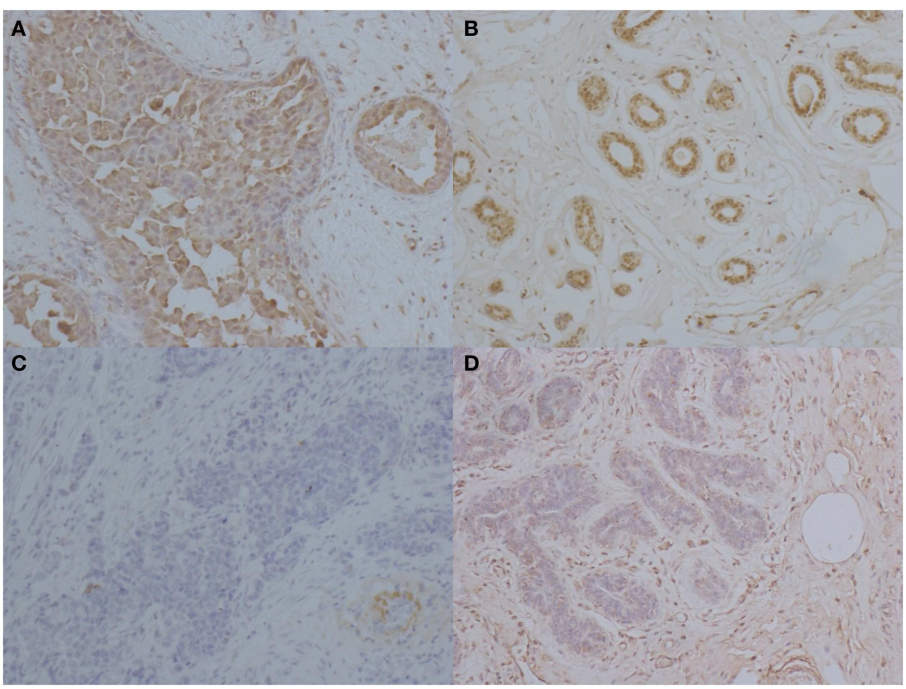

FIGURE 2 | Immunohistochemical staining of IL-33 in patients with breast cancers. Representative images of immunohistochemical staining for IL-33 in breast carcinoma, adjacent tissue to tumor, and normal tissue. **(A)** High IL-33 expression in carcinoma tissues. **(B)** IL-33 expression in tissue adjacent to tumors. **(C)** Low IL-33 expression in carcinoma tissue. **(D)** IL-33 expression in normal breast tissue from BC patients (magnification: ×400).

Table 3 | Clinicopathological analysis of cytoplasmic IL-33 expression in breast carcinoma.

Variables	IL-33 (%)			
	N	Mean	SD	P value
Age				
≤50 years	13	83.85	27.25	0.103
>50 years	16	63.44	37.71	
Menopausal status				
No	13	83.85	27.25	0.114
Yes	16	63.44	37.72	
Size				
≤2 cm	13	68.85	32.28	0.607
>2 cm	16	75.63	36.87	
AJCC stage				
I + II	22	69.09	34.21	0.342
III	7	83.57	35.44	
Histological grade[a]				
1 + 2	15	87.33	18.70	0.027
3	11	58.64	41.96	
ER expression				
Negative	14	76.8	34.8	0.536
Positive	15	68.7	34.82	
PR expression				
Negative	16	73.44	34.09	0.887
Positive	13	71.54	36.25	
HER2 expression				
No	18	62.50	38.90	0.017
Yes	11	89.09	16.40	
Ki-67 expression				
Low	6	75.00	24.29	0.851
High	23	71.96	37.07	
Lymph node metastasis				
≤3	24	67.71	35.87	0.002
>3	5	96.00	8.94	
Family history				
No	26	69.81	35.34	0.002
Yes	3	96.67	5.77	

[a]*Two cases with invasive lobular carcinomas and one case with an intraductal papillary carcinoma.*

restoration/repair (18). IL-33/ST2 axis can also promote Th1-type responses depending on the presence or absence of IL-12 (5). The influence of the IL-33/ST2 axis may be protective or pathogenic in various disease conditions, as it has a dual role in inflammatory disorders (8, 19). IL-33 plays a crucial role in inflammation and is associated with many diseases, such as giant cell arteritis (20), biliary atresia (21), and chronic obstructive lung disease (22). However, few data have been reported about the role of IL-33/ST2 axis in cancer, and little is known about the function of IL-33 in patients with BC.

In this study, we investigated the serum level and tissue expression of IL-33 in patients with BC. We found significantly higher serum levels of IL-33 in patients with BC, compared with patients with BBD, and higher expression of IL-33 in carcinomas and adjacent tissues to tumors, compared with normal breast tissue from the same patients.

The serum concentration of sST2, the soluble form of the receptor for both IL-33 and IL-1, has been shown to be elevated in patients with metastatic BC, and knockdown of the sST2 decreases ErbB2-induced cell motility in two different cell lines (23). However, there are no previous reports about IL-33 expression in serum or tissues of BC patients. In this study, serum levels of IL-33 are higher in patients with ER-positive tumors, predicting that the IL-33/ST2 axis may be involved in hormone receptor signaling. Moreover, in carcinoma tissues, IL-33 expression is significantly higher in HER2-overexpressing tissues, consistent with the report that its receptor sST2 is over-expressed to promote BC metastases upon ErbB2 activation in BC cell lines (23). In ST knock out mice models, lack of ST2 can suppress BC progression and metastasis, through enhanced cytotoxic activity of NK cells and increased systemic Th1/Th17 cytokines (24). Although we found no association between serum levels of IL-33 and in patients with more than three involved lymph nodes, the higher expression of IL-33 consistent with the IL-33/ST2 axis being involved in progression and metastasis of BC.

Of interest is that serum levels and carcinoma tissue expression of IL-33 are higher in patients with a family history of malignant breast carcinoma. As IL-33 may play an important role in immunosuppression of cancer for subsequent tumor progression and metastasis, and auto-immune diseases are usually hereditary, patients with a family history may be more likely to trigger or promote the process of immunosuppression (11). Whether and how IL-33 expression is linked auto-immune disease and familial cancers needs to be clarified.

Ki-67 is a cancer cell proliferation biomarker (25). IL-33 was higher in the low Ki-67 expression group, suggesting serum levels of IL-33 are negatively associated with BC proliferation. After analyzing cytokines associated with IL-33, only a decrease in IL-12 is observed in patients with BC, suggesting systemic IL-33 may not play an important role in BC immunity. In a murine model, the IL-33/ST2 axis has been demonstrated to facilitate intratumoral accumulation of immunosuppressive and innate lymphoid cells, and then promote BC growth and metastases (11). Similarly, in head and neck squamous cell carcinomas, administration of IL-33 promotes cancer cell migration and invasion through induction of epithelial-to-mesenchymal transition. Moreover, IL-33 has been shown to be a potential prognostic biomarker and target for new therapeutic strategies (26). Recent research suggests that in the breast tumor environment, tumor-infiltrating T lymphocytes (TILs) secrete IL-17A, to activate the MAPK pathway, promoting proliferation and resistance to conventional chemotherapeutic agents (27). The elevations of both IL-33 serum levels and immunohistochemical expression might promote BC progression and metastases through regulation of IL-12 pathway.

The local expression of IL-33 may be an important marker for differentiating malignant from normal/benign tissues. IL-33 expression in adjacent tissues also tends to be higher compared to normal tissues, suggesting that adjacent non-cancerous tissues may be similarly relevant to cancers in terms of anti-tumor immunity. Local IL-33 expression may also increase intratumoral

accumulation of immunosuppressive lymphoid cells in patients with BC. However, in high grade tumor tissue, the expression of IL-33 is decreased compared to low grade tumor tissues, indicating that IL-33 may be more important in HER2-over-expressing tumors, and other cytokines may be involved in this crosstalk of regulation. Interestingly, IL-33 expression in serum and cancer tissues was contrary when comparing with ER and HER2 expression, although with statistical significance (**Tables 1** and **3**). Up to now, there were no reports about the relationship between IL-33 and ER or HER2. So we supposed that IL-33 may play different roles in system and in local tissues under different hormone conditions. The IL-33 may be involved in the resistence to endocrine therapy and Herceptin therapy of ER/HER2 positive patients with BC.

It is confirmed that IL-33 could activate, Th1, NK, NKT, and $CD8^+$ T cells under certain pathophysiological conditions (11). On the other hand, IL-33 has a dual role in inflammatory disorders, anti- and pro-inflammatory. The tissue expression of IL-33 is significantly different, indicated that in carcinomas, immune cells may be recruited to anti-inflammatory and subsequent immunosuppression in HER2 overexpression tumors. In summary, this study indicated that serum IL-33 is higher in cancer patients compare to patients with BBD. Immunohistochemical staining demonstrated that IL-33 is higher in both cancerous and adjacent tissues compared to normal tissues, suggesting its role in BC progression and metastases. Thus, IL-33 may be a useful biomarker for prediction of malignant potential and immunosuppression of breast carcinomas.

AUTHOR CONTRIBUTIONS

Jing Liu and Guo-Jun Zhang conceived and designed the experiments; Jing Liu, Jia-Xin Shen, and Jia-Lin Hu performed the experiments; Jing Liu, Wen-He Huang, and Guo-Jun Zhang analyzed the data; Jing Liu, Jia-Xin Shen, Jia-Lin Hu, Wen-He Huang, and Guo-Jun Zhang contributed reagents and materials; Jing Liu, Jia-Xin Shen, and Guo-Jun Zhang wrote the paper.

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Embryonic stem cells promoting macrophage survival and function are crucial for teratoma development

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Stem cell therapies have had tremendous potential application for many diseases in recent years. However, the tumorigenic properties of stem cells restrict their potential clinical application; therefore, strategies for reducing the tumorigenic potential of stem cells must be established prior to transplantation. We have demonstrated that syngeneic transplantation of embryonic stem cells (ESCs) provokes an inflammatory response that involves the rapid recruitment of bone marrow-derived macrophages (BMDMs). ESCs are able to prevent mature macrophages from macrophage colony-stimulating factor (M-CSF) withdrawal-induced apoptosis, and thus prolong macrophage lifespan significantly by blocking various apoptotic pathways in an M-CSF-independent manner. ESCs express and secrete IL-34, which may be responsible for ESC-promoted macrophage survival. This anti-apoptotic effect of ESCs involves activation of extracellular signal-regulated kinase (ERK)1/2 and PI3K/Akt pathways and thus, inhibition of ERK1/2 and PI3K/AKT activation decreases ESC-induced macrophage survival. Functionally, ESC-treated macrophages also showed a higher level of phagocytic activity. ESCs further serve to polarize BMDMs into M2-like macrophages that exhibit most tumor-associated macrophage phenotypic and functional features. ESC-educated macrophages produce high levels of arginase-1, Tie-2, and TNF-α, which participate in angiogenesis and contribute to teratoma progression. Our study suggests that induction of M2-like macrophage activation is an important mechanism for teratoma development. Strategies targeting macrophages to inhibit teratoma development would increase the safety of ESC-based therapies, inasmuch as the depletion of macrophages completely inhibits ESC-induced angiogenesis and teratoma development.

Keywords: angiogenesis, apoptosis, embryonic stem cells, macrophages, teratoma

INTRODUCTION

Stem cell-based therapies possess promising outcomes for many conditions, including spinal cord injury and other neurological degenerative disorders. However, this powerful therapeutic strategy is problematic because the pluripotency of stem cells is accompanied by a large risk of tumor formation after transplantation. Theoretically, three classes of tumors can be envisaged to arise from pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs); viz. teratomas, teratocarcinomas, and secondary tumors (1). Teratoma is constituted by cells from endodermal, mesodermal, and ectodermal lineages (2–4). Not only ESCs but also ESC-derived neuronal progenitors can induce teratomas in animal models (5). The tumorigenicity of stem cells is the major obstacle to the successful application of stem cell-based therapies (6). The safety issue of stem cells must be evaluated properly and the adverse consequences of teratoma formation from these stem cells must be overcome before stem cell therapy can be used for clinical application.

Various strategies developed to reduce this risk of teratoma formation include prolonged pre-differentiation of ESCs *in vitro*, blocking signaling pathways that promote proliferation, induction of apoptosis of proliferative ESCs, sorting cells expressing precursor markers, and deleting undifferentiated ESCs immunologically, genetically, and chemically (7–18). However, it is difficult to obtain a yield of 100% pure differentiated stem cells for transplantation: the contamination of grafts with undifferentiated cells can give rise to teratoma formation (19–21). Furthermore, teratoma could potentially develop into highly malignant teratocarcinoma, which constitutes of persistent and undifferentiated stem cells (22). Therefore, efforts must be made to ensure safe transplantation of a PSC-based cell treatment. We used undifferentiated ESCs as a worst-case model for teratoma formation by stem cells

and studied the role of macrophages and niche microenvironment of stem cell growth in the progression of teratomas.

The interplay of immune cells, especially macrophages and ESCs, causes alterations in the microenvironment and potential for tumorigenicity, which regulate the initiation, progression, angiogenesis, and metastasis of tumor. Thus, targeting this immune response can significantly inhibit the evolution of tumors (23, 24). Our previous data also demonstrated that interaction between transplanted ESCs and macrophages creates a microenvironment that facilitates the initiation and progression of teratomas (24). Infiltrated macrophages deliver macrophage migration inhibitory factor (MIF) and other angiogenic factors to stimulate endothelial cell proliferation and pericyte differentiation (24). There is growing evidence to suggest that macrophages promote tumorigenesis and that the tumor microenvironment polarizes macrophages toward an M2 (pro-tumor) phenotype, with properties that differ from the M1 phenotype (25–28). However, the role of macrophages in ESC growth and teratoma development is not clear. In this study, we demonstrate that ESCs promote macrophage survival and M2-like activation are critically important for teratoma angiogenesis and development. Significantly, we show that depletion of macrophages inhibits teratoma growth tremendously. Therefore, ESC-educated macrophages are considered attractive targets for an anti-teratoma strategy after ESC transplantation.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

All the chemicals were purchased from Sigma (St. Louis, MO, USA) and cell culture media were purchased from Invitrogen (Carlsbad, CA, USA) unless specifically noted. The F4/80 hybridoma cell line was from American Tissue Culture Collection (ATCC, Manassas, VA, USA). Recombinant mouse IL-34 was from R&D Systems (Minneapolis, MN, USA). The primary antibodies used in the study are listed in **Table 1**. All Alexa Fluor- or HRP-conjugated secondary antibodies were from Invitrogen.

Table 1 | Antibodies included in the study.

Protein name	Antibody ID	Manufacturer
AKT	4685	Cell Signaling
Arginase-1	sc-18354	Santa Cruz
Caspase-9	9504	Cell Signaling
CD31	553708	BD Biosciences
CD45	103108	BioLegend
Cytochrome *c*	4272	Cell Signaling
ERK1/2	4695	Cell Signaling
GAPDH	2118	Cell Signaling
IBA-1	019-19741	Wako
IL-34	PAB13397	Abnova
M-CSF	3155	Cell Signaling
PI3K p85	4257	Cell Signaling
Iκbα (Ser32)	2859	Cell Signaling
Phospho-Akt	9271	Cell Signaling
Phospho-ERK1/2	4370	Cell Signaling
Phospho-PI3K p85	4228	Cell Signaling
Tie-2	sc-9026	Santa Cruz
YM1	01404	Stem Cell Technologies

MICE

C57BL/6 mice and transgenic CX3CR1^{GFP} mice from Jackson Laboratory (Bar Harbor, ME, USA) were maintained in the pathogen-free animal facility in Rutgers, the State University of New Jersey. All animal experimental protocols were authorized by the Animal Care and Facilities Committee of Rutgers, the State University of New Jersey and Florida State University.

PREPARATION OF MOUSE BONE MARROW-DERIVED MACROPHAGES

Mouse bone marrow-derived macrophages (BMDMs) from C57BL/6 mice were prepared as described (24). Briefly, BM cells from mice 6–8 weeks of age were collected from femoral shafts by flushing the marrow cavity of femurs with Dulbecco's modified eagle medium (DMEM) supplemented with 1% fetal bovine serum (FBS). The cell suspensions were passed through an 18-gage needle to disperse cell clumps. Cells were cultured for 7 days at a cell density of 1×10^6/ml in 100 mm polystyrene tissue culture dishes (BD Biosciences) containing DMEM supplemented with 15% conditioned medium from L929 cells [a source of macrophage colony-stimulating factor (M-CSF)] and 10% FBS. Cell morphology was analyzed by image capture (Carl Zeiss, Germany) and using LSM 510 software (Nikon, Japan). The long axis, defined as the longest length of the cells, was manually traced and measured.

ESC CULTURE AND PREPARATION OF ESC-CONDITIONED MEDIUM

The mouse green fluorescent protein (GFP)-expressing -ESC line (F12) derived from C57BL/6 mouse was a kind gift from Professor Melitta Schachner (Rutgers University). Freshly thawed ESCs (P0) were seeded into a 10-cm tissue culture dish in the presence of mitomycin-treated murine embryo fibroblast (MEF) feeder layer, in ESC media [10^3 U/ml LIF (Millipore, CA, USA), 15% FBS, 1% non-essential amino acids solution (MEM), 200 mM L-glutamine, 1% nucleoside solution, 1% 100 nM Na-Pyruvate, and 0.2% 2-Mercaptoethanol in DMEM]. LIF was added every day into the culture medium. After 3–4 passages, ESCs were maintained only on a 0.1% gelatin-coated tissue culture dish without a feeder layer. ESC colonies were sub-cultured for every 2–3 days and their supernatant was collected as conditioned medium. ESC-conditioned medium (ESC-CM) was prepared by spinning the ESCs at 1,000 rpm for 5 min to pellet the cells, while the supernatant was again spun at 2,500 rpm for 10 min to remove any debris. Supernatant was then filtered through a 0.4-μ filter (Corning, USA). Supernatant collected from multiple passages was pooled together and stored at −80°C. Regular culture medium without ESCs was incubated in the same way and used as control medium (Con-M).

HISTOLOGY AND IMMUNOFLUORESCENCE

Mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. A segment of tissue encompassing the transplantation site was removed and fixed in 4% paraformaldehyde for 3 h and then cryoprotected in 20% sucrose overnight at 4°C. For histologic examination, the sections were stained with hematoxylin and eosin (H&E). For immunofluorescence staining,

the sections were incubated with primary antibodies overnight at room temperature (RT) followed by secondary antibodies at RT for 2 h. Non-specific binding was excluded by using secondary antibody only. Samples were examined and microphotographs were taken using a Zeiss AxioCam microscope and an AxioPhot image collection system (Carl Zeiss, Germany), and Confocal Laser Scanning Microscopy (Nikon, Japan). Tumor volume was determined as follows: short diameter^2 × long diameter × 1/2.

TRANSPLANTATION AND ESCs IN SPINAL CORD AND LIVER

Laminectomy was performed on WT and chimerical mice at the T9–T10 level to expose the spinal cord. GFP–ESCs (50,000 in 1 μl DMEM) were injected slowly at this segment of each mouse's spinal cord using a microliter syringe (Hamilton Company, NV, USA) fixed in a stereotaxic frame. ESCs (100,000 in 5.0 μl DMEM) were also injected slowly via microliter syringe into the left lobe of mouse liver. Mice were sacrificed and perfused at different time points after cell transplantation.

MTT ASSAY

To determine cell viability the colorimetric MTT metabolic activity assay was used. BMDMs were seeded in a 96-well plate at a density of 8,000 cell per well and cells were treated with Con-M and ESC-M for 48 h. MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] was added to each well and plate was incubated for 4 h. Finally, the absorbance was measured at 540 nm by using a microplate reader. The relative MTT uptake (% cell viability) was expressed as a percentage relative to the control cells.

PHAGOCYTIC FUNCTION TEST

Mature BMDMs were seeded in a 96-well culture plate at a density of 1×10^4 cells/well and incubated with Con-M and ESC-M for 24 h. BMDMs were incubated with carboxylate-modified fluorescent red Latex beads and apoptotic cells for 1 h, respectively. Non-ingested particles were washed away and phagocytosis was imaged by a Zeiss Axiovert 200 M Microscope (Carl Zeiss) and software AxioVision 4.6 (Carl Zeiss).

ARGINASE ACTIVITY ASSAY

To prepare the cell lysate for assay of arginase activity, BMDMs were rinsed with PBS after each specific treatment and 1×10^5 BMDMs from each group were lysed in 100 μl of lysis buffer containing 10 mM Tris-HCl (pH 7.4), proteinase inhibitor cocktail, and 0.4% Triton-X 100 for 10 min. Arginase activity of various cell lysate was measured by quantitative colorimetric assay of arginase activity (Bioassay Systems, CA, USA) according to the manufacturer's instructions. One unit of arginase activity is defined as 1 μmol of L-arginine converted to ornithine and urea per minute at pH 9.5 and 37°C. Urea concentration, as the degree of arginase activity, was measured at 520 nm at RT by spectrometer.

ANNEXIN V AND PROPIDIUM IODIDE STAINING

Bone marrow-derived macrophages were rinsed with PBS after each specific treatment and the apoptosis of BMDMs was measured by PE Annexin V Apoptosis Detection Kit I (BD Biosciences, CA, USA) according to the manufacturer's instructions. Binding of Annexin V and PI was measured by a flow cytometer (BD Biosciences, CA, USA) and analyzed using FlowJo software (FlowJo, NJ, USA).

RNA ISOLATION AND QUANTITATIVE REAL-TIME-PCR

Bone marrow-derived macrophages were incubated with Con-M and ESC-M for 6 and 12 h, respectively. Total RNA was isolated by TRIZOL and reverse-transcribed into cDNA by using oligo-dT primers and SuperScript II Reverse Transcriptase. The TNF-α primer pair (5′-ATGCTGGGACAGTGACCTGG-3′ and 5′-CCTTGATGGTGGTGCATGAG-3′) was specifically designed for mRNA. The ABI7900HT detection system (Applied Biosystems, UK) was used for quantitative real-time (qRT)-PCR. SYBR Green dye (Applied Biosystems) was used to monitor the replication of PCR products. Quantification of products were obtained by standard curve and then normalized to GAPDH amount. The gene expression level was represented by the ratio of gene TNF-α/GAPDH.

WESTERN BLOT

Western blot assay was performed following the standard procedure. Briefly, after washing with ice-cold PBS, cells were lysed with RIPA buffer containing phosphatase inhibitor and proteinase inhibitor cocktail. Total cellular proteins were loaded onto SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (GE Healthcare, UK). After blocking in 5% milk or BSA (according to antibody manufacturer's instructions) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at RT, membranes were incubated with appropriate primary antibody solution overnight at 4°C. Membranes were placed in appropriate secondary antibody for 1 h after rinsing in TBST. Subsequently, proteins were visualized by ECL plus western blot detection system (GE Healthcare, UK).

EX VIVO MOUSE AORTIC RING ASSAY

Mouse aortic ring assay was carried out as described (29) using C57BL/6 mice (8–12 weeks). Briefly, thoracic aortic segments were cut into 1-mm rings and carefully placed with the lumen of the rings opened up on Matrigel (BD Biosciences) with Con-M or ESC-M and then overlaid with an additional Matrigel. Aortic rings were examined daily and digital images were taken at day 6 for quantitative analysis of the area of vessel outgrowth by the SPOT Advanced program (Media Cybernetics, Sterling Heights, MI, USA). Microvessel outgrowth was calculated by circling the extent of microvessel outgrowth at 6 days and subtracting the area of the aortic ring (29).

DEPLETION OF MACROPHAGES *IN VIVO*

Liposomes containing either dichloromethylene diphosphonate (clodronate, a gift from Roche Diagnostics, Germany) or PBS were prepared as described (30). Mice were injected with clodronate liposomes (CL-Lip) or PBS liposomes (PBS-Lip) at 100 mg/kg body weight by intraperitoneal (i.p.) injection twice a week for up to 4 weeks.

STATISTICAL ANALYSIS

Results showed in figures were presented as mean ± SEM with n representing the frequency of experiments. Student's unpaired

t-test was used to evaluate statistical significance with a p value <0.05 considered significant.

RESULTS

TERATOMA DEVELOPMENT AFTER ESC INJECTION INTO SPINAL CORD

Undifferentiated enhanced gene fluorescent protein (EGFP)–ESCs were stereotaxically injected into the spinal cord of mice exposed by a T9–T10 laminectomy. During the first week after ESC injection, hindlimb function, as reflected by the Basso Mouse Scale (BMS), was normal. However, the BMS score decreased rapidly at 10 days after ESC injection and all mice were paralyzed at day 17 after cell transplantation (**Figure 1A**) because of rapid tumor growth (**Figure 1B**). The mice survived for only 3 weeks after ESC transplantation (data not shown). Histological examination revealed that these tumors were teratomas since they consisted of structures derived from all three embryonic germ lineages (**Figure 1C**). While most teratomas are benign, malignant teratomas do occur. Prognosis is inversely related to stage and histological grade, which is based on the amount of neurepithelium and immature neural tubes present according to the World Health Organization (WHO) classification (31). Teratomas of grade 0–1 are classified as benign or low grade, while grade 3 is malignant. We found that the median teratoma grade in mice was 3.0 (**Figure 1D**), indicating that these teratomas in mice were teratocarcinomas.

ESCs STIMULATE MACROPHAGE INFILTRATION

We observed the early infiltration of a large population of Mac-2^+/IBA-1^+ macrophages within the teratomas (**Figure 1E**). Macrophages can be detected as early as 1 day after ESC injection, with peak macrophage infiltration occurring after 1 week (**Figures 1E,F**). **Figure 1F** shows the mean density of macrophages (IBA-1^+) recruited at the ESC implantation site at different time points after ESC injection. Interestingly, there was a significant reduction in macrophage distribution after 2 weeks. The numbers of macrophages at 2 and 3 weeks were significantly less than those at any earlier time points and the difference between 2 and 3 weeks was no longer significant (**Figure 1F**). This suggests that macrophages may play an important role in teratoma initiation. By contrast, injection of PBS alone in the spinal cord did not induce macrophage infiltration (data not shown).

TERATOMA DEVELOPMENT IN THE TISSUE OUTSIDE OF SPINAL CORD

To better exclude the effect of neural and glial cells in spinal cord on teratoma growth and differentiation, an examination of teratomas induced by ESC transplantation in non-neural sites could support the role of signals produced by macrophages vs. other tissue types. ESCs were injected into liver and representative teratoma at week 4 is shown in **Figure 1G**. A large teratoma had formed in the liver and an enormous number of macrophages was detected (**Figure 1G**).

ESC-SECRETED FACTORS ACT AS MACROPHAGE SURVIVAL FACTORS

The ESC-induced macrophage distribution could result from either increased recruitment of these cells into the teratoma or cell survival. We first examined the function of ESCs in macrophage growth. It has been well-documented that M-CSF is a hematopoietic growth factor necessary for monocyte survival, proliferation, and differentiation (32, 33). L929 conditioned medium is the source for M-CSF. Mouse ESC-CM (without direct cell–cell contact) and control medium (Con-M, medium to culture ESCs) were used in the study. Day 7 BMDMs were cultured with DMEM alone, Con-M and ESC-M and DMEM with M-CSF for 48 h and cell viability was measured by monitoring metabolic activity of the cells using MTT assay. Incubation of BMDMs in the presence of M-CSF and ESC-M caused a significant increase in cell viability compared to medium without M-CSF (DMEM alone) and Con-M treatment (**Figure 2A**). These data suggest that ESC-M can partially prevent the loss of macrophage viability after M-CSF withdrawal, although to a lesser extent than did M-CSF. Furthermore, heat-inactivated ESC-M by boiling for 10 min failed to increase macrophage viability (data not shown).

ESCs PROTECT MACROPHAGES FROM M-CSF WITHDRAWAL-INDUCED APOPTOSIS *IN VITRO*

To further evaluate whether the survival effect of ESCs was due to the inhibition of apoptosis, apoptosis of macrophages was assessed by surface Annexin V staining using FACS and caspase-9 activation. When mature BMDMs were cultured with M-CSF, $<10\%$ of cells were Annexin V-positive (**Figures 2B,C**). However, when cells were subjected to medium without M-CSF or Con-M, more than 25% of the cells underwent apoptosis (**Figures 2B,C**). Propidium iodide (PI) staining revealed that M-CSF withdrawal or Con-M treatment for 24 h did not increase PI-positive macrophages significantly compared to M-CSF or ESC-M treatment (**Figure 2C**). Furthermore, Con-M treatment induced activation of caspase-9, assessed by the appearance of the cleaved caspase-9 (**Figure 2D**). Treatment of cells with ESC-M significantly protected M-CSF withdrawal-induced caspase-9 activation and apoptosis (**Figures 2B–D**). These results suggest that the anti-apoptotic effect of ESCs on macrophages resulted in enhanced cell survival. In order to identify whether ESC-mediated cell survival is induced directly by mediators in ESC-M or indirectly by stimulating the release of secondary mediators acting in an autocrine manner, we detected M-CSF and IL-34, the two most well-documented cytokines that regulate macrophage survival and differentiation (34). ESCs did not produce M-CSF (data not shown) but expressed a high level of IL-34 (**Figure 2E**) and secreted into the ESC-M (16.034 ± 4.56 ng/ml, $n = 3$). BMDM treated with ESCs did not further increase M-CSF and IL-34 expression (data not shown), suggesting that ESC-induced BMDM survival is M-CSF-independent and that IL-34 from ESCs may promote macrophage survival.

ACTIVATION OF PI3K/Akt AND ERK IS NECESSARY FOR ESC-INDUCED MACROPHAGE SURVIVAL

The PI3K pathway is one of the most potent intracellular mechanisms for promoting cell survival, and PI3K/Akt and extracellular regulated kinase1/2 (ERK1/2) regulate macrophage survival in response to M-CSF and IL-34 (35–37). We therefore examined whether activation of PI3K and ERK1/2 was required for ESC-mediated macrophage survival. As shown in **Figures 3A,B**, ESC-M stimulated phosphorylation of p85 regulatory subunit of PI3K and ERK1/2 as early as 3 min, and a persistent phosphorylation level was maintained up to 30 min. ESCs also induced activation of

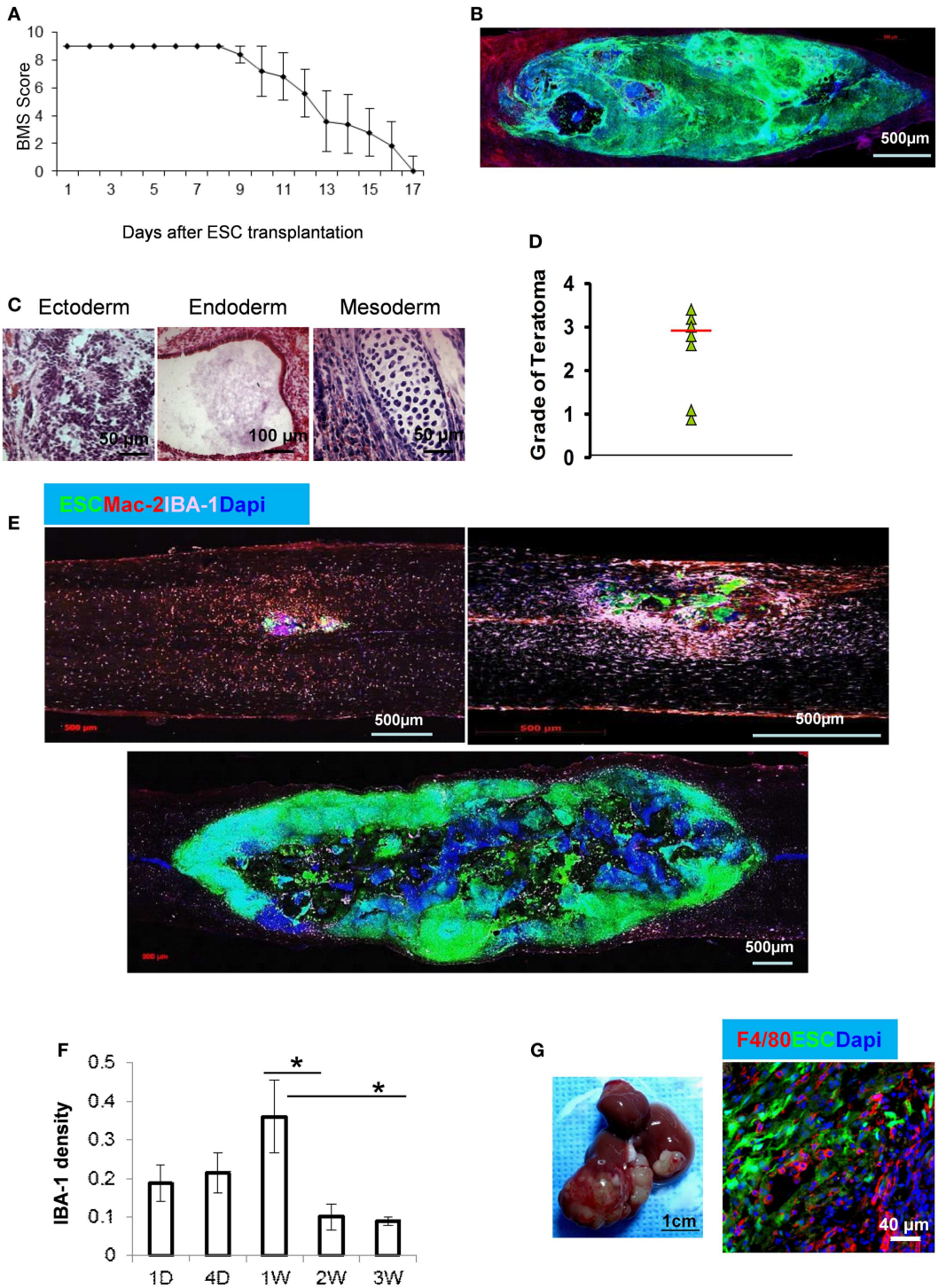

FIGURE 1 | Teratoma formation and macrophage infiltration after ESC injection into spinal cord. **(A)** ESCs were stereotaxically injected into the spinal cord in C57BL/6 mice and the function of the hindlimbs was evaluated by BMS score ($n = 7$, data are represented as mean ± SEM). A score of 0 indicates complete paralysis of the hind limbs and 9 denotes full mobility. **(B)** Tumor formation in spinal cord at 3 weeks after GFP–ESC injection. **(C)** Histological staining of spinal cord sections at 2 weeks after ESC injection showing structures derived from three embryonic germ lineages. **(D)** Median of teratoma grade in mice ($n = 6$). **(E)** Representative micrographs showing macrophage recruitment during teratoma progression. Macrophages in the sections of spinal cord at 1 day (upper left), 1 week (upper right), and 3 weeks after ESC transplantation (lower) were detected by antibodies to IBA-1 (purple) and Mac-2 (red). **(F)** Quantification of IBA-1$^+$ macrophages at indicated time points after ESC transplantation ($n = 10$, $^*p < 0.05$, two-sided Wilcoxon test. Data are represented as mean ± SEM). **(G)** Representative gross morphology of teratoma in liver (left) and F4/80$^+$ macrophages in teratoma at 4 weeks after ESC transplantation in liver.

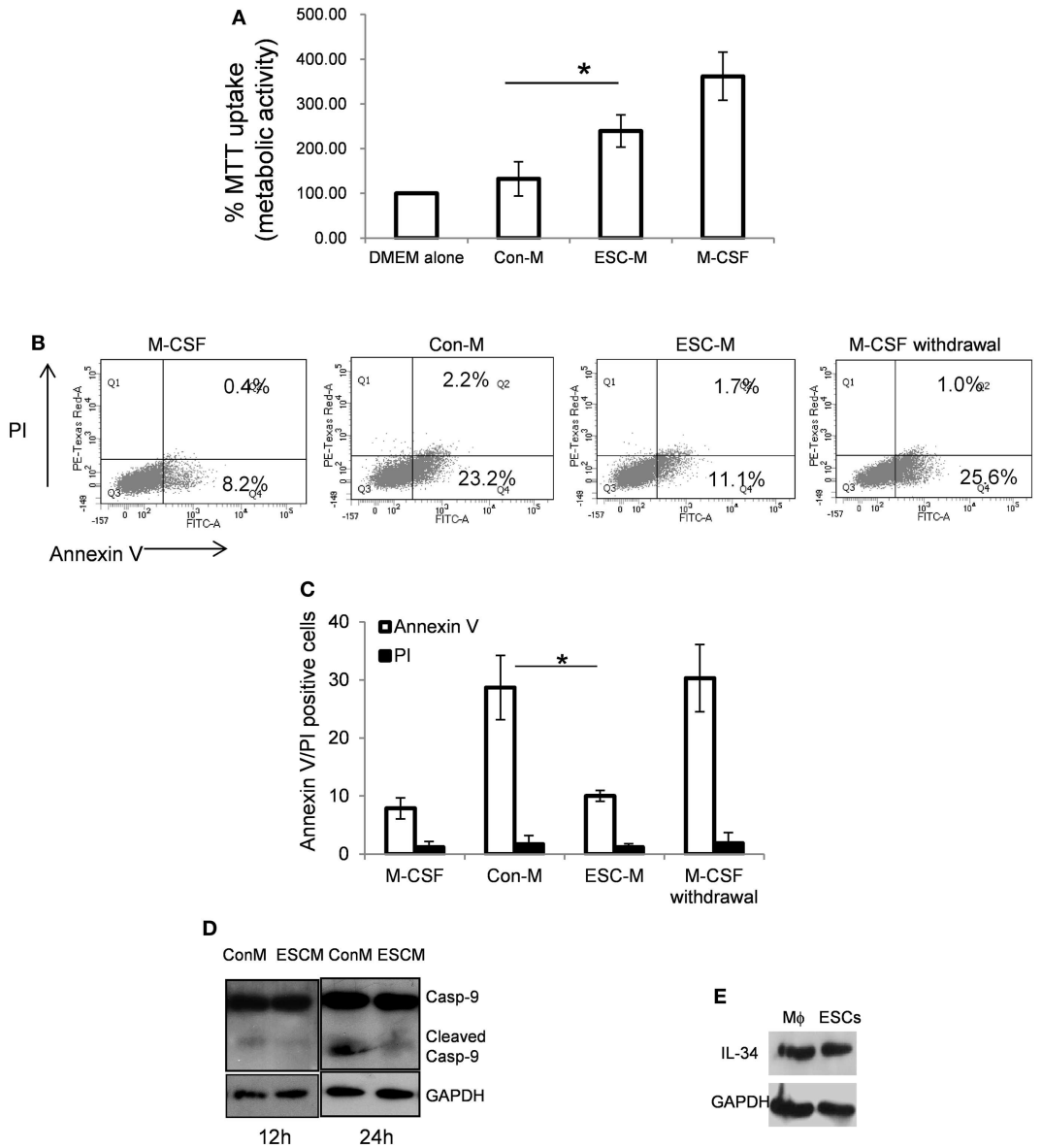

FIGURE 2 | Embryonic stem cells enhance BMDM survival. **(A)** Day 7 BMDMs were incubated with DMEM alone in absence of M-CSF, Con-M, ESC-M, and DMEM in the presence of M-CSF for 48 h. The metabolic activity of the cells was analyzed by the MTT assay and is presented relative to the activity of cells treated with DMEM alone ($n = 3$). **(B,C)** Flow cytometric analysis of annexin V/propidium iodide (PI) staining of BMDMs treated with ESC-M, Con-M, DMEM with M-CSF, and DMEM alone for 24 h, respectively ($n = 3$). **(D)** Effect of ESCs on caspase-9 activation. BMDMs were treated with Con-M and ESC-M for 12 and 24 h, respectively and whole cell lysates were analyzed by Western blotting for caspase-9 activation. **(E)** IL34 in BMDMs and ESCs was detected by Western blot analysis. $^*p < 0.05$, two-sided Wilcoxon test. Data are represented as mean ± SEM.

the major downstream kinase Akt of PI3K by phosphorylating residue Ser473 (**Figure 3C**). It is interesting to note that ESC-M also induced NF-κB activation by enhancing IκBα phosphorylation (**Figure 3C**). We further determined whether LY-294002 and PD98059, specific inhibitors of PI3K and ERK1/2, can reverse the protective role of ESC-M on macrophage survival. Both LY-294002 and PD98059 significantly reduced ESC-mediated macrophage survival (**Figure 3D**), suggesting that PI3K and ERK1/2 activation are required for ESC-mediated macrophage survival. Moreover, the activation of PI3K and ERK1/2 was inhibited by LY-294002 and PD98059, respectively, which parallels their effect on macrophage survival (**Figure 3E**).

We showed that ESCs produce IL-34 (**Figure 2E**) and the concentration of IL-34 in ESC-M was 16.034 ± 4.56 ng/ml. We therefore examined the ability of mouse recombinant IL-34 to promote macrophage survival on BMDMs. IL-34 simulated macrophage survival in a dose-dependent manner (**Figure 3F**). IL-34 at low as 10 ng/ml increased macrophage survival. Furthermore, IL-34

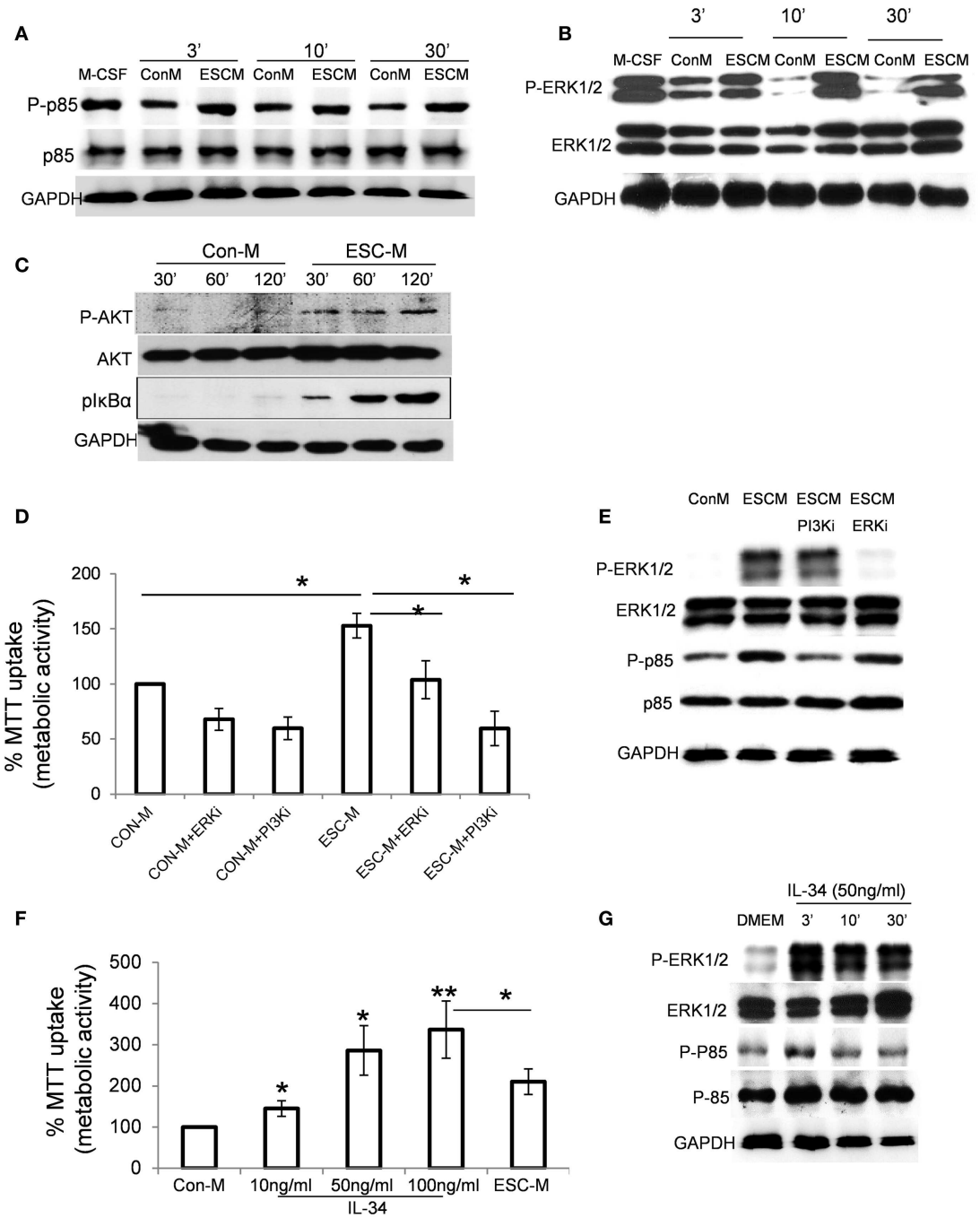

FIGURE 3 | Effect of ESCs on activation of PI3K/Akt, ERK1/2, and NF-κB pathways. Phosphorylation of p85 **(A)**, p-ERK1/2 **(B)**, and pAkt and NF-κB **(C)** in BMDMs treated with Con-M and ESC-M for the indicated time. **(D)** Effect of inactivation of PI3K and ERK1/2 in ESC-induced cell survival. BMDMs were pretreated with PI3K inhibitor LY-294002 (20 μM) and ERK inhibitor PD98059 (10 μM) for 30 min and then treated with Con-M and ESC-M for 48 h. The metabolic activity of the cells was analyzed by the MTT assay and is presented relative to the activity of cells treated with Con-M ($n = 3$, $^*p < 0.05$, two-sided Wilcoxon test. Data are represented as mean ± SEM). **(E)** BMDMs were pretreated with PI3K inhibitor LY-294002 (20 μM) and ERK inhibitor PD98059 (10 μM) for 30 min and then treated with Con-M and ESC-M for 30 min. Phosphorylation of p85 and p-ERK1/2 was detected by Western Blot assay. **(F)** Effect of IL-34 on macrophage survival. BMDMs were incubated with mouse recombinant IL-34 at the indicated concentration for 48 h. The metabolic activity of the cells was analyzed by the MTT assay and is presented relative to the activity of cells treated with Con-M ($n = 3$, $^*p < 0.05$; $^{**}p < 0.001$, ANOVA, data are represented as mean ± SEM). **(G)** Phosphorylation of p85 and p-ERK1/2 in BMDMs treated with IL-34 (50 ng/ml) for the indicated time.

stimulated phosphorylation of ERK1/2 and PI3K in macrophages to a similar degree, with similar kinetics, compared to ESC-M treatment (**Figure 3G**).

ESCs INDUCES TYPICAL SHAPE CHANGE

We next examined how ESCs modulate macrophage function. We exposed mature BMDMs to interferon γ (IFN-γ), IL-4, ESC-M, Con-M, and IL-34 for 72 h, respectively. BMDMs present a unique morphology, depending on the stimulation used. Cells treated with IL-4, which stimulates M2 activation, adopted a spindle-shape morphology (**Figure 4A**). M1 macrophages induced by IFN-γ had a relatively round shape with large filopodia. Con-M treatment exhibited the typical bipolar, spindle-shaped morphology of BMDMs. In contrast, ESC-M led to a majority of elongated fibroblast-like-shaped cells and some of the macrophages showed a long, single process or bipolar processes (**Figure 4A**). IL-34 treated cells demonstrated a wider range of cell length whereas the ESC-M treated cells displayed a relatively shorter range because the cells were all approximately the same length (**Figure 4A**). Quantitative analysis showed that ESC-M-treated macrophages exhibited a

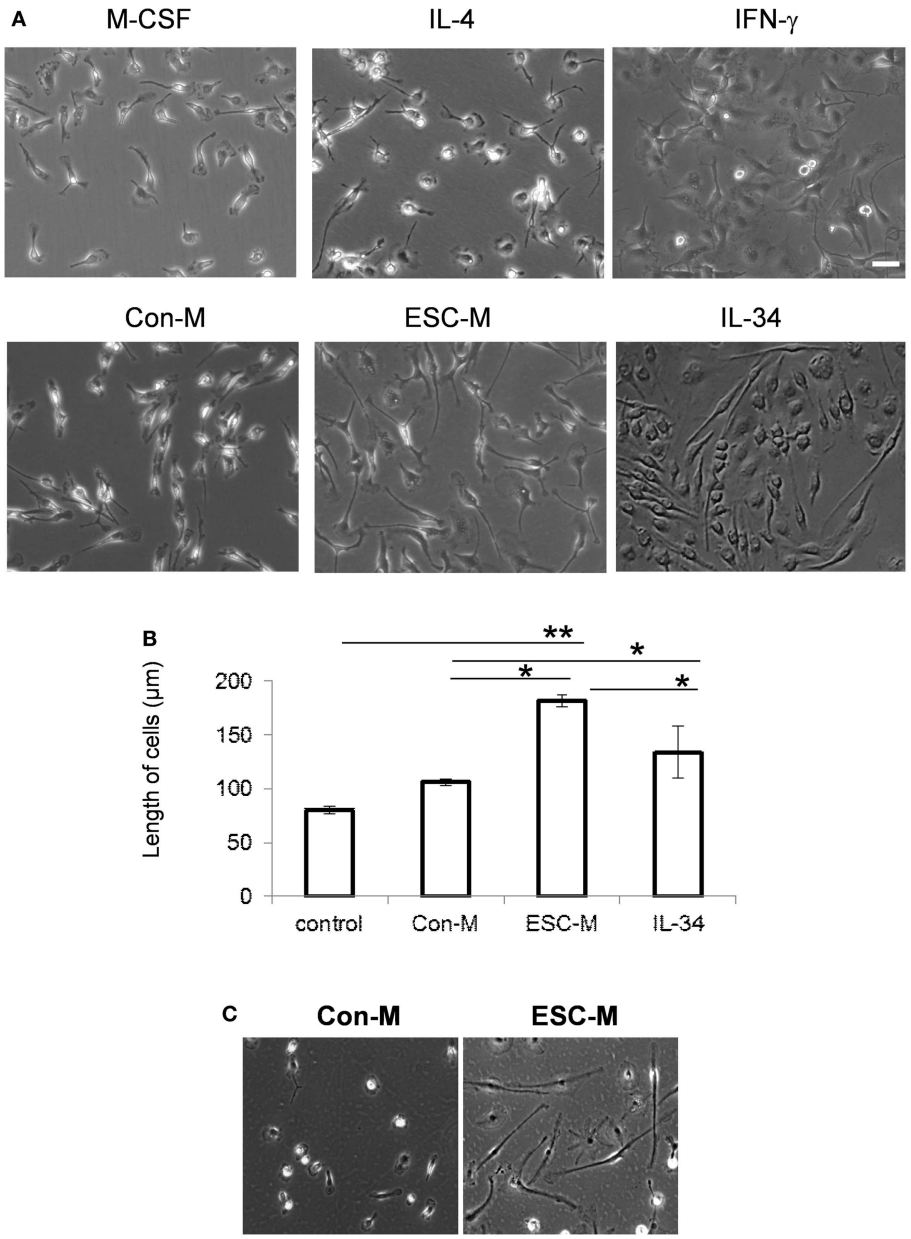

FIGURE 4 | Effect of ESCs on macrophage activation. **(A)** Representative phase-contrast photomicrographs of BMDMs (original magnification, ×400). BMDMs were cultured with M-CSF, IL-4, IFN-γ, IL-34, Con-M, and ESC-M for 72 h. **(B)** Length of BMDMs incubated with IL-34, Con-M, and ESC-M for 72 h was measured by ImageJ ($n = 6$, $^*p < 0.05$, $^{**}p < 0.001$, two-sided Wilcoxon test. Data are represented as mean ± SEM). **(C)** Representative phase-contrast photomicrographs of primary microglial cells incubated with Con-M and ESC-M for 72 h. Original magnification, ×400.

significantly higher degree of elongation compared either to Con-M treated or untreated macrophages (**Figure 4B**). The average length of cells treated with IL-34 was longer than that of Con-M treated cells but shorter than that of ESC-M, suggesting that other factors produced by ESC-M may have contributed to cell elongation. To further demonstrate that the ESC-induced phenotypic characteristic was not restricted to BMDMs, we isolated primary microglial cells from brain. ESC-M also resulted in remarkable elongation in microglial cells (**Figure 4C**).

ESCs MAINTAIN MACROPHAGE PHENOTYPE AND FUNCTION

In order to know whether ESC treatment would maintain macrophage phenotype and function, we analyzed the expression of macrophage markers such as F4/80 and Mac-2 on cells treated with ESC-M. BMDMs were treated with Con-M and ESC-M for 3 days and expression of macrophage markers was confirmed by flow cytometry and Western Blot analysis, respectively. Both Con-M and ESC-M treatment maintained macrophages expressing a high level of F4/80 (**Figure 5A**). Mac-2 expression was enhanced by ESC-M treatment and the expression level was higher compared with Con-M treatment (**Figure 5B**). To further study, whether ESC-M-treated macrophages were biologically functional, we incubated BMDMs with apoptotic neutrophils (PMNs) and latex beads for 30 min. The results showed that ESC-M-treated BMDMs displayed active functional phagocytosis of apoptotic cells and latex beads (**Figures 5C,D**).

ESCs POLARIZE MACROPHAGES INTO UNIQUE M2-LIKE CELLS

In addition to the role of ESCs promoting macrophage infiltration and survival, ESCs are able to activate BMDMs and stew them toward the M2 phenotype (Arginase-1^{high}YM1^{high}) via activation of STAT3 and STAT6 pathways (24). A recent study showed that macrophage elongation enhanced the effect of M2-inducing cytokines and inhibited the effect of M1-inducing cytokines, suggesting that cell shape has an important role in modulating macrophage activation (38). We demonstrated in the present study that the phenotypic characterizations of ESC-treated macrophages were distinct from classic M2 macrophages induced by IL-4 (**Figure 4A**). We thus further evaluated whether ESC-treated macrophages are different from "alternatively activated" M2 macrophages. Treatment with ESC-M significantly enhanced arginase-1 (Arg-1) activity in BMDMs in a time-dependent manner, compared to treatment with Con-M using a colorimetric assay that detects production of urea (**Figure 5E**). The distributions of angiopoietin (Ang) receptor (Tie)-2^{+} (Tie-2) cells and F4/80^{+}/arginase-1^{+} macrophages were detected in the teratoma *in vivo* (**Figure 5F**). It has been shown that M2 express a very low level of TNF-α (39, 40). However, we showed that macrophages expressed only minimal TNF-α mRNA in the absence of ESC-M (**Figure 5G**). Upon co-culture with ESC-M, TNF-α expression increased significantly in macrophages (**Figure 5G**). Furthermore, the amount of TNF-α secreted into the culture medium was significantly increased in BMDMs treated with ESC-M compared to the amount present in supernatants of Con-M-treated macrophages (**Figure 5H**). In summary, ESC-macrophages exhibited an Arg-1^{high}Tie-2^{high}TNF-α^{high} phenotype, which differs from conventional M2 phenotypes.

ESCs EXERTS ANGIOGENIC ACTIVITY *EX VIVO* AND *IN VIVO*

It is widely accepted that tumor growth requires angiogenesis. Therefore, fast teratoma growth is supposedly induced by increased angiogenesis. ESCs were injected into the spinal cord and images were taken at 3 weeks after cell transplantation. Spinal cords with teratoma appeared reddish or brownish, suggesting an increased permeability (**Figure 6A**). Immunohistochemical analysis of teratoma tissue with anti-CD31 antibody showed a massively branched intratumoral vascular network at 3 weeks after cell injection (**Figure 6A**). This high density of "plexus-like" vascularity in teratoma may be important for teratoma growth. By contrast, injection of PBS alone in the spinal cord did not produce neovascularization (data not shown).

To better understand the contribution of macrophages to vascular development during teratoma progression, we performed a ring sprouting *ex vivo* assay. A 3D-culture of aortic rings in Matrigel was used to evaluate the outgrowth of linear endothelial structures from the preexisting vessel (41). The aorta ring assay is thought to more closely mimic multiple stages of *in vivo* angiogenesis, including endothelial cell proliferation, migration, and tube formation. Mouse thoracic aorta was sectioned into 1-mm rings, and incubated in growth factor-reduced matrigel with Con-M or ESC-M for 6 days. Sprouting from the rings was photographed and outgrowth area was quantitated. ESC-M treatment significantly increased the areas of sprouting ($1.81 \pm 0.03\ mm^2$) at 6 days, whereas Con-M-treated ring segments showed little sprouting (**Figures 6B,C**). Qualitatively, the arborization of endothelial networks emanating from aortic rings was also more complex in the rings treated with ESC-M. Together, these data demonstrated an important role for ESC-mediated angiogenesis in aortic rings *ex vivo*.

We showed that ESC-educated macrophages (SEM) exhibited an Arg-1^{high}Tie-2^{high}TNF-α^{high} phenotype (**Figures 5E–H**). In order to know whether TNF-α is responsible for ESC-enhanced angiogenesis, aortic rings were cultured with TNF-α at 10 ng/ml for 6 days. A significant increase in angiogenic spouting was observed in aortic rings in response to TNF-α treatment (**Figures 6B,C**). Therefore, we consider that TNF-α contributed, at least partially, to ESC-induced angiogenesis.

It has been shown that macrophages are found around sprouting neovessels and are particularly abundant at the root of the vascular outgrowth (42). Pharmacologic ablation of macrophages from aortic explants blocked formation of neovessels *in vitro* and reduced aortic ring-induced angiogenesis *in vivo* (42). We further determined how crucial macrophages are to the enhanced angiogenic ability of ESCs. We applied a well-documented approach to deleted macrophages by treating mice with liposome-encapsulated clodronate (Cl-Lip) or control liposomes (PBS-Lip) (43). We used CX3CR1 $^{GFP/-}$ mice, in which one (CX3CR$1^{GFP/+}$) copy of the CX3CR1 gene was interrupted by EGFP (44). CX3CR1 is highly expressed by human and mouse macrophages (45). Intraperitoneal injection of mice with Cl-Lip but not control liposomes (PBS-Lip) resulted in complete depletion of macrophages in the aortic ring tissue (**Figure 6D**). Depletion of macrophages by Cl-Lip led to a markedly reduced angiogenic response to ESC-M (**Figure 6E**). However, we cannot rule out the direct effect of ESC-M on aortic ring sprouting, because Cl-Lip treatment did not completely inhibit the vascularization (**Figure 6E**).

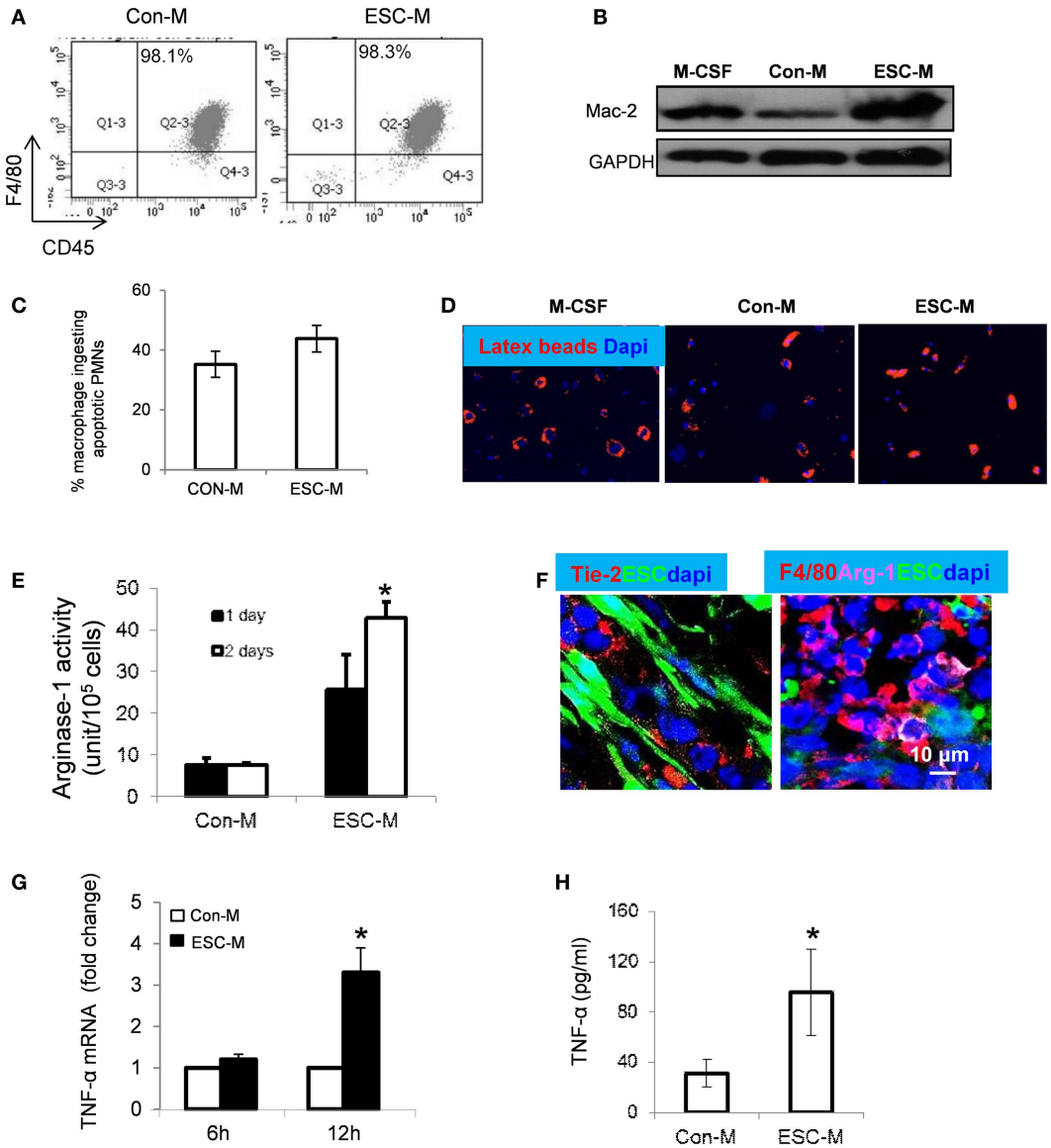

FIGURE 5 | Effect of ESCs on macrophage activation. BMDMs were incubated with Con-M or ESC-M for 72 h and F4/80 (macrophage marker), CD45 (hematopoietic marker), and Mac-2 (macrophage marker) were assessed by flow cytometry **(A)** and Western Blot **(B)**, respectively. **(C)** BMDMs were pretreated with Con-M and ESC-M for 48 h and then incubated with apoptotic neutrophils for 30 min. The number of macrophages ingesting apoptotic cells was counted ($n = 4$, data are represented as mean ± SEM). **(D)** BMDMs pretreated with Con-M and ESC-M ingestion of latex beads (red, original magnification, ×400). **(E)** BMDMs were treated with Con-M and ESC-M for 1 and 2 days and arginase-1 activity was detected by colorimetric assay ($n = 3$, $^*p < 0.05$, two-sided Wilcoxon test. Data are represented as mean ± SEM). **(F)** Representative confocal images of immunostaining of sections from mice at 2 weeks after ESC injection showing positive staining for Tie-2 (red, left) and F4/80 (red, right) Arginase-1 (purple), respectively. **(G)** TNF-α mRNA in BMDMs treated with Con-M and ESC-M for 6 and 12 h was detected by real time RT-PCR ($n = 4$, $^*p < 0.05$, two-sided Wilcoxon test. Data are represented as mean ± SEM). **(H)** TNF-α in the supernatants of BMDMs treated with Con-M and ESC-M for 48 h was detected by ELISA ($n = 3$, $^*p < 0.05$, two-sided Wilcoxon test. Data are represented as mean ± SEM).

TARGETING MACROPHAGES INHIBITS ESC-INDUCED ANGIOGENESIS AND TERATOMA DEVELOPMENT

A large amount of macrophage infiltration and phenotype of M2-like macrophages in the teratoma suggested that macrophages may create a microenvironment for teratoma development. We depleted macrophage populations from mice to verify the contribution of macrophages to teratoma growth. We demonstrated that i.p. injection of Cl-Lip twice a week after ESC transplantation into liver resulted in near-complete depletion of macrophages in liver and teratoma when assayed at 4 weeks (**Figures 7A,B**). We also quantified the blood vessel density by counting the percentage of the area occupied by cross-section of all blood vessels in an image. We observed that blood vessel density in teratoma from mice treated with

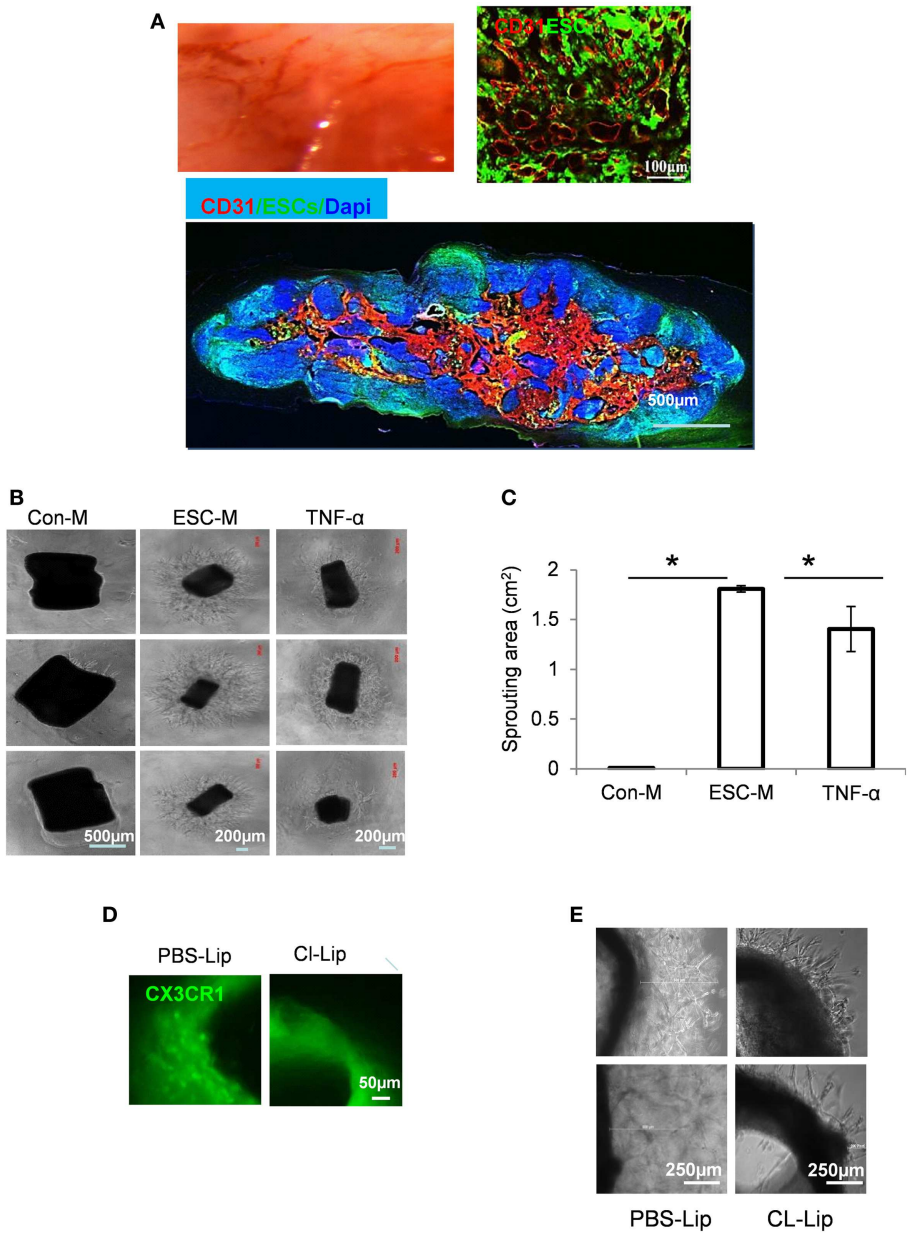

FIGURE 6 | Embryonic stem cells induced an angiogenic switch in macrophages. **(A)** Representative gross morphology micrograph showing blood vessel development during teratoma progression (top left) and immunostaining by endothelial marker CD31 (red) in sections from mice at 3 weeks after ESC injection (top right and bottom). **(B)** Representative gross morphology of aortic rings in Matrigel containing Con-M (left panel), ESC-M (middle panel), or TNF-α at 10 ng/ml (right panel) at day 6. **(C)** Area of vascular sprouting at day 6 in Con-M, ESC-M, and TNF-α was measured by ImageJ ($n = 6$, $^*p < 0.05$, two-sided Wilcoxon test. Data are represented as mean ± SEM). **(D)** Representative gross morphology of aortic ring from CX3CR1$^{GFP/+}$ mice treated with PBS-Lip (left) and CL-Lip for 4 weeks (right). **(E)** Mice were treated with PBS-Lip (left) and CL-Lip (right) for 4 weeks and aortic rings were cultured in Matrigel containing ESC-M for 6 days. Representative gross morphology of aortic ring sprouting was taken.

Cl-Lip was significantly lower than that of control treatment (**Figure 7C**). Blood vessels were significantly smaller in Cl-Lip-treated teratoma compared to control treatment (**Figure 7C**). Depletion of macrophages did not affect the pluripotency of ESCs, as all three germ layers can be observed in macrophage-deleted teratoma (**Figure 7D**). Teratomas from mice treated with PBS-Lip appeared much darker and were filled with blood, indicating that functional vasculature had formed via angiogenesis (**Figure 7E**). In contrast, tumor tissue from mice treated with Cl-Lip was transparent (**Figure 7E**). Subsequently, ablation of macrophages significantly inhibited teratoma growth, with a mean tumor size of $83.13 \pm 60.81\ mm^3$ in Cl-Lip group vs. $2502.75 \pm 1410.02\ mm^3$ in mice treated with PBS-Lip ($n = 5$, $p < 0.05$, **Figure 7F**).

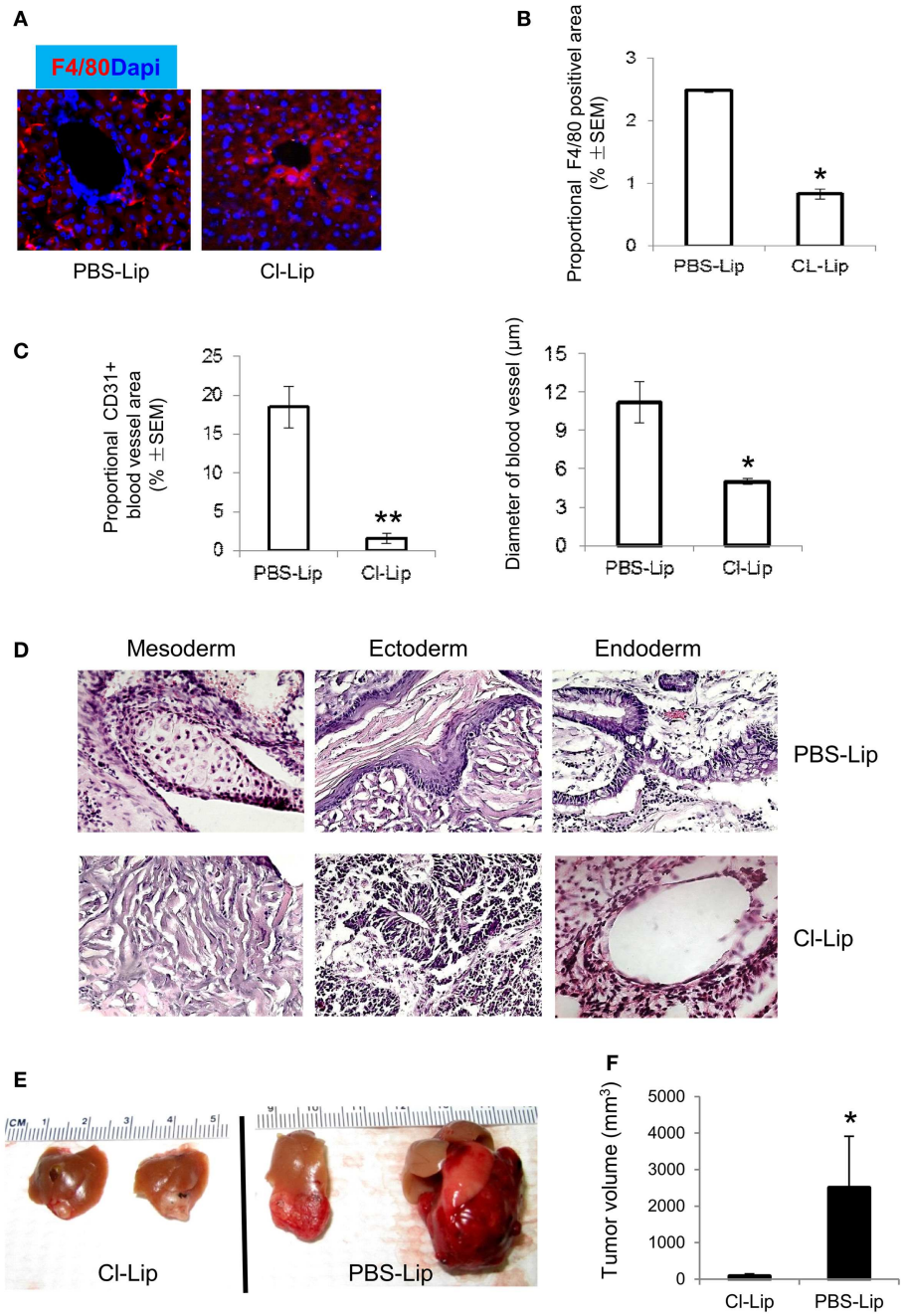

FIGURE 7 | Depletion of macrophages inhibiting angiogenesis and teratoma growth. **(A)** Cl-Lip was administrated i.p. at the day for ESC injection and twice a week for 4 weeks. PBS-Lip was used as control. Liver tissues were stained with F4/80. Note that Cl-Lip treatment completely depleted F4/80$^+$ macrophages in liver harvested from the Cl-Lip-treated group (left) and control group (treated with PBS-Lip) at 4 weeks after ESC transplantation. Quantification of F4/80$^+$ macrophages **(B)**, density of blood vessels stained for CD31 [**(C)**, left], size of blood vessels [**(C)**, right], HE staining **(D)**, and representative images of teratomas **(E)** in teratomas from Cl-Lip- or PBS-Lip-treated mice at 4 weeks after ESC transplantation. **(F)** Average teratoma size at 4 weeks after ESC transplantation in the Cl-Lip and PBS-Lip treated groups. $*p < 0.05$, $**p < 0.001$, $n = 5$, two-sided Wilcoxon test. Data are represented as mean ± SEM.

DISCUSSION

In this study, we demonstrated for the first time that BMDMs can be guided by ESCs to migrate to the site of ESC implantation while soluble factor(s) produced by ESCs polarize macrophages into a novel Arg-1^{high}Tie-2^{high}TNF-α^{high} phenotype. Furthermore, ESCs can prevent BMDM apoptosis induced by M-CSF-withdrawal through PI3K/Akt and ERK1/2 activation. We demonstrated that these ESC-educated macrophages (SEM) exhibit an

elongated morphology and produce high level of TNF-α, which participates in angiogenesis and contributes to teratoma progression. Depletion of macrophages completely inhibits ESC-induced angiogenesis and teratoma development. These studies provide a novel rationale for the control of teratoma development by targeting macrophage growth or activation.

Macrophage colony-stimulating factor is known to regulate monocyte/macrophage survival (32, 33). PI3K/Akt and ERK or MARK are the major signaling pathways triggered by M-CSF stimulation (35, 46, 47). We showed that ESCs promote the survival of cultured BMDMs after M-CSF withdrawal, and that activation of both PI3K/Akt and ERK1/2 MAP kinase are required for the survival effect of ESCs, which is similar to that activated by M-CSF. ESC-maintained macrophage survival and function was independent of M-CSF, because M-CSF was detectable neither in ESC-M nor in ESC-M-treated macrophages (24). In the present study, we studied whether other factors are involved as autocrine or paracrine effectors to induce macrophage survival. For example, GM-CSF and IL-4 are known to regulate monocyte/macrophage survival (48). Our results ruled out the requirement for GM-CSF and IL-4, as these cytokines were undetectable in ESC-M (24). Recent studies reported that IL-34 is an alternative ligand for M-CSF receptor (CSF-1R) (34, 49). IL-34 binds specifically to human and mouse myeloid cells, induces ERK1/2 activation, and supports macrophage proliferation and differentiation (34). We showed that IL-34 is highly expressed by ESCs. IL-34 promotes macrophage survival and activates ERK and PI3K pathways. Although IL-34 also results in cell elongation, its effect is not as strong as that of ESC-M. It is possible that other soluble factors produced by ESCs play a role in cell shape change. It has been reported recently that IL-34-activated macrophages exhibit an IL-10^{high} IL-12^{low} M2 profile in response to LPS stimulation (50). Therefore, it is likely that IL-34 produced by ESCs play a pivotal role in ESC-induced macrophage survival and M2 polarization. More studies are needed to investigate whether IL-34 secretion and M2-like polarization of macrophages are general features of ESCs.

It is interesting to note that a large number of macrophages exist in the early stage of teratoma development and the number of macrophages rapidly declines at 2 weeks after ESC injection. We reasoned that ESCs have ability to regulate macrophage survival and activation. The inhibition of macrophage apoptosis by ESCs at an early stage may favor teratoma initiation and development. However, the effect of ESCs on macrophage may be reduced once ESCs are differentiated into its three germ layer structures. It will be important to determine whether fully differentiated tissues lose the ability to maintain macrophage function or have the capacity to inhibit macrophage survival. Beside IL-34, the factors produced by ESCs that promote macrophage growth and program M2-like phenotype remain unknown. It is possible that the function/phenotypes of macrophages are regulated by coordinated action of different classes of molecules secreted by ESCs. However, more detailed studies will be necessary to determine whether additional molecules either alone or in combination contribute to macrophage survival. The identification of these factors appears crucial in the development of strategies to prevent and/or reverse macrophage phenotype and thereby increase the safety of stem cell applications in clinical settings.

Beside mediation of macrophage survival, ESCs are able to regulate macrophage activation. It is well-documented that M1 macrophages express high levels of nitric oxide (NO), reactive oxygen species (ROS), and TNF-α, contributing to tissue inflammation and damage. In contrast, M2 macrophages produce anti-inflammatory factors and have a reduced capacity to produce pro-inflammatory molecules, thereby contributing to wound healing and tissue remodeling, as well as tumor progression (51–53). Tumor microenvironment educates macrophages to perform supportive roles that initiate and promote tumor progression (28). Tumor-associated macrophages (TAMs) have many properties of M2 phenotype such as impaired expression of IL-12 and TNF-α, and up-regulated levels of M2 markers including Arg-1 and YM1 (54–58). However, TAMs from several tumor models also exhibit typical M1 cytokines such as TNF-α and IL-1β (59). We previously showed that ESC-treated macrophages express higher levels of M2 markers such as PPAR-γ, Arg-1, YM1, as well as M2 cytokines including VEGF, MMP9, and MCP-1 (24), suggesting that ESCs induced the M2-like phenotype. In the present study, we demonstrated that ESCs not only increased Arg-1 and Tie-2^{+} expression but also triggered TNF-α expression, implying that ESC-educated macrophages are different from classic M2 macrophages and resemble more TAMs. ESCs–macrophages not only exhibited M2 characteristics (expression of M2 markers and STAT3/6 activation) but also acquired properties of M1 macrophages (activation of NF-κB) and TAMs (high levels of Tie-2 and TNF-α), exhibiting enhanced neovasculation in an *in vitro* and *ex vivo* angiogenesis assay. Another point of interest is that ESCs also activate the NF-κB pathway. Defective NF-κB activation within macrophages leads to the development of an M2 activation. Although ESCs are not oncogenically transformed, they have potent ability to regulate macrophage function and induce the unique Arg-1^{high}Tie-2^{high}TNF-α^{high} phenotype. Tie-2-expressing monocytes/macrophages (TEM) share some characteristics with M2 macrophages and are highly pro-angiogenic cells critical for tumor vascularization (59, 60). Tie-2 expression can be up-regulated by TNF-α (61). Specific depletion of TEM or conditional *Tie2* knockdown inhibits tumor angiogenesis. (62–64). Within the ESC implantation site, the presence of Tie-2^{+} macrophages and TNF-α secreted by ESC-macrophages stimulates angiogenesis and supports teratoma growth. Several angiogenic molecules may be linked to ESC-induced angiogenesis. We previously showed that ESCs increased macrophage MIF, MMP9, VEGF, and MCP-1. MIF secretion is tightly regulated by TNF-α (65, 66) and MIF can also increase TNF-α expression (67, 68). Therefore, TNF-α may be the key factor in ESC-induced angiogenesis. Anti-TNF-α agents such as infliximab (IFX), etanercept (ETA), adalimumab (ADA), golimumab (GLM), and certolizumab pegol (CZP) have been widely used for the treatment of a variety of chronic inflammatory diseases. Thus targeting TNF-α by administration of TNF-α antagonists may be a promising option to suppress teratoma angiogenesis. However, side effects such as increasing frequency of infection and promoting tumor growth by induction of T cell apoptosis make anti-TNF-α treatment a difficult balance. Administration of CZP can minimize this side effect since it does not induce T cell apoptosis but remains an efficacious treatment for inflammatory diseases because of the lack of an Fc region (69).

Embryonic stem cells can attract macrophages, induce M2 activation, and promote macrophage survival, and consequently, inhibition of any of these three functions could potentially offer a therapeutic solution to prevent teratoma development. A few strategies are developed to target macrophages: inhibiting macrophage migration, suppressing macrophage survival, promoting M1 activation, and blocking M2 polarization (70). Our data suggested that IL-34 maybe important to maintain macrophage survival via activation of PI3K and ERK1/2. Therefore, targeting IL-34 and the PI3K/ERK pathways could decrease macrophage number effectively and alter the microenvironment involved in teratoma angiogenesis and development. Because IL-34 was recently discovered (34), no antagonists are currently available to inhibit IL-34 activity. Thus, in turn, the antagonists of IL-34 receptor (CSF-1R) can be applied to block IL-34 binding to its receptor. Anti-CSF-1R treatment to inhibit tumor growth *in vitro* and *in vivo* has been well-documented (71, 72). Similar strategies can be applied to target macrophages in teratoma models. In addition, combined targeting of the ERK1/2 and PI3K pathways in teratoma may be a potential therapeutic strategy. Numerous small molecule inhibitors of specific PI3K and ERK1/2 pathways have been developed to exhibit promising anti-tumor activity *in vitro* and *in vivo* (73). For example, therapy with a dual PI3K (ZSTK474) and MEK inhibitor (CI-1040) combination is more effective than either inhibitor alone in cancer treatment (74). Combination of the PI3K inhibitor GDC-094 and the MEK inhibitor PD 0325901 induced marked tumor growth inhibition *in vivo* (75). Further study is required to demonstrate whether the dual PI3K and ERK inhibition have anti-teratoma activity *in vivo*, either through direct inhibition of macrophage survival, or ESC growth because PI3K is implicated in regulation of ESC proliferation (76).

Specifically, targeting M2 or TAM-like cells remains challenging. It has been shown that pharmacological skewing of TAM polarization from an M2 macrophage phenotype to a full M1 macrophage phenotype sustains anti-tumor immunity (57). It is possible to re-polarize TAMs. The recent report showed that M2pep, a peptide, can preferentially binds to M2 macrophages with low affinity for other leukocytes. Systemical administration of an M2pep fusion peptide with a proapoptotic peptide specifically reduced M2-like macrophages (77). A combination of CpG oligodeoxynucleotides and an IL-10 receptor-specific antibody switched TAMs from an M2 to an M1 type and triggered an innate response that was able to cure the majority of mice bearing large tumors (78).

One therapeutic option is to target STAT3. Numerous studies demonstrated that constitutive activation of STAT3 promotes initiation and development of tumors by inducing cell proliferation, angiogenesis, and metastasis in a wide variety of tumors (79). Furthermore, STAT3 is a critical mediator of LIF-induced signaling pathways that regulate ES cell self-renewal and proliferation (80). STAT3 also contributes to M2 macrophage activation. Therefore, STAT3 could be an attractive target to control teratoma development by direct effects on ESC growth and macrophage M2-like activation. Numerous strategies to suppress STAT3 activation have been developed such as anti-sense oligonucleotide targeting STAT3, synthetic drugs, small molecules, and gene therapy techniques (79).

In conclusion, our present findings show an important link between ESC-induced macrophage infiltration, growth and activation, initiation of angiogenesis, and teratoma development. ESCs induce BMDM accumulation and stew novel pro-angiogenic phenotype and thus accelerate teratoma development. A better understanding of the regulation and function of macrophages in the tumorigenicity of ESCs may yield useful therapies for the safe transplantation of ESCs. Targeting of the host microenvironment of the transplantation site such as modulating macrophage phenotype and function rather than ESCs directly could be a more efficient approach for suppressing angiogenesis and teratoma progression without affecting the pluripotency of ESCs.

AUTHOR CONTRIBUTIONS

The author(s) have made the following declarations about their contributions: conceived and designed the experiments: Yi Ren. Performed the experiments: Tianxiang Chen, Xi Wang, Lei Guo, Mingmei Wu, Zhaoxia Duan, Jing Lv, Wenjiao Tai, Hemamalini Renganathan, and Ruth Didier. Analyzed the data: Tianxiang Chen, Xi Wang, Jianqing Fan, and Yi Ren. Contributed reagents/materials/analysis tools: Jinhua Li, Xiaoming Chen, Dongming Sun, Xijing He, Jianqing Fan, and Wise Young. Wrote the first draft of the paper: Yi Ren. Contributed to the writing of the paper: Yi Ren.

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Fallen angels or risen apes? A tale of the intricate complexities of imbalanced immune responses in the pathogenesis and progression of immune-mediated and viral cancers

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Excessive immune responses directed against foreign pathogens, self-antigens, or commensal microflora can cause cancer establishment and progression if the execution of tight immuno-regulatory mechanisms fails. On the other hand, induction of potent tumor antigen-specific immune responses together with stimulation of the innate immune system is a pre-requisite for effective anti-tumor immunity, and if suppressed by the strong immuno-regulatory mechanisms can lead to cancer progression. Therefore, it is crucial that the inevitable co-existence of these fundamental, yet conflicting roles of immune-regulatory cells is carefully streamlined as imbalances can be detrimental to the host. Infection with chronic persistent viruses is characterized by severe immune dysfunction resulting in T cell exhaustion and sometimes deletion of antigen-specific T cells. More often, this is due to increased immuno-regulatory processes, which are triggered to down-regulate immune responses and limit immunopathology. However, such heightened levels of immune disruption cause a concomitant loss of tumor immune-surveillance and create a permissive microenvironment for cancer establishment and progression, as demonstrated by increased incidences of cancer in immunosuppressed hosts. Paradoxically, while some cancers arise as a consequence of increased immuno-regulatory mechanisms that inhibit protective immune responses and impinge on tumor surveillance, other cancers arise due to impaired immuno-regulatory mechanisms and failure to limit pathogenic inflammatory responses. This intricate complexity, where immuno-regulatory cells can be beneficial in certain immune settings but detrimental in other settings underscores the need for carefully formulated interventions to equilibrate the balance between immuno-stimulatory and immuno-regulatory processes.

Keywords: regulatory T cells, immune dysfunction, immune-regulation, inflammation, cancer, HIV-1

INTRODUCTION

The observation that a sustained and potent immune response to a foreign pathogen, self-antigen, or normal microflora can be the root cause of uncontrolled cancer outgrowth and progression underscores the need for tight immuno-regulatory interventions that could be harnessed for the development of cancer vaccines and cell-based immunotherapies. On the other hand, inflammatory responses characterized by infiltration of tumor-associated antigen (TAA)-specific T cells and other components of the innate immune system are a pre-requisite for effective anti-tumor immunity. Therefore, it is crucial that the inevitable co-existence of these opposing forces is carefully streamlined as imbalances can be detrimental to the host.

Oncogenic viruses such as Epstein–Barr virus (EBV), human papilloma virus (HPV), and Kaposi sarcoma herpes virus (KSHV) express viral oncogenes, which can directly induce tumorigenic cell transformations and initiate the carcinogenesis process. In the case of non-oncogenic viruses such as hepatitis B (HBV) and hepatitis C (HCV), chronic infection and inflammation can lead to carcinogenic mutations in host cells (1), which are manifested by the increased incidences of liver cancer in chronic HBV and HCV patients. In both of these scenarios, the arising transformed tumor cells are genetically altered in a manner that distinguishes them from ordinary healthy self-cells thus conferring the ability to trigger effector immune responses, which in some cases are capable of controlling tumor growth (2, 3). In other instances, however, such modifications may lead to altered antigenicity and escape from immune-surveillance whereby the newly transformed cells are no longer recognized by their original cognate antigen-specific immune cells, thus leading to uncontrolled cancer progression. On a different platform, continuous antigenic stimulation that occurs during chronic virus infections causes severe immune dysfunction characterized by T cell exhaustion, anergy and in some cases deletion of antigen-specific B and T cells (4–6), and a concomitant induction of immuno-regulatory processes, which all result in the loss of tumor immune-surveillance and lead to cancer establishment. This is indeed supported by epidemiological data showing increased incidences of malignancies such as Kaposi sarcoma (KS)

and cervical cancer, as well as EBV-associated malignancies such as non-Hodgkin lymphoma (NHL) and Burkitt lymphoma in immunosuppressed HIV/AIDS (7) and transplant patients.

Cancer can also arise due to dysfunctional immuno-regulatory mechanisms that result in uncontrolled excessive inflammatory immune responses. For example, pathogenic immune responses directed at commensal intestinal microflora during inflammatory bowel disease (IBD) are known to increase the risk of colon cancer (8, 9). Indeed prolonged periods of ulcerative colitis (UC) and Crohn's disease (CD) are associated with impaired immuno-regulatory mechanisms, which are in turn linked to colitis-associated colon carcinogenesis (10–12). Under normal circumstances both intrinsic and extrinsic regulatory pathways come into force to limit excessive immune activation and inflammation thus preventing tissue pathology and subsequent risk of cancer. However, as in many cases, failures of these control measures, including reduced frequencies or altered phenotype and function of regulatory T cells (Treg) means that this inflammation progresses in leaps and bounds. These paradoxical scenarios highlight a disruption in the natural homeostatic immuno-regulatory mechanisms that can be switched on to prevent excessive immune activation or turned off to allow execution of effector immune responses and tumor immune-surveillance. However, the exact timing of when a "good" immune response aimed at pathogen or tumor clearance can become a very "bad" response that creates an environment conducive for cancer growth and dissemination remains elusive. Understanding the intricate complexities and the timings of these events will be crucial in designing interventions for immune-mediated and viral cancers.

EXTRINSIC AND INTRINSIC IMMUNO-REGULATORY PATHWAYS

A complex network of finely tuned immune-regulation pathways exists to actively inhibit excessive immune responses during chronic viral infections and inflammation. This is essential for preventing the hyper-proliferation of antigen-specific T cells that could cause immunopathology due to increased release of inflammatory cytokines and targeted killing of infected or antigen-expressing tumor cells by CD8+ T cells. Immuno-regulatory pathways can broadly be divided into extrinsic or intrinsic pathways as depicted in **Figure 1**. Intrinsic mechanisms derive from within the effector cell and usually involve down-regulation of activating receptors and up-regulation of inhibitory receptors as well as activation of antagonist mechanisms, as discussed in the next section. Extrinsic pathways on the other hand usually involve other cells, which exert regulatory functions by cell-to-cell contact or via release of suppressive cytokines and biochemical compounds that inhibit cellular functions.

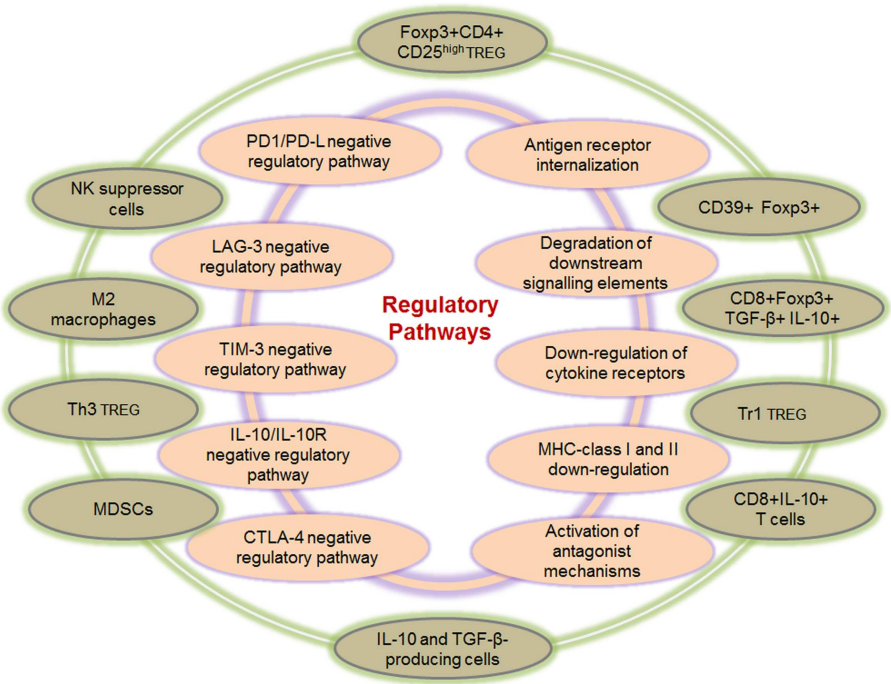

FIGURE 1 | Intrinsic and extrinsic immune-regulatory pathways. Several pathways of immune-regulation exist, and these comprise intrinsic and extrinsic mechanisms. Intrinsic pathways (inner circle) derive from within the effector cell and usually involve up-regulation of inhibitory receptors, down-regulation of cytokine and T cell activation receptors, down-regulation of MHC molecules, as well as the degradation of downstream signaling elements. Although the intrinsic pathways derive mainly from within the effector or antigen presenting cells, interactions with external elements do play a significant role, for instance the down-regulation of MHC class I, which is directly mediated by the HIV-1 Nef protein (13, 14). Extrinsic pathways (outer circle) involve several other cell types that exert immune suppression via cell-to-cell contact or through the release of suppressive cytokines and other biochemical compounds with suppressive activity. These include the various types of regulatory T cells in addition to the Foxp3+ Treg, CD8+ regulatory T cells, MDSCs as well as M2 macrophages and suppressive NK cells.

Of the extrinsic immuno-regulatory pathways, CD4+CD25^{high} Foxp3+ Treg are the most extensively studied and their suppressive mechanisms have been elucidated in greater detail. Existence of other types of immune cells with regulatory functions has been documented, for example CD4+Foxp3− Treg with suppressor functions such as the IL-10 producing Tr1 cells (15) and TGF-β producing Th3 cells (16) have been found in inflammatory environments. Tr1 cells secrete high levels of IL-10 and moderate amounts of TGF-β, and mainly suppress vial IL-10 release, as IL-10 neutralization abrogates their suppressive function (17, 18). On the converse, Th3 cells secrete high levels of TGF-β and low levels of IL-10 and can suppress both Th1 and Th2 responses (16, 17). Other cells with regulatory properties include myeloid-derived suppressor cells (MDSC), which can be induced by cytokines such as IL-6 and growth factors including G-CSF and GM-CSF (19), CD8+Foxp3+ Treg producing both IL-10 and TGF-β, or IL-10-producing CD8+ T cells (20–22), as well as NK cells that possess suppressor functions (23, 24). Activated MDSC can suppress via several mechanisms including IL-10 production as well as via compounds such as arginase 1, reactive oxygen species (ROS), and nitric oxide (NO) among others (19). Moreover, MDSC can indirectly contribute to immuno-regulatory functions by inducing Treg differentiation and expansion.

IMMUNE DYSREGULATION DURING PERSISTENT VIRUS INFECTIONS AND CHRONIC INFLAMMATION

T cells are the key players in many infectious diseases and in eradication of malignant cells. This is well-demonstrated in acute infections where T cells become activated and acquire effector functions, with subsequent clearance of infection and formation of stable memory populations. Moreover, tumors heavily infiltrated with fully functional effector T cells progress less rapidly and in some cases regression can be achieved. However, in the case of persistent antigen stimulation in a chronic setting, memory T cell formation and effector functions are altered, resulting in exhausted, functionally impaired defective T cells incapable of conferring protection. The characteristic properties of these defective cells include diminished cytokine production, decreased cytotoxicity, and reduced proliferative and self-renewal potential. In some cases, mutational escape and/or physical deletion of antigen-specific T cells occurs resulting in inadequate immune control, hence chronic persistence of the viruses. Furthermore, some chronic pathogens directly infect the immune cells, e.g., HIV-1 (CD4+ T cells) and EBV (B cells) leading to loss of immune functions. This state of immunological dysfunction is consistently found in chronic virus infections including HIV, HBV, and HCV (25–27) and is also prevalent in cancer patients. Immune dysregulation can be manifested in several forms, some of which are summarized in **Figure 2** and described below.

T CELL EXHAUSTION

T cell exhaustion refers to a state of progressive loss of immune function, which in some cases, can result in physical deletion of responding cells due to imbalances in the expression of pro-apoptotic and anti-apoptotic factors and the inability to respond to IL-7 and IL-15 (26–28). The dominant mechanism of T cell exhaustion is the up-regulation of several inhibitory receptors, although down-regulation of cytokine receptors such as IL-7Rα and IL-15Rα by exhausted memory T cells is frequently observed. Lower levels of IL-7Rα and IL-15Rα can lead to defective cytokine signaling and consequently impaired homeostatic self-renewal and suboptimal numbers of functional memory T cells (27, 28). Up-regulation of inhibitory receptors such as programed-death 1 (PD-1), T cell immunoglobulin mucin 3 (TIM-3), lymphocyte activation gene 3 (LAG-3), and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) is a characteristic feature of exhausted T cells. PD-1, an inhibitory receptor of the CD28 superfamily is highly expressed on exhausted CD8+ T cells during progressive chronic viral infections and uncontrolled cancer, making it a major factor in T cell exhaustion. Under normal circumstances, PD-1 is induced following T cell activation to inhibit the TCR signaling cascade and prevent excessive T cell activation, but is then down-regulated following pathogen clearance. In peripheral tolerance, PD-1 is important in inhibiting potentially pathogenic self-reactive T cells as well as promoting Treg development (29, 30) and mice lacking PD-1 succumb to autoimmune diseases (31, 32). However, in chronic infection, the PD-1 pathway mediates pathogen-specific CD8+ T cell dysfunction as demonstrated in HIV (33–35), HCV (36, 37), and HBV (38, 39) infections. For example, the frequency of PD-1+CD8+ T cells is highly elevated in HIV-1 patients where it correlates significantly with viral load and declining CD4+ T cell numbers (33, 40). PD-1 is also up-regulated on HIV-specific CD4+ T cells (40, 41) and inhibits CD4+ T cell responses including proliferation. Interestingly, PD-1 levels are significantly reduced in HIV-1 progressors who initiate highly active antiretroviral therapy (HAART) or in long-term non-progressors (LTNPs), suggesting that antigen persistence drives T cells to exhaustion (33, 34). In chronic HCV infection, increased PD-1 expression on HCV-specific CD8+ T cells is associated with impaired proliferation and cytokine production (37). A part from inhibition of T cell function, PD-1 expression can also lead to spontaneous or FAS-mediated apoptosis of virus-specific T cells (42).

Besides PD-1, other inhibitory receptors such as TIM-3, 2B4 (natural killer cell receptor), and LAG-3 are also up-regulated on virus-specific T cells, and the expression of multiple inhibitory receptors correlates with a severely dysfunctional state (43–45). For example, co-expression of PD-1 and TIM-3 is associated with severely exhausted HIV-specific CD8+ T cells (45) and majority of these also co-express PD-1 and 2B4 (46). CTLA-4 is another inhibitory receptor expressed by activated CD4+ and CD8+ T cells. It has a higher affinity for the B7 ligands (CD80 and CD86) allowing it to out-compete CD28, hence it is a powerful negative regulator of CD28-dependent T cell responses. It is significantly up-regulated on CD4+ T cells during HIV-1 and HCV infections where it correlates positively with disease progression and negatively with antigen-specific IL-2 production (41, 47). CTLA-4 is abundantly expressed on Treg as it is required for optimum suppressive function.

IMPAIRED APC FUNCTION

The fact that fully functional pathogen-specific T cells are rarely found in chronic infections suggests impaired antigen presentation, which could be attributed to either inadequate priming by

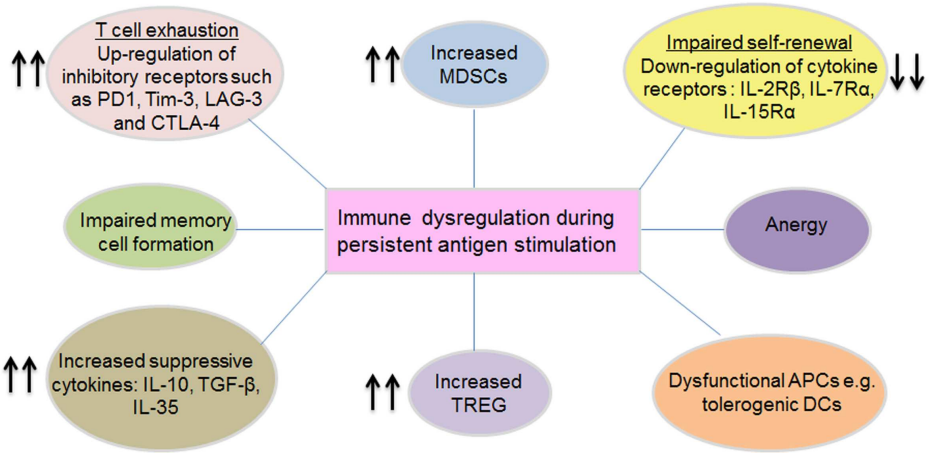

FIGURE 2 | Mechanisms of immune dysregulation during persistent antigen stimulation. Immune dysregulation manifests in several distinct forms, which can occur in isolation or in combination. Persistent antigenic stimulation, especially in settings with high antigen loads can lead to T cell exhaustion (characterized by the up-regulation of several inhibitory receptors and down-regulation of specific T cell activation receptors), anergy (generalized unresponsiveness), impaired memory formation, impaired proliferation, and self-renewal capacity. Besides these, chronic viruses trigger various biochemical pathways that lead to increased frequencies of MDSCs and Treg, which actively suppress effector immune responses via a variety of mechanisms. Immune dysregulation occurring at the T cell priming stage is linked to dysfunctional APCs (for example inadequately activated or tolerogenic DCs), which are likely to skew the immune response toward tolerance. Conceivably, such regulatory mechanisms serve to prevent tissue damage and aberrant immune activation, but they inevitably contribute to the chronic persistence state as a result of inhibiting effector immune responses.

non-professional APCs or possibly altered function of professional APCs during the chronic stages of disease. Indeed, functional impairment of DCs has been associated with T cell exhaustion and progression of disease during HIV, HBV, HCV, and LCMV infection (48–51). Decreased expression of co-stimulatory molecules and lower production of immuno-stimulatory cytokines by APCs can result in functionally tolerant or anergic T cells. Furthermore, chronic infections are associated with loss of DCs, possibly due to direct infection by viruses such as HIV and LCMV. Moreover, DCs can induce T cell exhaustion or tolerance by signaling through inhibitory receptors such as PD-1 and CTLA-4, and also acting via indoleamine 2,3-dioxygenase (IDO)-dependent mechanisms to induce Treg, which further suppress immune responses (52). However, other factors such as virus-induced modulation of the expression of MHC or co-stimulatory molecules have been described and may also significantly affect the generation of fully functional T cells (13, 14).

INCREASED FREQUENCY OF TREG AND MDSC

Increased frequencies of Treg and MDSC are a common feature of persistent chronic viral infections, which is well-documented in infections with HBV (53–55), HCV (56–58), and HIV (52, 59–61). These chronic persistent viruses trigger the production of IL-10 and TGF-β, which in turn increase the frequency and suppressive function of Treg, such as observed in HCV-infected hepatocytes (62). Alternatively, these cytokines may promote the induction of adaptive Treg further reinforcing the immune barrier at sites of infection. HIV and HCV infections also induce plasmacytoid dendritic cells (pDCs) known to induce IL-10-producing Treg via IDO-dependent mechanisms (52, 58). Additionally, the chronic micro-environments created by virus persistence contribute to enhanced Treg proliferation and suppressive function by secreting cytokines and other factors on which Treg thrive. The high frequencies of Treg and MDSC serve an important role of preventing excessive antigen stimulation, persistent inflammatory responses, and viral mediated immunopathology in the chronic stages of viral disease (56, 63). However, the elevated frequencies and enhanced suppressive capacity of Treg and MDSC also contribute to suppression of effector T cells in an antigen-specific or bystander mechanism (64) thus promoting prolonged viral persistence (65, 66) characterized by secondary T cell impairment and exhaustion (67). Thus, counterintuitively, increased expansion and survival of regulatory cells serve to establish, propagate, and maintain the chronic infection state.

INCREASED SUPPRESSIVE CYTOKINES

Apart from Treg and MDSC, increased IL-10 production is another powerful immuno-regulatory mechanism that negatively impacts on the quantity and quality of antigen-specific immune responses. IL-10 is an immuno-regulatory cytokine produced by many cell types and has multiple functions including inhibition of pro-inflammatory cytokine production, dampening T cell responses, blocking APC functions, and also causing B cell dysregulation. Increased IL-10 production is seen in several chronic viruses including HIV, EBV, HCV, HBV, and LCMV (68–75), and IL-10R blockade can induce rapid virus control indicating that excessive levels of IL-10 have a negative influence on the quality of immune responses and disease course (68, 69). TGF-β is yet another immunosuppressive cytokine whose role in limiting immune responses is documented in a number of disease settings (76). Both IL-10 and TGF-β are known to establish highly suppressive micro-environments that are suitable for cancer progression.

DISRUPTION OF IMMUNE-REGULATORY T CELLS IN INFLAMMATORY ENVIRONMENTS

Resolution of inflammation requires swift execution of functional regulatory mechanisms such as the expansion of Treg, a lineage of lymphocytes committed to suppressive functions that maintain self-tolerance and immune homeostasis. Dysregulation of Treg function or induction is linked to a number of chronic inflammatory disorders such as IBD and also fatal autoimmune diseases. Thus, interventions which can restore functional regulation without inducing effector immune responses would be beneficial in such settings. Dysfunctional regulation can manifest as reduced Treg numbers (either due to defective Treg induction or loss of Treg), defective suppressive function (due to loss of Foxp3 expression or reduced production of suppressive cytokines), and impaired migration (due to altered expression of adhesion molecules and chemokine receptors). This section gives a brief description of these mechanisms and the various inflammatory conditions that drive phenotypic and functional modification of Treg.

TREG INSTABILITY: PHENOTYPIC ALTERATION AND FUNCTIONAL IMPAIRMENT

Despite the widely held view of thymic imprinting of Treg cell functions, recent studies indicate developmental plasticity and instability, whereby Treg lose Foxp3 expression and convert to Foxp3− helper T cells (exFoxp3) (77, 78) in certain inflammatory or lymphopenic environments (**Figure 3**). Although exFoxp3 Treg may largely arise from a few promiscuous uncommitted Treg (79), their comparatively higher potential to expand, coupled with the fact that a majority of them are skewed toward self-reactivity suggests potential pathogenicity as a result of altered regulatory functions such as secretion of pro-inflammatory cytokines directed against self-antigens (77). Adoptive transfer studies showed that a large fraction of Treg transferred to lymphopenic recipients lost Foxp3 expression alongside other Treg cell surface markers, and that this was accompanied by acquisition of effector functions including IFN-γ, IL-2, and IL-17-production and a concomitant loss of suppressive function (80–82). Other reports indicated that Foxp3+ Treg effectively lost Foxp3 expression and converted to T helper-type 2 phenotype cells expressing IL-13 and IL-5 (78, 83) or differentiated into follicular helper T cells (Foxp3–T_{FH}-like cells) under the influence of IL-6 and IL-21 (84). Acquisition of T helper features without the simultaneous loss of Foxp3 expression has also been observed. This results in hybrid Treg, which display an activated-memory T cell phenotype and pro-inflammatory properties, such as the IL-17-producing Foxp3+ROR-γt+ IL-17+ (85–90) and IFN-γ-producing Foxp3+T-bet+ IFN-γ+ (91, 92) Treg. Although this hybrid Treg phenotype can exert dual inflammatory and regulatory functions, it has been shown that the phenotypic and transcriptional modifications can reduce their overall suppressive function (81, 93). In other instances however, Treg have been shown to lose their suppressive function without necessarily converting to exFoxp3 or dual function (hybrid) inflammatory Treg (94–100). Such functionally impaired Treg show decreased expression of Foxp3, CTLA-4, and GITR, together with production of very low levels of IL-10 and TGF-β.

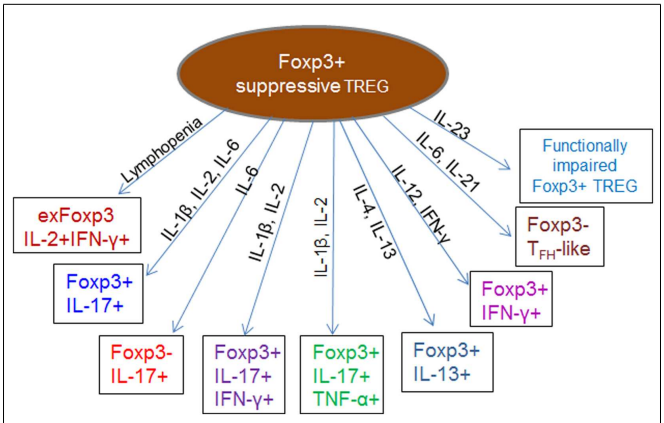

FIGURE 3 | Impaired or altered function of regulatory cells during inflammation. The cytokine and chemokine milieu of inflammatory micro-environments can induce phenotypic and functional modification in Treg, leading to generation of pathogenic exFoxp3 T cells, which express lower levels of Foxp3, CTLA-4, and GITR and produce a combination of Th1, Th2, or Th17 cytokines. Conversion of Foxp3+ Treg into pathogenic IFN-γ-producing Th1 cells or IL-17-producing Th17 exFoxp3 Treg cells is documented in various immunological settings (77, 80, 101). Conversion to a Th2 phenotype expressing IL-13 (Foxp3+IL-13+) and IL-5 (78, 83) as well as differentiation into follicular helper T cells (84) have also been reported. In most cases, the suppressive function of these altered phenotypes is significantly reduced due to decreased Foxp3 expression (81, 82). Certain inflammatory conditions can support the generation of hybrid phenotype Treg, which exhibit dual suppressive and pro-inflammatory functions such as the IL-17-producing Foxp3+IL-17+ (85–90), IFN-γ-producing Foxp3+ IFN-γ+ (91, 92), Foxp3+IL-17+ IFN-γ+, or Foxp3+IL-17+ TNF-α+ (102, 103) Treg. Generally, environments enriched with Th1 cytokines such as IFN-γ, IL-2, and IL-12 favor generation of exFoxp3 Treg producing IFN-γ, those enriched with Th2 cytokines such as IL-4 and IL-13 favor generation of Th2 Treg, while IL-6 favors conversion into the IL-17+Foxp3+ and IL-17+Foxp3− phenotypes.

Several lines of evidence indicate that functional and phenotypic plasticity of Foxp3+ Treg is largely governed by extrinsic signals provided by the inflammatory milieu of their surrounding environments. Increased levels of pro-inflammatory cytokines such as IL-12 or IFN-γ correlate with the frequency of functionally impaired Th1-like Treg (104). In this setting, the Treg suppressive functions were effectively restored by IL-12 withdrawal or IFN-γ blockade suggesting that a pro-inflammatory cytokine milieu not only promotes the Th1-like phenotype, but also inhibits Treg suppressor functions. Overall, inflammatory environments enriched with cytokines such as IL-1β, IL-4, IL-6, IL-21, and IL-23 drive conversion of Foxp3+ Treg into T helper phenotypes (80, 105, 106). As an example, stimulation of peripheral Treg in the presence of IL-6 was shown to result in loss of Foxp3 expression and production of IL-17 (105, 106). Inflammatory environments with IL-1β, IL-2, IL-6, IL-21, IL-23, and TGF-β have been shown to drive conversion of Foxp3+ Treg into IL-17 producing Treg (87, 107), whereas TGF-β, IL-10, and IL-2 help to maintain continued Foxp3 expression, Treg stability, and suppressive function (80, 81, 92, 108–110). Therefore, stable Foxp3 expression and maintenance of optimal Treg suppressive function require the continuous presence of specific signals within the inflammatory environment, without which

conversion of Treg into functionally impaired exFoxp3 T cells or hybrid phenotype Treg occurs (102, 103).

Although the various Treg phenomena described above are well-documented in autoimmune settings, it remains possible that the chronic inflammatory environments created by persistent viral infections can also support phenotypic and functional modifications that would render Treg dysfunctional. In favor of this speculation, a recent study has demonstrated that Treg infected with HIV display increased CpG methylation of the Foxp3 locus and a deregulated functional profile, which was characterized by down-regulation of Foxp3 expression, reduced suppressive capacity, and altered cytokine secretion pattern (111). These Treg showed decreased production of TGF-β and increased IL-4 secretion, a characteristic which is thought to orchestrate severe systemic immune hyper-activation that is observed during progressive HIV disease. In chronic infection with HCV, PD-L1 was found to negatively regulate both the function and proliferation of Treg by controlling STAT-5 phosphorylation (112). Although PD-1 was expressed on both Treg and effector T cells, Treg showed significantly higher up-regulation of PD-1, which was correlated with disease progression. These studies highlight the potential of viruses to subvert the induction and function of Treg, but clearly further research is needed to unravel the mechanisms underlying defective regulation during chronic virus infections.

IMPAIRED OR ALTERED MIGRATION OF TREG

Another crucial aspect contributing to Treg dysfunction is their ability to migrate to peripheral sites of chronic inflammation such as the skin, urogenital mucosa, gut-associated lymphoid tissues (GALT), transplanted organs, or tumors for appropriate localization, in close proximity with effector immune cells as suppression is mostly contact-dependent. To do this effectively, activated Treg up-regulate distinct site-specific inflammatory chemokine receptors and adhesion ligands, which facilitate their migration into the inflamed tissues, usually in response to a variety of inflammatory chemokines that serve as migrational cues (113–117). Therefore, altered chemokine receptor and adhesion molecule expression can affect the migrational properties of Treg and impact on their ability to access sites of chronic inflammation. Such attenuated Treg migration can in turn lead to sustained inflammation and increased risk of inflammation-driven cancer in the Treg inaccessible areas, owing to reduced frequency and suppressive activities.

The crucial role of chemokine-receptor-dependent migration in functional regulation is demonstrated in several experiments including a mouse model of colitis and IBD, where CCR4-deficient Treg had impaired migration to the mesenteric lymph nodes and therefore failed to prevent colitis (118). In other settings, a number of chemokine receptors including CCR2, CCR4, CCR5, CCR6, CCR7, and CXCR3 have been implicated in the selective and preferential recruitment of Treg to sites of chronic inflammation and/or tumors (115, 119, 120), thus indicating that alteration in chemokine receptor patterns or blockade of chemokine receptor signaling would have a significant impact on their migration and immuno-regulatory activities. Tumors and their associated stroma are known to express elevated levels of specific inflammatory chemokines, which serve to chemoattract various leukocytes including Treg (121, 122). Although the overall recruitment is also significantly influenced by the type of chemokine receptors expressed by the leukocytes, Treg, especially the "inflammation-seeking" phenotype usually up-regulate multiple chemokine receptors (116), which allow them access to a variety of tumors, where they preferentially accumulate (119). Some studies demonstrate that disruption of key chemokine receptor signaling axes such as CCR4 or CCR5, or the depletion of chemokine receptor-specific Treg can significantly inhibit their migration and prevent accumulation in tumors (123, 124), thus influencing the overall prognosis. Conceivably, while impaired migration and reduced Treg access to tumors would be an awesome advantage in the majority of cancer settings where they impinge on anti-tumor immune responses, it may however be a major setback in certain other settings, which require Treg to limit excessive immune responses, such as in IBD and chronic virus infections.

LOSS OF TREG (IMPAIRED TREG INDUCTION OR TREG DELETION)

In certain disease settings, physical deletion of Treg can result in reduced frequencies. For instance, it is postulated that by virtue of their activated nature, Treg express higher levels of CCR5 and CXCR4, the co-receptors for HIV-1 thus making them preferential targets for HIV-1 infection (125, 126). Since Foxp3+ Treg represent a high proportion of CD4+ T cells (up to 50%) found in mucosal lymphoid organs of HIV-infected individuals (60), it is plausible that HIV infection can subsequently lead to significant depletion of these cells and impaired immuno-regulatory functions (127–129). Furthermore, Treg also express both Fas and Fas ligand and can be targeted and killed by effector T cells without necessarily being infected. Several studies indicate that myeloid dendritic cells (mDCs) can contribute to Treg induction by promoting conversion of conventional CD4+ T cells into Treg (130, 131). However, it has been shown that *in vitro* HIV infection of mDCs not only impairs their capacity to induce Treg but can also trigger preferential targeting and killing of Treg via a caspase-dependent pathway (132), thus contributing to numerical loss of Treg. Changes in the levels of chemokines expressed within certain tissues, together with diminished levels of TGF-β and IL-2 can also result in the loss of Treg in that particular organ. For example, altered expression of ligands for CXCR3, CCR4, and CCR7 was associated with a loss of Treg in lymph nodes during simian immunodeficiency virus (SIV) infection (133). Other mechanisms for reduced Treg frequencies may include increased apoptosis, reduced proliferation and survival, as well as impaired peripheral Treg induction. As discussed earlier, Treg may also be lost by conversion to exFoxp3 T cells under certain inflammatory cytokine milieu.

IMBALANCES IN IMMUNO-REGULATORY AND IMMUNO-STIMULATORY PROCESSES CAN CAUSE CANCER

Increased risk of cancer is often associated with poorly regulated immune responses (**Figure 4**) constituting unresolved inflammation as a result of perturbations in the balance of tumoricidal and tumorigenic activities (134, 135). Treg play a crucial role in maintaining optimum balance between these two arms of the immune response and persistent viruses are known to trigger production of IL-10 and TGF-β (136) to ensure induction and maintenance

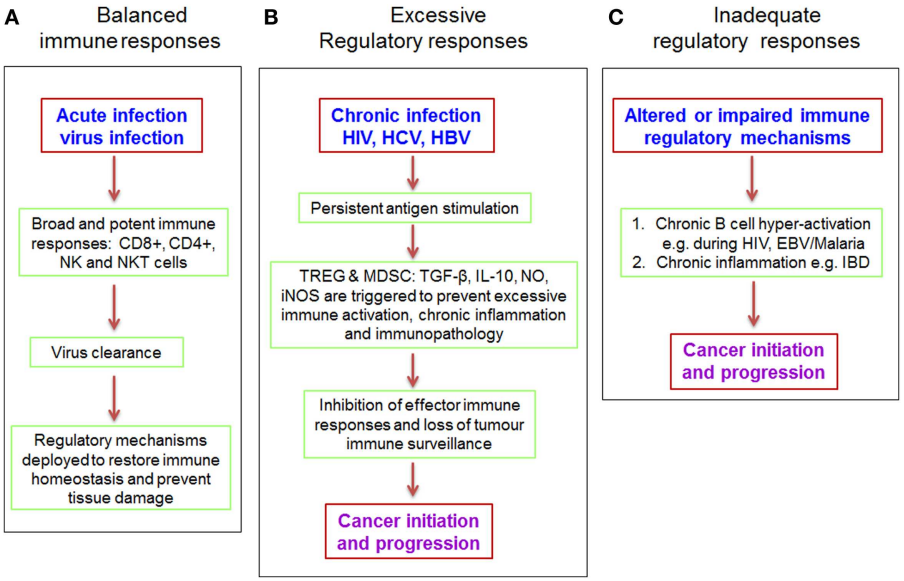

FIGURE 4 | Dysregulated immune responses create a microenvironment suitable for cancer initiation and progression. Perturbations of the balance between effector and regulatory immune responses are often the cause of chronic inflammation and increased risk of cancer (146). Under normal circumstances **(A)**, the immune system mounts a potent and broadly directed immune response following acute virus infection. This immune response, comprising both the innate and adaptive components leads to effective virus clearance and stable memory cell formation, which is effective at rapidly countering subsequent infections. Under this scenario, regulatory mechanisms kick in to prevent tissue damage after virus clearance. However, in the case of persistent antigenic stimulation **(B)** such as caused by HIV, HCV, and HBV, there is continuous generation of effector immune cells that are incapable of clearing the pathogen. This leads to a state of chronic inflammation that in turn triggers regulatory pathways such as increased production of suppressive cytokines and recruitment of Treg and MDSC to dampen excessive immune responses and prevent tissue damage (136). But as fate would have it, such potent regulatory responses also inhibit anti-tumor effector responses leading to loss of tumor immune-surveillance and subsequently cancer initiation and progression. In certain contrasting scenarios such as IBD, impaired regulatory mechanisms **(C)** can result in chronic inflammation, which initiates carcinogenesis. Furthermore, uncontrolled B cell activation during HIV and EBV infections is associated with increased risk of non-Hodgkin's lymphoma (147). NO, nitric oxide; iNOS, nitric oxide synthase.

of adequate numbers of Treg in circulation. In some cases, viruses express homologs of immunosuppressive cytokines or cytokine receptors, such as the well-described human cytomegalovirus (HCMV)-IL-10 and EBV-IL-10 homologs (137, 138), which allow them to directly influence Treg induction or modulate the immune system via other mechanisms including impaired production of pro-inflammatory cytokines and chemokines, as well as MHC class II down-regulation (136). As mentioned earlier, viruses can also promote Treg induction by disrupting the normal activation cascade of dendritic cells and other antigen presenting cells. Furthermore, inflammatory micro-environments are enriched with type 2 macrophages (M2) and MDSC, which also enhance recruitment of Treg, besides directly suppressing antigen-specific effector T cells (19, 139, 140). Additionally, antigen-specific CD8+ Treg are frequently detected in chronic HIV (141, 142), HCV (57, 143), and herpes virus infections (144, 145). The increased numbers of Treg and other immunosuppressive mechanisms serve to actively prevent excessive immune activation and the associated immunopathology, but by so doing, they block antigen-specific effector immune responses that are essential for clearing the pathogen and for tumor immune-surveillance. The resulting immune impairment allows chronic pathogen persistence and an overwhelming state of recurrent inflammation, thus favoring cancer establishment.

Besides the direct disruption of tumor immune-surveillance, establishment of chronic inflammation creates a suppressive tumor-promoting microenvironment, which is enriched with IL-10, TGF-β, and other pro-inflammatory cytokines such as IL-17, known to be angiogenic and to contribute to tumor cell survival and growth (148, 149). In the presence of IL-6, TGF-β can further up-regulate ROR-γt expression leading to enhanced Th17 differentiation (150–152) and increased risk of cancer progression (148, 153–155). Moreover, as discussed earlier, Foxp3+ Treg in certain inflammatory environments can express IL-17, which together with hypoxic conditions could play a role in generation of cancer initiating cells (156). As highlighted in earlier sections, inflammatory environments can also induce phenotypic and functional impairments in immuno-regulatory cells thus leading to dysfunctional immune-regulation and increased risk of cancer. However, whether increased incidence of cancer in individuals with chronic virus infection and inflammation is due to increased suppression of tumor immunity as a result of increased frequency and suppressive activity of immuno-regulatory cells, due to failure of regulatory cells to prevent excessive immune activation and inflammation, or due to enhanced oncogenic potential of the carcinogen remains a subject of intense debate. In this review, I will focus on the contradictory roles of immuno-regulatory cells where they can cause cancer by either exerting

potent suppression of effector immune responses that inhibit tumor immune-surveillance (e.g., during chronic virus infections) or through their functional impairment and inability to execute effective suppression of pathogenic effector immune responses (e.g., during IBD).

THE DELICATE BALANCE BETWEEN IMMUNITY AND REGULATION IN HIV INFECTION AND DISEASE

Although HIV can exist in latent reservoirs for many years, it is a chronic persistent virus characterized by the continuous presence of infectious virus and thus chronic immune activation, persistent inflammation, and concurrent CD4+ T cell loss are all observed (157–159). Thus, increased numbers of fully functional regulatory mechanisms become necessary to counteract the ongoing inflammatory processes. However, Treg, which are instrumental in counteracting immune activation and inflammation by actively suppressing effector immune responses can also be detrimental by inhibiting T cell responses that control HIV replication. An appropriate immune response must therefore not disturb this delicate balance, by aiming to maximize the "good" immune responses, which control the virus while minimizing the "bad" immune responses that cause pathology.

Although still a subject of intense debate, a number of studies have demonstrated increased frequencies of Treg during HIV-1 infection and more especially in the chronic stages that mark progression to AIDS (128, 160–163). Studies looking at tissue distribution revealed accumulation of Treg at sites of HIV infection and replication such as the gastrointestinal mucosa and lymph nodes (59, 60, 128, 164). With such increased frequencies and especially given the suppressive role of Treg, it follows that progression of HIV-1 infection to chronic disease could in fact be a consequence of suppressed T cell function. Indeed, robust CD8+ and CD4+ T cell responses (characterized by high proliferation, IFN-γ production, and cytotoxicity), which correlate with HIV control in a minority of infected people usually diminish during chronic infection, coinciding with increased Treg numbers. Depleting Treg was shown to restore the *in vitro* effector immune functions of these cells (59, 165). Lower levels of Treg and a corresponding higher level of HIV-specific T cell responses have been observed in individuals who naturally control HIV-1 in the absence of HAART, i.e., the LTNPs and Elite controllers (160). Furthermore, depletion of Treg in cord blood samples of HIV-exposed uninfected neonates (166) was shown to augment both CD4+ and CD8+ HIV-specific T cell responses. These findings, together with the observation that Treg frequencies are reduced in HIV-infected patients on HAART (160, 164, 167, 168) provide compelling evidence that Treg impinge on immune control of HIV and strongly support immunotherapeutic interventions that reduce their numbers or impair their functions.

Whereas depleting Treg or interfering with their suppressive function might seem plausible in the context of immune function restoration, in fact several studies indicate that reduced Treg frequencies correlate with increased immune activation, which is in turn significantly associated with higher plasma viral loads (169, 170). Treg can therefore prevent collateral damage during chronic HIV infection by limiting immune activation, while at the same time reducing the pool of activated CD4+ T cell targets that would become susceptible to HIV infection. Accordingly, it is thought that the high frequencies of Treg found in highly exposed persistently sero-negative (HESN) individuals (171) and in the *in utero* HIV-exposed uninfected neonates (166) contribute to resistance to HIV infection by significantly reducing the numbers of activated target CD4+ T cells. These studies suggest that Treg may be beneficial at least to some extent, not only in HIV-infected individuals where they could limit immune activation, but also in highly exposed individuals with a greater risk of HIV infection. However, given that LTNPs and elite controllers exhibit both lower levels of immune activation and lower Treg frequencies (172), while at the same time mounting robust HIV-specific immune responses that inhibit virus replication, it is plausible to suggest that Treg are dispensable in HIV immunity, although caution must be exercised as LTNPs and elite controllers represent a very small proportion of HIV-infected individuals, in whom protective HLA alleles are over-represented.

Contrary to these findings, many studies document persistence of immune activation in the presence of elevated Treg frequencies, suggesting that perhaps the suppressive activities of the Treg found in chronic HIV infection are not sufficient to completely reverse the state of chronic immune activation. Indeed, it has been demonstrated that higher frequencies of Treg exist in HIV-infected individuals with progressive disease (173), but their ability to suppress HIV-specific T cells is significantly reduced, which in turn leads to inability to control HIV-associated aberrant immune activation (111, 161, 174). This is in fact discredited by studies demonstrating the existence of functionally suppressive Treg in progressive HIV-1 disease (59, 165, 175), hence suggesting that failure to reduce immune activation may be due to overwhelming levels of persistent stimulation rather than functional impairment of Treg. Thus, it is possible that high Treg frequencies found in chronic HIV infection are a result of failed attempts to reduce the state of chronic persistent antigenic stimulation (176, 177).

Faced with this paradox, timings of when to initiate interventions remain critical to achieving desirable outcomes. Whereas, immune-based therapies aimed at increasing the frequencies of Treg such as IL-2 therapy may only serve to suppress anti-HIV immunity and provide more targets for HIV, and thus not offer clinical benefit earlier in HIV infection (178, 179), they might indeed become useful during the chronic stages in order to limit immune activation (169, 170). Conversely, depleting Treg during the early stages of infection will allow for generation of robust immune responses capable of controlling virus replication and preventing establishment of latent reservoirs (126).

HIV-ASSOCIATED IMMUNE DYSFUNCTION PREDISPOSES TO MALIGNANCIES

The existence of a few HIV-infected individuals with robust HIV-specific immune responses who maintain very low virus loads for many years without treatment and only progress to AIDS following viral immune escape demonstrates constant immune-surveillance that keeps the virus in check. In these individuals, a normal balance between the effector and regulatory immune responses exists, whereby effective immune responses occur without excessive immune hyper-activation that causes T cell exhaustion and functional impairment. However, in a majority of HIV-infected

people, the immune system does not control virus replication, leading to continuous immune stimulation with high antigen loads and generates a large pool of immune-effector cells that are by far inadequate in controlling the virus. This can be either due to anergy, functional exhaustion, or immune escape (157), as described earlier. Furthermore, HIV directly infects CD4+ T cells and this leads to the progressive diminution of T helper functions and immune incapacitation that marks progression to AIDS.

Besides these, a number of immuno-regulatory mechanisms triggered to prevent immune activation and inflammation also suppress immune-effector functions and sustain chronic virus persistence. For example, during chronic HIV infection, the expansion of Treg (180) with potent suppressive activity within mucosal tissues not only contributes to persistence of HIV, but also reduces immune vigilance and predisposes to HPV and cervical cancer. Moreover, HIV-1 gp120 has recently been shown to induce IL-6 and a concomitant expansion of MDSC (181), which contribute to immune suppression by modulating cytokine and cellular responses as well as inducing the differentiation and expansion of Treg (182). Large amounts of B cell activation-associated cytokines such as IL-6 and IL-10 are produced during chronic HIV infection and can also increase the numbers and suppressive capacity of MDSC leading to further suppression of effective immune responses. Indeed, higher levels of MDSC are associated with chronic progressive HIV disease (183). The decline of both IL-6 levels and Treg numbers following HAART-mediated immune restoration strongly supports their role in immune modulation during HIV-1 progression. A highly immunosuppressive environment with increased numbers of Treg, MDSC, and suppressive cytokines such as IL-10 and TGF-β is strongly associated with increased risk of cancer (19, 182, 184, 185). Thus, HIV-1 can be classified as indirect carcinogen that perturbs immune balance through immune suppression and a concomitant loss of tumor immune-surveillance to set the stage for oncogenic tumor viruses (186).

Another consequence of HIV-driven impairment of the immune system is the hyper-activation and uncontrolled proliferation of B cells, which not only favors secondary infection by oncogenic viruses (187) such as KSHV and EBV but also increases significantly the potential of chromosomal translocations and oncogenic mutations. A few studies have linked HIV infection with chronic B cell hyper-activation (147, 188) and lymphomagenesis, for example, increased incidence of Burkitt lymphoma in HIV-infected individuals or those persistently exposed to *Plasmodium falciparum* in malaria endemic regions where their B cells are in constant stimulation by these antigens (189). HIV-1 can also act directly via gp120 to induce B cell activation and subsequent development of lymphomas (190). Moreover, incorporation of CD40L into HIV virions stimulates B cell activation via interactions with CD40, resulting in production of B cell activating cytokines such as IL-6, IL-8, IL-10, and GM-CSF (191, 192). Indeed, HIV-associated lymphomas are often the aggressive B cell lymphomas, directly supporting a role for HIV in altering the B cell phenotypic and proliferative characteristics.

Therefore, the profound T cell dysfunction, progressive depletion of CD4+ T cells, B cell hyper-activation, together with the increased immuno-regulatory mechanisms all collude to actively impede tumor immune-surveillance and create a permissive environment for cancer initiation and progression. This is a classic example of a "vicious cycle of immune responses" where an effector immune response to a pathogen (in this case HIV) is induced during the initial stages of infection, but somehow fails to eliminate the pathogen, and regulatory mechanisms are triggered in order to restore immune balance and limit excessive inflammation and pathology, yet such regulatory mechanisms actively suppress the anti-tumor immune-surveillance processes and predispose to increased risk of cancer.

HIV-ASSOCIATED MALIGNANCIES

Cancer is a complex multistep process involving many molecular events, which together with the carcinogen or oncogenic virus infection work in concert to generate a transformed cellular phenotype. However, immune response is an important extrinsic factor that determines whether or not cancer occurs following exposure to potential carcinogens. While the immune system of healthy individuals limits proliferation of pre-malignant cells by recognizing and deleting cells that express potentially oncogenic viral proteins, these pre-transformed cells go unchecked and become malignant in immuno-compromised individuals, hence the increased incidence of cancer in transplant patients and those with congenital or secondary immunodeficiency disorders. HIV is not directly oncogenic but it is significantly associated with several lymphoid malignancies known to arise in immuno-compromised individuals who become infected with oncogenic viruses such as HPV, EBV, or KSHV (7). Surveillance data estimates the risk of developing NHL at 60- to 200-fold in people with progressive HIV disease compared to the uninfected population, while that of Hodgkin lymphoma (HL) is 8- to 10-fold, thus supporting the active role of the immune system in controlling cancer. Plausibly, HIV-mediated immune dysregulation contributes to immune escape of these viruses thus allowing for proliferation and emergence of stable populations of virally transformed cells that are not efficiently recognized and eliminated by the host's immune system (187, 193, 194). A wide body of literature documents several AIDS-defining malignancies in the pre-HAART era, but for the purposes of illustrating how immune dysregulation sets a microenvironment conducive for cancer development, this section will draw examples from HIV-associated predisposition to cervical cancer and KS.

HIV-1, KSHV, AND KAPOSI SARCOMA

The non-redundant role of host immunity in the control of viral cancers is well-illustrated by KS, which is more prevalent in untreated HIV/AIDS patients, mainly due to immunosuppression (195). KSHV was discovered as the causative agent of KS in 1994 (196), however, infection with this virus alone is not sufficient to cause KS in healthy immuno-competent individuals. Indeed, the incidence of KS in the general population remains very low (around 1/100,000), but increases dramatically to around 1/20 amongst HIV-infected people (197) and almost 1/3 HIV-infected homosexual men in the pre-HAART era (198). Furthermore, countries in which KS was endemic before the AIDS epidemic have seen a sharp increase in the incidence, with almost half of HIV-infected individuals who acquire KSHV infection going on

to develop KS (199). However, within the endemic areas or in the high risk groups, most HIV-negative KSHV-infected individuals do not develop KS, indicating that HIV-associated immune impairment predisposes to KS development.

HIV-1, HPV, AND CERVICAL CANCER

Human papilloma viruses are the main etiological factor for cervical cancer (200). Of these, HPV-16 and HPV-18 are linked with cervical and anogenital cancers hence are classified as high risk genotypes. As with other cancers, the immune system is central in the pathogenesis of HPV and cervical cancer. In immunocompetent individuals, robust HPV-specific immune responses comprising B and T cells are generated and these correlate with spontaneous resolution of HPV (201, 202), demonstrating that host immunity can be sufficient to clear HPV infection. In particular, a Th1 cytokine profile is instrumental in HPV clearance and prevention of viral persistence. Thus, detection of both humoral and cellular responses including T helper cells induces regression of cervical lesions (203, 204), whereas T helper cell impairment leads to cancer development (205). Natural killer cells also play a protective role by directly lysing HPV-infected cells and initiating regression of squamous intraepithelial lesions (SIL) (206, 207).

Despite the existence of strong HPV-specific immune responses in HPV-infected individuals, progression to HPV-associated malignancies does occur in some individuals due to escape from immune-surveillance caused by immune dysfunction as discussed earlier. Central to this is the systemic enrichment of Treg, which correlates with HPV persistence and is frequently detected in patients who develop high grade cervical intraepithelial neoplasia (208, 209). Furthermore, mucosal enrichment of Treg, which is often associated with diminished cellular immunity in the cervical mucosa has been observed and is linked with the severe forms of cervical carcinoma (210, 211). Higher frequencies of HPV-specific Treg are found in the stroma, intraepithelial tissues and tumor draining lymph nodes of cervical cancer patients where they suppress alloreactive CD4+ responder T cells (212, 213). Depletion of Treg in the *in vitro* experiments resulted in increased production of IFN-γ. Besides enhanced Treg-mediated immunosuppression, the profound immune dysfunction resulting from HIV-1 infection and the concomitant loss of CD4+ T cells collude to create an environment permissive for HPV persistence and cervical cancer. This can be directly deduced from the increased incidence of cervical cancer and prolonged persistence of SIL in immunosuppressed women with progressive HIV disease (214–216). In fact, cervical cancer was designated as an AIDS-defining illness in 1993 (217), strongly implicating HIV-driven immune impairment as a major factor favoring the progression from HPV infection to cancer development.

IMMUNE RESTORATION OR HIV SUPPRESSION REDUCES HIV-ASSOCIATED MALIGNANCIES

There is consensus that HIV-associated malignancies arise mainly due to loss of immune-surveillance caused by a dysfunctional immune system. Indeed, the severity of these malignancies correlates positively with the degree of immune impairment as measured by the extent of CD4+ T cell depletion and HIV viral burden. Moreover, the incidence of AIDS-defining malignancies has significantly reduced since the wide-scale implementation of HAART, strongly suggesting better immune control following reconstitution by HAART or perhaps a direct impact of HAART on the replication of EBV, HPV, and KSHV. Therefore, it seems that interventions which limit virus production and prevent chronic antigenic stimulation can effectively reduce immune activation and inflammation, restore effector immune functions through homeostatic equilibration of immuno-stimulatory and regulatory mechanisms, and lead to reduced incidences of HIV-associated malignancies. Recent studies indicate that the increased frequency and suppressive function of Treg observed during chronic HIV infection decreases significantly following HAART initiation (167). This is accompanied by reduced levels of immune activation and enhanced immune-effector functions, which are in turn associated with decreased prevalence and increased regression of cervical lesions in HAART-treated HPV-infected patients (218–220), thus supporting a role for immune reconstitution in the control of HPV and associated cancers. These observations provide evidence for a strong causative link between HIV-mediated immune dysregulation and the onset of HIV-associated cancers (NHL, KS, and cervical cancer) whose incidence has reduced significantly since the introduction of HAART.

HCV/HBV-DRIVEN IMMUNE DYSREGULATION PREDISPOSES TO HEPATOCELLULAR CARCINOMA

Unlike HIV, which directly targets the immune cells (CD4+ T cells) causing their deletion and loss of T helper functions, HBV and HCV target the liver and replicate in hepatocytes. These viruses have also evolved multiple mechanisms to escape immune elimination and can establish chronic persistence and replicate in infected hosts for many years. Epidemiological studies indicate a strong link between chronic HBV/HCV persistence with the development of liver disease, initially manifesting as chronic hepatitis, and leading on to nodular fibrosis that can progress to cirrhosis and eventually hepatocellular carcinoma (HCC). These processes are characterized by inflammation and oxidative stress owing to the influx of several cell types including NK, NKT, and PMN leukocytes, which accumulate in inflammatory lesions in the liver and contribute to inflammation and liver damage. In a majority of infected individuals, robust and poly functional T cell responses are generated causing clearance of acute infection, while in a minority of those infected, both low frequencies and narrowly focused virus-specific CD8+ T cell responses in the liver correlate with persistent chronic infection and increased risk of HCC (221). Furthermore, defects in HBV-specific CD8+ T cells characterized by exhaustion and increased expression of pro-apoptotic mediators have been reported (222). Thus, although virus-specific lymphocytes can be readily detected in inflammatory lesions in the liver, they are often defective and not sufficient to clear virus infection (223). Moreover, weaker CD4+ T cell proliferative responses have been reported (224).

Infection with HBV and HCV is known to induce IL-10 and TGF-β (72, 73), which in turn induce the expansion of Treg to maintain a tolerogenic environment in the liver. HCV-specific impairment of dendritic cell function can also lead to increased numbers of Treg, and these have been found in both the blood and liver of patients with chronic HBV and HCV infection and HCC

(185), where they correlate with *in vitro* suppression of antigen-specific effector responses (225). These effector responses were enhanced by depleting Treg (54, 226). Overall, immune function restoration and inhibition of viral replication following treatment with anti-HBV drugs is associated with diminished Treg expression (227). Thus, persistence of weak, defective, and narrowly directed T cell responses coupled with high numbers of immune-regulatory cells and increased levels of suppressive cytokines act to promote chronic liver disease and progression to HCC. Indeed, patients with HCC often have increased Treg numbers in blood and within tumors, and the tumor-infiltrating CD8+ and CD4+ T cells have been found to be dysfunctional (228), suggesting a possible link between immune disruption and the pathogenesis of HCC. Other factors such as chronic unresolved inflammation can further support tumor growth via induction of angiogenic and tumor survival signals (229).

IMMUNE DYSREGULATION IN INFLAMMATORY BOWEL DISEASE AND COLORECTAL CANCER

Inflammatory bowel disease is characterized by an uncontrolled, microbe-induced chronic inflammatory state that increases the risk of colorectal cancer (CRC) by twofold (8, 9). These chronic inflammatory responses also drive carcinogenesis of colitis-associated cancer (230). Various cell types infiltrate the inflamed mucosa including MDSC, M2 macrophages, and Th17 cells, which promote tumor growth, and NK and CD8+ T cells, which either target and destroy or inhibit proliferation of CRC cells. These effects are mediated by cytokines such as IL-17A, IL-21, IL-6, and TNF-α that create a tumor-permissive environment versus IFN-γ, which exerts tumor-suppressive functions (231). IFN-γ protects from carcinogenesis by activating cytotoxic T cells as well as increasing the susceptibility of pre-malignant cells to cell-mediated cytotoxicity, thus IFN-γ-producing Th1 cells correlate with increased immune-surveillance and better prognosis in CRC patients (232).

Although IL-4- and IL-13-producing Th2 cells have been associated with increased tumor growth in humans (233) and in animal models using IFN-γ−/− and IL-4−/− deficient mice (234, 235), Th17 cells seem to be the most aggressive orchestrators of chronic inflammation during IBD and have a significant role in the initiation of CRC. This has been linked to IL-23, a cytokine known to induce high numbers of Th17 cells and a concomitant accumulation of pathogenic IL-17A+ IFN-γ+ effector T cells, which cause intestinal pathology and correlate with poor prognosis in CRC (153, 236–238). Indeed, high frequencies of activated Th17 cells together with their signature cytokines are found in the intestinal and serum samples of patients with IBD, and also within the colon and blood samples from patients with CD. Furthermore, IL-23-mediated accumulation of IL-17+IL-22+ innate lymphoid cells (ILCs) in inflamed colons is associated with development of invasive colon cancer (239–241), while increased frequencies of IL-17+ILCs are often found in the intestines of patients with CD (242). The tumor-promoting feature of Th17 cells largely arises from secretion of large amounts of IL-17, which in turn induces expression of pro-inflammatory factors such as TNF-α, IL-6, IL-1, and iNOS, known to play a role in CRC pathogenesis (243). Mice that are deficient in ROR-γt, the transcription factor of Th17 cells were shown to be resistant to chronic inflammation in models of colitis (244). Thus, immuno-regulatory pathways capable of limiting the induction and function of pathogenic Th17 effectors cells are required.

TREG PLAY A CRITICAL ROLE IN THE PATHOGENESIS OF IBD AND CRC

The pro-tumoral role of Treg in cancer establishment and progression is well-documented, and in fact a number of interventions that deplete Treg lead to improved prognosis of cancer patients. Furthermore, Treg depletion increases vaccine-mediated anti-tumor immunity (245) and can lead to eradication of established experimental tumors (210, 246). However, Treg play such a critical role in the maintenance of normal gut mucosal immunity by preventing chronic inflammatory responses to food antigens and commensal microflora (247), that inhibition of their function is associated with development of IBD (11, 12). Most astoundingly, increased infiltration of Treg in CRC correlates with a favorable prognosis (10), with several studies in experimental animal models providing evidence that Treg can prevent establishment of CRC (248, 249). This is thought to be through initiation of potent immuno-regulatory functions that prevent chronic inflammation, which would otherwise predispose to cancer establishment and growth. Under normal homeostatic conditions, high frequencies of Treg are found in the gut as it is a preferential site for peripheral Treg induction due to the abundant commensal micro-biota and CD103-producing DCs, which are specialized in inducing the differentiation of Treg from naïve CD4+ T cells (250, 251). However, inadequate regulatory functions are a major characteristic defect during IBD, suggesting alterations in the induction, maintenance, or even suppressive function of Treg. This section highlights some of the mechanisms of immune dysregulation that exacerbate the inflammatory state of IBD to set a stage for CRC.

TREG INDUCTION AND FUNCTION ARE IMPAIRED IN IBD AND CRC

Impaired frequency and function of Treg is one of the mechanisms of immune dysregulation that plays a central role in the pathogenesis of IBD. This is strongly associated with IL-23, a cytokine whose expression is increased in several human cancers including CRC (252). IL-23R signaling suppresses both the differentiation of Treg and IL-10 production by T cells, hence leading to intestinal pathology (236). Such pathology could be prevented by transfer of Treg or administration of Treg-related cytokines such as IL-10 and TGF-β1 (253). TGF-β signaling in tumor-infiltrating lymphocytes is associated with reduced tumor growth in animal models of CRC (254). Crucially, the frequency of Foxp3+ Treg in the colon increases in the absence of IL-23R signaling, indicating a role for IL-23 in controlling the induction and expansion of Treg (255). Since Treg are a source of both IL-10 and TGF-β, the key cytokines in immuno-regulation, it is plausible that IL-23-driven loss of Treg contributes significantly to immune dysregulation by overriding the immunosuppressive pathways in the intestine and favoring IBD and CRC development via generation of pathogenic Th17 effectors cells. Besides reduced numbers, Treg in IBD show altered phenotype and function, attributed to the local cytokine milieu arising from chronic inflammation of the intestinal mucosa. Perhaps, normal Treg in circulation migrate to the lamina propria during active inflammation in order to maintain homeostasis, but

on encountering various cytokines within the inflamed mucosa, they undergo phenotypic and functional modifications turning into dual inflammatory and regulatory Foxp3+IL-17+ Treg, which produce large amounts of IFN-γ and IL-17, and moderate amounts of TNF-α and IL-2 (102, 103).

IL-10 PROTECTS AGAINST IBD AND CRC

IL-10 deficiency increases susceptibility to IBD-associated CRC, where it is associated with poor prognosis (256). Mice lacking IL-10 were shown to be highly susceptible to colitis-associated CRC following *Helicobacter hepaticus* infection, and this could be prevented by exogenous administration of IL-10 (257–259), further demonstrating a critical role for IL-10 in the pathogenesis of CRC. It is thought that IL-10 deficiency leads to elevated levels of TNF-α, IL-6, and IL-17, which in turn allow persistence of chronic inflammation (260) thus promoting tumor growth.

BI-FUNCTIONAL IMMUNE-EFFECTOR CELLS CAN PROMOTE IBD AND CRC

Intriguingly, a single cell type can exhibit bi-functional immune characteristics by co-producing effector and suppressor cytokines, thus may have the potential to exert both tumor-promoting and tumor-suppressive functions, depending on the microenvironment. For example, as mentioned above, CD8+ T cells express cytotoxic molecules, which kill CRC cells in addition to secreting IFN-γ, which augments the anti-tumor response (261, 262). However, in some cases of IBD, infiltration of CD8+ T cells does not correlate with improved prognosis (263) and this is linked to elevated perforin and granzyme levels, which sustain the tumor-promoting chronic inflammation (264). Accordingly, perforin deficient mice develop less severe colitis and much fewer tumors in experimental models of colitis-associated CRC (265). Similar bi-functional characteristics have been observed in NKT cells, which exert protective cytotoxic functions, but also secrete Th1, Th2, and Th17 cytokines that could act as enhancers or suppressors of tumor immunity. Increased infiltration of IFN-γ-secreting NKT cells correlates with tumor immunity, which is reflected in increased disease-free survival of CRC patients (266, 267). Conversely, Th2 NKT cells that secrete the immunosuppressive cytokine IL-13, may contribute to colitis-associated CRC (268, 269). These studies demonstrate that CD8+ T cells and NKT cells can simultaneously exert pro-tumoral and anti-tumoral responses, and that perhaps pro-tumoral responses predominate during progressive IBD and CRC. Arguably, intervention strategies targeted at these bi-functional effector cells may result in undesirable outcomes.

INTERVENTIONS

As discussed earlier, some settings such as inflammatory autoimmune diseases will require interventions that boost the immunoregulatory arm of the immune response. Such may include immunotherapeutic agents that expand Treg numbers and enhance suppressive function to effectively curtail chronic inflammation. Therapeutic vaccines to restore immune tolerance could benefit from adjuvants that induce adaptive Treg without generating functional effector cells (270). Other measures such as restoration of TGF-β and IL-10, together with IL-2 administration can help to maintain Treg numbers and Foxp3 expression, thus sustaining functional regulation. In other inflammatory settings such as IBD and colon cancer, measures that enhance Treg differentiation and expansion and restore suppressive function, for example, blockade of IL-23 signaling with the concurrent depletion of IFN-γ and IL-2 to impede generation of pathogenic exFoxp3 Treg might be desirable. Additionally, induction of stable expression of site-specific homing and chemokine receptors in Treg can confer the ability to migrate to preferential sites of chronic inflammation, for example, CCR4 for migration to the lung airways during allergic inflammation, CXCR4 for migration to the bone marrow, and CCR4/CCR9/CD62L/$\alpha_4\beta_7$/α_E(CD103)β_7 for migration to the intestinal mucosa of IBD patients. However, in cases where immuno-regulatory responses are detrimental then immune deregulation interventions are required. Such can include administration of cytokines and/or antibodies that inhibit Treg induction and expansion, suppressive function, and recruitment via blockade of chemokine receptors (124). Interventions such as concurrent CTLA-4 blockade and vaccination (271–273), combined CTLA-4 and PD-1/PD-L1 blockade (274), and Treg depletion (275–277) have been successfully used to ameliorate Treg-mediated immune pathologies and cancer. Measures to reverse exhaustion and restore immune function in chronic infections include blockade of the PD-1:PD-L1/PD-L2 pathway and MDSC development. PD-1/PD-L1 blockade restores HIV-specific T cell function *in vitro* (33, 34, 278), and clinical benefit is also documented in cancer patients (279, 280). In some instances, combined blockade of PD-1 and LAG-3 or PD-1 and TIM-3 synergistically improves T cell responses leading to better virus control (43, 45). Very recently, a study utilizing a mouse model of retrovirus infection showed that combining the blockade of inhibitory receptors PD-1 and Tim-3, together with Treg ablation was more efficient in reducing chronic virus load compared with either strategy on its own (281). Functional blockade, developmental inhibition, or physical deletion of MDSC was shown to enhance the efficacy of cancer vaccines in animal models (282–284).

CONCLUSION

The role of the immune system in inflammation and carcinogenesis is highly influenced by the microenvironment, thus some disease settings can display unique characteristics where immunoregulatory processes are highly beneficial to the host but in other cases quite detrimental and predispose to pathogen persistence and increased risk of cancer. This calls for tailor-matched interventions, which are quite promising, however caution must be exercised since blocking an inhibitory pathway might re-invigorate the immune system to achieve disease control on one hand, but exacerbate immune activation and inflammation on the other. Overall, the timings of these interventions will be crucial in order to achieve favorable outcomes.

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How do CD4$^+$ T cells detect and eliminate tumor cells that either lack or express MHC class II molecules?

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CD4$^+$ T cells contribute to tumor eradication, even in the absence of CD8$^+$ T cells. Cytotoxic CD4$^+$ T cells can directly kill MHC class II positive tumor cells. More surprisingly, CD4$^+$ T cells can indirectly eliminate tumor cells that lack MHC class II expression. Here, we review the mechanisms of direct and indirect CD4$^+$ T cell-mediated elimination of tumor cells. An emphasis is put on T cell receptor (TCR) transgenic models, where anti-tumor responses of naïve CD4$^+$ T cells of defined specificity can be tracked. Some generalizations can tentatively be made. For both MHCII^{POS} and MHCII^{NEG} tumors, presentation of tumor-specific antigen by host antigen-presenting cells (APCs) appears to be required for CD4$^+$ T cell priming. This has been extensively studied in a myeloma model (MOPC315), where host APCs in tumor-draining lymph nodes are primed with secreted tumor antigen. Upon antigen recognition, naïve CD4$^+$ T cells differentiate into Th1 cells and migrate to the tumor. At the tumor site, the mechanisms for elimination of MHCII^{POS} and MHCII^{NEG} tumor cells differ. In a TCR-transgenic B16 melanoma model, MHCII^{POS} melanoma cells are directly killed by cytotoxic CD4$^+$ T cells in a perforin/granzyme B-dependent manner. By contrast, MHCII^{NEG} myeloma cells are killed by IFN-γ stimulated M1-like macrophages. In summary, while the priming phase of CD4$^+$ T cells appears similar for MHCII^{POS} and MHCII^{NEG} tumors, the killing mechanisms are different. Unresolved issues and directions for future research are addressed.

Keywords: tumor immunology, CD4$^+$ T cells, MHC class II, T cell receptor transgenic, transgenic mouse models, tumor antigen, T helper 1, multiple myeloma

RECENT ADVANCES IN TUMOR IMMUNOLOGY

The field of tumor immunology has come a long way since the formulation of the tumor immunosurveillance hypothesis by Thomas and Burnet (1–4). Although still debated, increasing evidence suggests that the immune system can detect and reject incipient tumors, and that CD4$^+$ and CD8$^+$ T cells play an important role as mediators of immunosurveillance (5). Furthermore, there is accumulating evidence that the immune system is not completely tolerant even to established tumors, based on the observation that tumor-infiltrating T cells, when expanded *in vitro* and injected back to lymphopenic patients, have a clinical effect in some patients (6). Further supporting the notion of ongoing immune responses to tumors, antibodies that block inhibitory molecules on T cells induce long-term remission in a subset of cancer patients (7). Finally, parameters that indicate immune activation in tumors are associated with improved prognosis (8).

Abbreviations: APC, antigen-presenting cell; CIITA, class II trans-activator; FasL, Fas ligand; HA, hemagglutinin; Id, idiotype; IFN-γ, interferon gamma; Ig, immunoglobulin; MHC, major histocompatibility complex; MM, multiple myeloma; MOPC, mineral oil-induced plasmacytoma; OVA, ovalbumin; s.c., subcutaneous; TCR-Tg, T cell receptor transgenic; TIL, tumor-infiltrating lymphocyte; Trp1, tyrosinase-related protein 1; SCID, severe combined immunodeficiency.

CD4$^+$ VERSUS CD8$^+$ T CELLS IN TUMOR IMMUNOLOGY

Traditionally, CD8$^+$ T cells have been thought to be the major mediators of effective anti-tumor T cell responses. Such a view is supported by the pronounced cytotoxic activity of CD8$^+$ T cells *in vitro*, and the observation that tumors that escape CD8$^+$ T cells onslaught may have altered or downregulated MHC class I antigen expression (9–11). Moreover, studies done in an MHC class I-restricted T cell receptor (TCR) transgenic mouse showed that CD8$^+$ T cells, in the absence of CD4$^+$ T cells, maintained their anti-tumor effect (12). Despite these observations, several studies indicate limited anti-tumor effects of CD8$^+$ T cells alone (6, 13–16).

The helper function of tumor-reactive CD4$^+$ T cells improves the efficacy of tumor-reactive CD8$^+$ T cells (17–20). Similarly, treatment of a patient with metastatic melanoma with autologous CD4$^+$ T cells specific for the tumor-associated antigen NY-ESO-1 resulted in sustained clinical remissions with evidence

of endogenous immune responses against other tumor-derived antigens (21). In support of these findings, transfection of tumor cells with MHC class II genes resulted in increased protective immune responses against tumors (22, 23). Collectively, these results indicate an augmenting effect of $CD4^+$ T cells on $CD8^+$ T cell responses against tumors.

On the other hand, $CD4^+$ T cells alone, in the absence of $CD8^+$ T cells, have also been demonstrated to eliminate tumor cells. Thus, adoptive transfer experiments using primed $CD4^+$ T cells generated by immunization with tumor cells conferred protection against a subsequent tumor challenge (24, 25). Moreover, naïve $CD4^+$ T cells in TCR-transgenic mice conferred protection against tumor development upon subcutaneous (s.c.) injection of tumor cells (26, 27). Finally, using MHC class I-molecule and MHC class II-molecule restricted TCR-transgenic mice specific for the Dby H–Y antigen, $CD4^+$ T cells were found to be more efficient at eradicating cancer cells than $CD8^+$ T cells in a side-by-side comparison (28). Here, we will focus on the anti-tumor properties of $CD4^+$ T cells in the absence of $CD8^+$ T cells.

PIONEERING EXPERIMENTS ON THE ROLE OF $CD4^+$ T CELLS IN ERADICATION OF TUMORS

The role of $CD4^+$ T cells was initially investigated in experiments where tumor-bearing mice were treated by adoptive transfer of T cells obtained from syngeneic mice immunized with irradiated tumor cells (25, 29), or with living tumor cells followed by surgical resection (24). It was shown that when T cells from tumor-immunized donors were purified prior to adoptive transfer, $Lyt1^+$ 2^- ($CD4^+$) T cells had a superior ability to cure FBL-3 erythroleukemic tumors compared to $Lyt1^-$ 2^+ ($CD8^+$) T cells (29). Treatment with cyclophosphamide was required for the curative effect of $CD4^+$ T cells to be observed. However, in the first reported experiments (29), a role of endogenous $CD8^+$ T cells in the tumor-bearing host was not ruled out. In follow-up experiments, this possibility was formally excluded by the use of T cell deficient tumor-bearing recipients (25). Similar results were obtained using the X5563 plasmacytoma model (24), where transfer of purified $Lyt1^+$ 2^- ($CD4^+$) T cells had superior therapeutic potential. In the following decades, experimental evidence supporting the anti-tumor properties of tumor-specific $CD4^+$ T cells alone has accumulated (27, 28, 30–39).

TCR-TRANSGENIC MODELS FOR $CD4^+$ T CELL-MEDIATED REJECTION OF TUMORS

The experiments referred to in the preceding section had features that prohibited detailed studies of the mechanisms of $CD4^+$ T cell-mediated tumor protection. First, the $CD4^+$ T cells were polyclonal. Second, $CD4^+$ T cells were pre-primed cells obtained after immunization, making it impossible to study naïve $CD4^+$ T cells in primary anti-tumor responses. Third, the relevant tumor-specific antigens were often not known.

The generation of TCR-transgenic mice that recognize tumor antigens presented on MHC class II molecules (**Table 1**) offered a novel approach to bypass these difficulties. In two models, these antigens are *bona fide* cancer antigens; the tumor-specific myeloma protein V region idiotype (Id) (26, 27) and the melanoma-associated tyrosinase-related protein 1 (Trp1) (35). In other TCR-transgenic models, the antigens are either minor histocompatibility antigen Dby (H-Y) (28), viral antigens such as the hemagglutinin (HA) (40–42), or xenogeneic proteins such as ovalbumin (OVA) (17, 43, 44). While the transgenic TCR specific for the mutated myeloma antigen was obtained after immunization of mice syngeneic to the tumor (45, 46), the transgenic TCR specific for the non-mutated antigen was obtained after immunization of Trp1-deficient mice. Thus, in the latter model, Trp1 represents a foreign antigen to which high-affinity TCRs are induced (due to a lack of T cell tolerance) (35).

Table 1 | TCR-transgenic models employed in studies of anti-tumor $CD4^+$ T cell responses.

TCR-Tg model	Antigen	Classification of antigen	Antigen location	MHC II restriction	Peptide	Reference
4B2A1 ($\lambda 2^{315}$)	Light chain idiotype (Id) of mouse M315 myeloma protein	Mutated tumor-specific antigen	Secreted, plasma membrane (52, 53)	$I\text{–}E^d$	aa91–101	(46)
7A6 (Trp1)	Mouse tyrosinase-related protein 1	Melanocyte-specific differentiation antigen	Secreted, melanosome membrane (54)	$I\text{–}A^b$	aa113–125	(35)
Marilyn (H–Y)	Minor histocompatibility antigen (Dby)	Tissue antigen	Secreted, cell membrane (55, 56)	$I\text{–}A^b$	aa608–622	(47)
T2.5-5 (HA)	Influenza PR8 hemagglutinin	Viral antigen	Varying (construct dependent)[1]	$I\text{–}A^d$	aa126–138	(48)
14.3.d (HA)	Influenza PR8 hemagglutinin	Viral antigen	Varying (construct dependent)[1]	$I\text{–}E^d$	aa110–120	(49)
DO11.10 (OVA)	Chicken ovalbumin	Xenogeneic model antigen	Varying (construct dependent)[2]	$I\text{–}A^d$	aa323–339	(50)
OT-II (OVA)	Chicken ovalbumin	Xenogeneic model antigen	Varying (construct dependent)[2]	$I\text{–}A^b$	aa323–339	(51)

[1]*Varyingly expressed by fusion to other proteins, which control cellular distribution. The viral protein, as such, localizes to the cell surface (57).*

[2]*Varyingly expressed as full-length cDNA [containing signal sequence for secretion (58)] or fused to other proteins, which control cellular distribution.*

MHC CLASS II STATUS OF TUMOR CELLS USED IN TUMOR IMMUNOLOGY STUDIES FOCUSED ON THE ROLE OF CD4+ T CELLS

CD4+ T cells recognize peptides (about 13–17aa long) bound to the groove of MHC class II molecules (59) on professional antigen-presenting cells (APCs) (B cells, dendritic cells, macrophages, in addition to thymic epithelial cells) (60–62). However, in certain cells, MHC class II molecules may be induced by interferon gamma (IFN-γ) stimulation (63, 64). Thus, in CD4+ T cell immune responses to tumors, the MHC class II status of the tumor cells is of importance. The MHC II expression status of tumor cells used in studies with CD4+ TCR-transgenic mice is summarized in **Table 2**.

DIRECT AND INDIRECT KILLING OF TUMOR CELLS BY CD4+ T CELLS

The antigen-specific interaction between CD4+ T cells and MHC II^{POS} tumor cells is conceptually easy to grasp. On the other hand, the basis for antigen presentation and anti-tumor effector mechanisms are less obvious in the context of MHC II^{NEG} tumors (25, 26, 31, 70) – simply because such cancer cells cannot directly stimulate MHC class II-restricted CD4+ T cells (**Figure 1**). In the following sections, we discuss mechanism of CD4+ T cell-mediated direct killing of MHC II^{POS} tumor cells and indirect killing of MHC II^{NEG} tumor cells. Emphasis is put on observations from TCR-transgenic models, where the T cell specificity is known and both naïve and primed CD4+ T cells are readily available.

Table 2 | Use of TCR-Tg models for studies of anti-tumor CD4+ T cell immune responses.

TCR-Tg model (antigen)	Tumor cell line	Ectopic antigen expr.[a]	MHC II expr.	Antigen secreted?	T cell source	Reference
4B2A1 (λ2^{315})	MOPC315 (plasmacytoma)	No	–	Yes	Naïve (endogenous)[b]	(26, 27, 34, 65)
	MOPC315.37[c]	No	–	No	Naive (endogenous)	(36)
	A20 (B lymphoma)	Yes	+	Yes	Naive (endogenous) Adoptive transfer, naive	(26, 33, 66)
	A20 (B lymphoma)[d]	Yes	+	No	Naive (endogenous)	(26)
7A6 (Trp1)	B16/CIITA (melanoma)	No	+[e]	N/D	Naive (endogenous) Adoptive transfer, activated	(35)
	B16 (melanoma)	No	+[f]	N/D	Adoptive transfer, naïve Adoptive transfer, activated	(37, 38)
Marilyn (H–Y)	MB49 (bladder)	No	+[f]	N/D	Adoptive transfer, naive	(28)
	TRAMP-C2 (prostate)	No	–	N/D	Adoptive transfer, activated	
	βTC-TET	No	–	N/D		
	WR21 (salivary gland)	No	–	N/D		
T2.5-5 (HA)	AB1 (mesothelioma)	Yes	–	N/D[g]	Naive (endogenous) Adoptive transfer, naive	(40)
14.3d (HA)	CT26 (colon)	Yes	N/D[h]	N/D[i]	Naive (endogenous) Adoptive transfer, naive	(41, 42)
DO11.10 (OVA)	A20 (B lymphoma)	Yes	+	N/D[j]	Adoptive transfer, activated	(17)
	A20 (B lymphoma)	Yes	+	No[k]	Naive (endogenous) Adoptive transfer, activated	(44)
OT-II (OVA)	EG-7 (thymoma)	Yes	–	Yes[l]	Adoptive transfer, activated	(43)

N/D, not determined.

[a] Ectopic antigen expression signifies that the tumor cell line was transfected for expression of the relevant antigen.

[b] The designation naive (endogenous) is used to describe tumor challenge experiments in TCR-Tg mice in which no prior priming of antigen-specific T cells was performed.

[c] MOPC315.37 contains a Gly15→ Arg15 mutation within the λ2 gene that causes intracellular retention (67).

[d] Cells were transfected with a mutated λ2^{315} variant that causes retention within the endoplasmic reticulum, precluding secretion (67).

[e] Cells were transfected to overexpress MHC class II trans-activator (CIITA) to ensure high levels of expression of MHC II (35).

[f] Inducible expression by interferon gamma stimulation.

[g] Only cell surface expression was tested (40).

[h] A previous publication reports constitutive MHC II expression in vitro (68).

[i] Cells were transfected with HA fused to EGFP. Only surface expression was tested (41).

[j] Secretion expected; cells were transduced with constructs containing the full-length OVA cDNA sequence, which contains signal element for secretion (58).

[k] Cells were transfected with OVA fused to the trans-membrane domain of transferrin receptor, causing membrane expression (44).

[l] Earlier report demonstrates secretion from the same cell line (69).

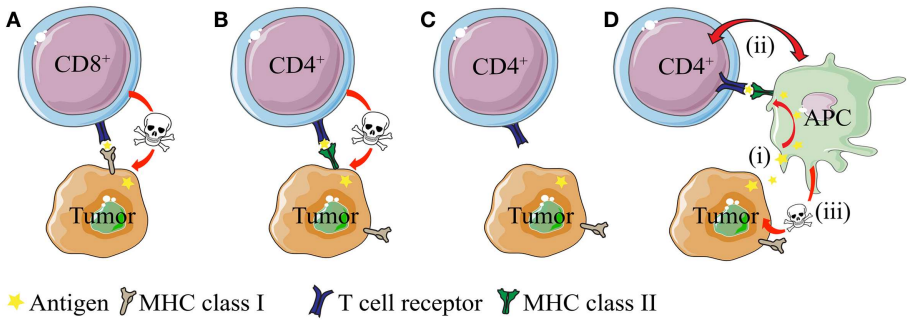

FIGURE 1 | Direct and indirect killing of tumor cells by CD4+ T cells. **(A)** $CD8^+$ T cells can directly kill tumor cells that express MHC class I molecules, whereas **(B)** cytotoxic $CD4^+$ T cells can kill tumor cells that express MHC class II molecules. **(C)** While most tumor types express MHC class I molecules, they often lack expression of MHC class II. How do $CD4^+$ T cells recognize and eliminate $MHCII^{NEG}$ tumor cells? **(D)** $CD4^+$ T cells may kill MHC class II negative ($MHC\ II^{NEG}$) tumors by a mechanism where (i) tumor antigen secreted by tumor cells is processed and presented by $MHCII^{POS}$ macrophages to $CD4^+$ T cells. (ii) Bi-directional interaction/activation of macrophages and $CD4^+$ T cells (iii) activates tumoricidal macrophages that in turn kill the tumor cells (In addition, activated $CD4^+$ T cells themselves could possibly directly kill tumor cell in a TCR/MHC II-independent manner.).

DIRECT KILLING OF MHC CLASS II^{POS} TUMOR CELLS

The existence of $CD4^+$ T cells with cytotoxic properties has been increasingly recognized throughout the last three decades. Such cells are thought to function in a fashion analogous to cytotoxic $CD8^+$ T cells, with antigen recognition triggering the release of cytotoxic mediators. $CD4^+$ T cells displaying direct cytotoxicity *in vitro* toward MHC II^{POS} targets, including tumor cells, have been described by several authors (37, 45, 70, 71). Correspondingly, efficient elimination of MHC II^{POS} tumors by T cells with such properties is also observed *in vivo* (26, 28, 33, 35, 37, 38, 72).

Several effector mechanisms have been implicated for tumor-specific cytotoxic $CD4^+$ T cells. In a model of Id-specific $CD4^+$ T cell responses against an MHC II^{POS} B lymphoma, *in vitro* cytotoxicity was shown to be dependent on signaling mediated by binding of Fas ligand (FasL) on $CD4^+$ T cells to the death receptor Fas on tumor cells (66). Naïve T cells showed little killing activity, whereas Th1 differentiation greatly enhanced cytotoxicity. However, *in vivo* elimination of tumor cells was not affected in FasL-deficient ($gld^{-/-}$) Id-specific TCR-Tg mice, suggesting that signaling through the Fas pathway is dispensable for tumor killing and that additional mechanisms are operational *in vivo* (66). Indeed, if the tumor antigen is secreted as is the case in the studies of Lundin et al. (33, 66), the indirect mechanism via Th1/M1 macrophages described below could also be active, and might play a prominent role in tumor rejection. In the Trp1-specific TCR-transgenic model, it was demonstrated that the rejection of B16 melanoma cells was abrogated in mice deficient for either granzyme B or perforin, indicating that these molecules are important for $CD4^+$ T cell-mediated killing of MHC II^{POS} tumor cells (37). In summary, different MHC II^{POS} tumors may vary in susceptibility to various effector mechanisms of $CD4^+$ T cells, as indicated by the observations addressed above.

INDIRECT KILLING OF MHC CLASS II^{NEG} TUMOR CELLS

In general, antibody-secreting plasma cells are MHC class II negative due to silencing of the MHC Class II trans-activator (CIITA) occurring during plasma cell differentiation (73, 74). Multiple myeloma (MM) is the malignant counterpart of plasma cells and usually express little if any MHC class II molecules. MHC class II negativity due to loss of CIITA expression appears to be a stable phenotype, although some studies have reported MHC II upregulation in MM cells exposed to retinoic acid (75) or IFN-γ (76, 77).

The work of our research group is based on experiments using the mineral oil-induced BALB/c plasmacytoma (MOPC)315 (52, 70). MOPC315 cells secrete a highly mutated and unique monoclonal IgA (myeloma protein). The λ2 light chain of the myeloma protein contains somatic mutations in positions 38, 50, 94, 95, and 96 that are unique to MOPC315 (78). Thus, the myeloma protein light chain is referred to as $\lambda 2^{315}$ (**Figure 2A**).

By immunization of BALB/c mice with free $\lambda 2^{315}$ L chain, known from previous studies to stimulate T cells (81), I-E^d-restricted, Id-specific $CD4^+$ T cell clones were generated (**Figure 2A**) (45). These clones recognize a unique Id-epitope, which depends on the somatic mutations in codons 94, 95, and 96 within the CDR3 loop of the $\lambda 2^{315}$ light chain (79). As would be expected, MOPC315 derived $\lambda 2^{315}$-immunoglobulin has to be endocytosed and processed by APCs prior to MHC class II presentation of the Id-peptide (80).

MOPC315 is found to be MHC class II negative by a number of criteria: (i) Negative staining with anti-MHC class II antibodies both *in vitro*, *ex vivo* (70), and *in vivo* (65). Lack of expression of MHC II molecules on MOPC315 was independently reported by others (82). (ii) Exposure to high amounts (500 ng/ml) of IFN-γ IL-4, or supernatant from activated Th1 cells, all failed to induce any detectable expression of MHC class II *in vitro* (70). (iii) Both *in vitro*-cultured (70) and *ex vivo* (65) MOPC315 cells failed to stimulate Id-specific MHC class II-restricted T cells in proliferation and cytokine secretion assays.

IDIOTYPE-SPECIFIC $CD4^+$ T CELL CLONES INDUCE KILLING OF MHC CLASS II NEGATIVE MYELOMA CELLS *IN VITRO* – BUT ONLY IN THE PRESENCE OF MHC-COMPATIBLE APCs

A weak cytotoxicity that was greatly augmented by addition of high amounts of myeloma protein was observed when Id-specific $CD4^+$

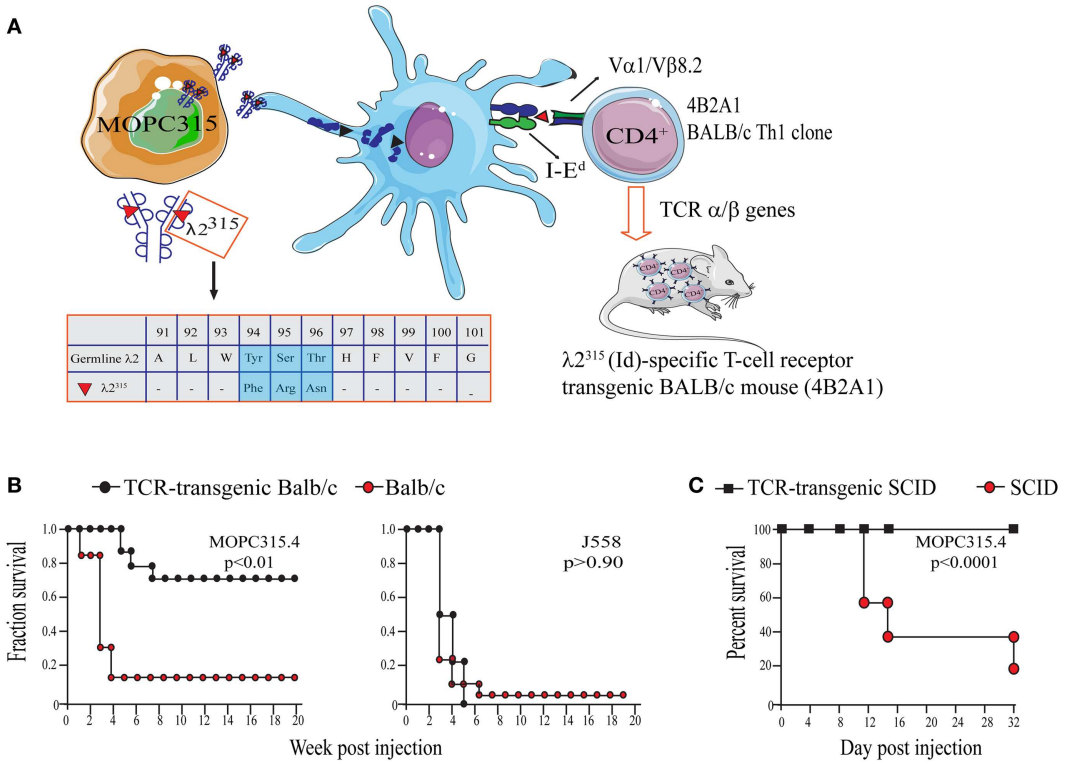

FIGURE 2 | The MOPC315 myeloma model. Naïve tumor-specific CD4+ T cells protect against MHC II^{NEG} tumor challenge in the absence of other T cells and B cells. **(A)** MOPC315 myeloma cells of BALB/c origin secrete an IgA M315 myeloma protein with a mutated λ2 light chain referred to as λ2^{315}. M315 is endocytosed and processed by BALB/c APCs, and a CDR3 sequence that includes residues 91–101 of λ2^{315} is presented on the MHC class II molecule I-E^d to Id-specific CD4+ T cells. The peptide that is recognized by Id-specific CD4+ T cells contains somatic mutations in positions 94, 95, and 96 (45, 79, 80). Based on the αβ TCR of the Id-recognizing 4B2A1 clone, a TCR-transgenic mouse was generated (46). Most CD4+ T cells in this mouse express a transgenic TCR that can be tracked by a clonotype-specific mAb [Nomenclature: antigenic determinants in immunoglobulin variable (V) regions are called idiotopes (Id). The 91–101 peptide is thus an Id-peptide, and the CD4+ T cells that recognize this Id-peptide presented by I-E^d are called Id-specific]. **(B)** Id-specific TCR-transgenic mice on an immunosufficient background (BALB/c) are resistant to a challenge with Id^{POS} MOPC315 cells but succumb to Id^{NEG} J558 myeloma cells [reproduced with permission from Proc Natl Acad Sci (26), Copyright 1994 National Academy of Sciences, U.S.A.]. **(C)**. Id-specific TCR-transgenic mice on an immunodeficient background (SCID), lacking other T and B cells than Id-specific CD4+ T cells, are also resistant to MOPC315 tumor development [reproduced with permission from Immunity (34)]. Tumor resistance could be transferred with purified Id-specific CD4+ T cells to SCID mice (27).

T cells were co-cultured with MHC-compatible spleen cells from BALB/c (H-2^d) MHC II^{NEG} MOPC315. Importantly, MHC II incompatible spleen cells from C57BL/6 failed to support cytotoxicity (70). Moreover, the cytotoxic effect could not be transferred by supernatants of activated T cells. It was suggested that some of the spleen cells, e.g., macrophages (Mϕ) stimulated by activated T cells, were important as cytotoxic effector cells in the *in vitro* cultures (70).

NAÏVE Id-SPECIFIC CD4+ T CELLS IN T CELL RECEPTOR TRANSGENIC MICE PROTECT AGAINST Id+ MYELOMA CELLS IN THE ABSENCE OF CD8+ T CELLS AND B CELLS

To facilitate studies of the role of Id-specific CD4+ T cells in tumor protection against MHC II negative MOPC315, an Id-specific TCR-transgenic mouse on syngeneic BALB/c background was established (46).

In initial experiments, naïve Id-specific T cells from TCR-transgenic mice did not respond to MOPC315 *in vitro*. Despite this, Id-specific TCR-transgenic mice were specifically protected against s.c. challenge with MOPC315 cells (26) (**Figure 2B**). Eradication of MOPC315 cells resulted in a change of T cell phenotype, since T cells of surviving TCR-transgenic mice had increased cytotoxicity against Id+ MHC II^{POS} B lymphomas, and since they upon stimulation produced much IFNγ and some IL-4.

By breeding the TCR-Tg mice onto a SCID background, it was demonstrated that rejection of MOPC315 was independent of CD8+ T cells and B cells/antibodies (27, 34) (**Figure 2C**). Additionally, tumor protection could be transferred to SCID mice with adoptive transfer of purified Id-specific CD4+ T cells (27).

Id-PRIMED APC CAN BE DETECTED IN TUMOR TISSUE OF LARGE ESTABLISHED MYELOMAS

The finding that naïve CD4+ T cells could initiate rejection of a MHC II negative tumor indicated that host cells expressing MHC class II molecules were responsible for the presentation of Id to CD4+ T cells. In a subsequent study, it was demonstrated that s.c.

MOPC315 tumors contained APCs that were able to stimulate Id-specific CD4$^+$ T cells *in vitro* in an MHC-restricted manner (65). The great majority of MHC II^{POS} tumor-infiltrating APCs were CD11b$^+$CD11c^{LOW}CD80$^+$CD86$^+$. These studies demonstrated that MHC class II negative MOPC315 tumors were infiltrated with Id-primed APCs with macrophage-like characteristics.

Id-SPECIFIC CD4$^+$ T CELLS ARE PRESENT AND ACTIVATED IN TUMOR TISSUE

Given that Id-primed APC could be demonstrated in MOPC315 tumors, it was investigated if Id-specific CD4$^+$ T cells were also present, and whether they were activated. In these experiments, a high amount of MOPC315 cells were injected in order to overcome the resistance of TCR-transgenic mice. A number of observations indicated that Id-specific CD4$^+$ T cells were specifically activated in small s.c. MOPC315 tumors established in Id-specific TCR-transgenic mice: (i) The CD4$^+$/CD8$^+$ ratio was skewed toward CD4$^+$ in tumor tissue. (ii) CD4$^+$ blasts within the tumor were selectively enriched for cells expressing the Id-specific TCR. (iii) Id-specific CD4$^+$ tumor-infiltrating lymphocytes (TIL) were activated (CD69$^+$ CD25$^+$), and proliferated (BrdU$^+$) in clusters associated with MHC II^{POS} tumor-infiltrating APC (65).

SECRETION OF TUMOR-SPECIFIC ANTIGEN IS REQUIRED FOR CD4$^+$ T CELL-MEDIATED REJECTION OF MHC II^{NEG} TUMORS

While it was clear that tumor-infiltrating APCs and lymph node cells take up the $\lambda 2^{315}$ antigen and display the Id-peptide on MHC class II molecules (34, 65), the precise source of the priming Id antigen was not established. To address this question, we used two secretory variants of MOPC315: one that secretes the complete M315 myeloma protein composed of α H chain and $\lambda 2^{315}$ L chain (MOPC315), and another that only secrets the free $\lambda 2^{315}$ L chain (MOPC315.26). In addition, we used two non-secretory variants: one where the free $\lambda 2^{315}$ L chain is retained intracellularly due to a point mutation (MOPC315.37) and another where no Ig is produced (MOPC315.36) (67, 83).

When Id-specific TCR-transgenic SCID mice were challenged with the four variants, protection was observed for the $\lambda 2^{315}$-secreting variants MOPC315 and MOPC315.26, while there was no protection against the antigen-negative MOPC315.36. Tumor take was significantly delayed, but still complete, in mice challenged with the MOPC315.37, which retains $\lambda 2^{315}$ intracellularly. This result was surprising since in MOPC315.37-containing Matrigels, macrophages were MHC II^{HI}, and Id-specific T cells were activated (CD69$^+$). The only striking deficiency observed with MOPC315.37 *in vivo* was deficient T cell activation in draining lymph nodes, presumably due to poor local availability of the intracellularly retained tumor antigen. These results indicate that the extracellular concentration of secreted tumor-specific antigen is important for protection against an MHC II^{NEG} tumor, most likely due to enhanced priming of APCs in draining lymph nodes as well as macrophages in tumors (36, 84).

DETECTION OF TUMOR-SPECIFIC CD4$^+$ T CELLS AND MACROPHAGES IN EARLY STAGES AFTER TUMOR CELL CHALLENGE: THE MATRIGEL METHOD

To study local events at the injection site at the early stages of the anti-tumor immune response, we injected the tumor cells suspended in a Matrigel solution (**Figures 3** and **4**). Matrigel is a liquid basement membrane preparation that jellifies rapidly at body temperature. Thus, a tumor bed of a defined size was generated that could be isolated and assayed to characterize infiltrating cells at any time point following tumor cell injection (**Figure 3**). Moreover, the defined volume of the gel plug allows quantitative assays of secreted factors within the tumor microenvironment (39). Initial experiments demonstrated that tumor cells embedded in Matrigel were rejected by TCR-transgenic SCID mice, although less efficiently than in the absence of Matrigel (34). Thus, events in the tumor cell-containing Matrigel most likely reflected those taking place during successful immunosurveillance of MHC II negative tumor cells by CD4$^+$ T cells.

Using this system, a longitudinal characterization of the immune response within the tumor microenvironment and draining lymph nodes was undertaken (34, 36, 39, 85). The findings are summarized in **Figure 4**. Briefly, secreted myeloma protein is presented by APC in tumor-draining lymph nodes to Id-specific CD4$^+$ T cells. Upon recognition, T cells are activated, polarize into Th1 cells, and migrate to the Matrigel/tumor. In

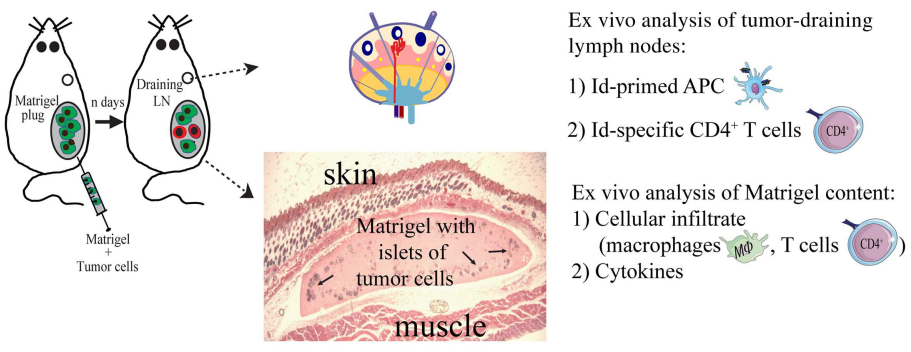

FIGURE 3 | The Matrigel assay. A novel approach to unravel the dynamics of CD4$^+$ T cell-mediated primary anti-tumor immune responses. At day 0, subcutaneous injections with MOPC315 tumor cells suspended in liquid Matrigel. When the Matrigel solution reaches body temperature, it jellifies and forms a plug containing the tumor cells. At various time points after injection (*n* days), the Matrigel plug and tumor-draining lymph nodes are dissected out and analyzed *ex vivo* for cellular content, function of cells, and cytokines (34, 36, 39, 85).

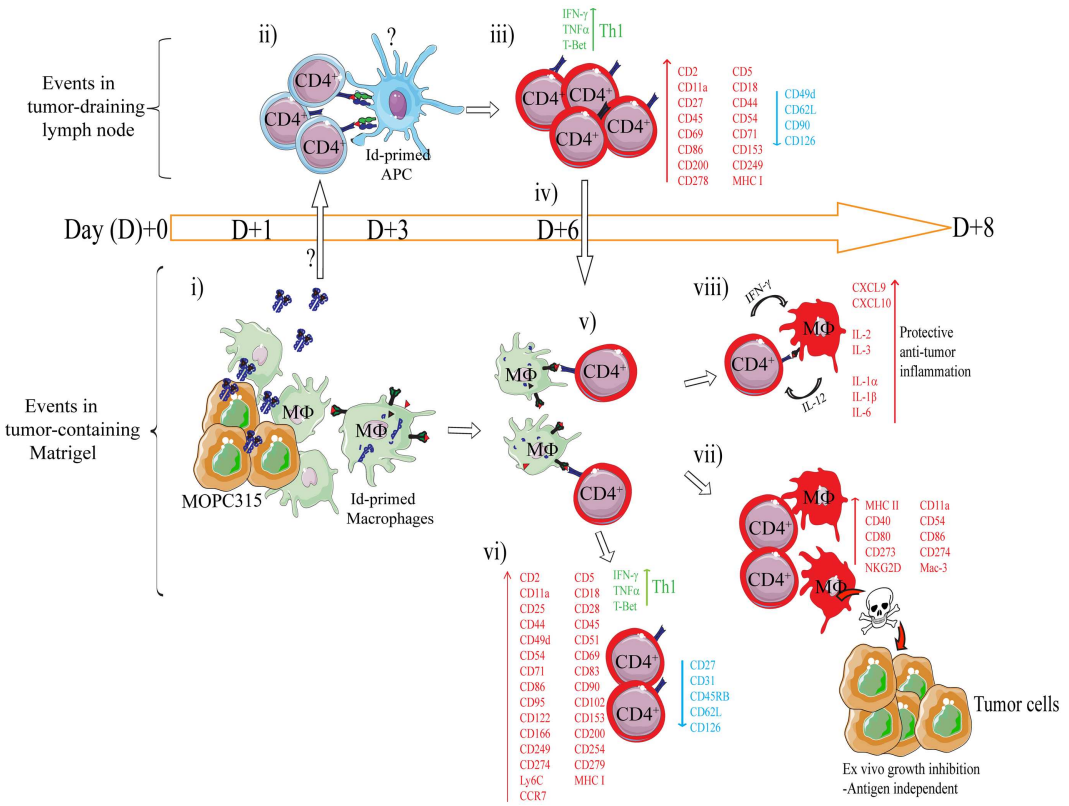

FIGURE 4 | Mechanism of rejection of MHCII^{NEG} myeloma cells by Id-specific CD4$^+$ T cells. The following events are based on experiments where Id-secreting MOPC315 suspended in liquid Matrigel was injected subcutaneously in TCR-transgenic mice. (i–viii). (i) At the incipient tumor site, macrophages [CD11b$^+$, CD11c$^-$, CD80/CD86$^+$ MHC II^{LO}, F4/80$^+$] start to infiltrate the tumor/Matrigel from day +1. Tumor-infiltrating macrophages become Id-primed by extracellular myeloma protein by the conventional MHC II presentation pathway (65). (ii) Extracellular Id+ myeloma protein (or possibly Id-primed tumor APCs) drain to lymph nodes where Id-primed APCs stimulate Id-specific CD4$^+$ T cells. Uncertainties as to the mechanism of Id+ Ag draining and the identity of Id-primed APCs are indicated by a question mark (?). (iii) Id-specific CD4$^+$ T cells become activated by day +3, are substantially expanded by day +6 (34), and polarize into Th1 cells by day +8 (39, 85). Upon activation in the tumor-draining lymph node, a number of molecules are significantly upregulated on the surface of the Id-specific CD4$^+$ T cells, while some are consistently downregulated (85). (iv) Activated CD4$^+$ T cells (CD62L^{LOW}) leave the lymph node and accumulate at the tumor site from day +6 (34, 86). (v) At the incipient tumor site, infiltrating Id-specific CD4$^+$ T cells are re-activated by Id-primed macrophages (34). (vi) Moreover, in addition to a sustained Th1 phenotype, the tumor-infiltrating CD4$^+$ T cells dramatically change expression of a number of surface molecules (85). Several molecules are upregulated on both activated CD4$^+$ T cells in the tumor-draining lymph node, and on tumor-infiltrating CD4$^+$ T cells, although at higher levels in the latter population. (vii) IFN-γ produced by tumor-infiltrating Th1 cells activates macrophages that up-regulate MHC class II on the cell surface and show increased expression of M1-associated surface molecules (34, 39). IFN-γ-activated macrophages acquire a tumoricidal phenotype with the upregulation of cytotoxicity-associated markers including granzyme A/B, and NKG2D (39). In addition, purified activated macrophages can directly inhibit myeloma growth *in vitro* (34, 36, 39). The mechanisms underlying M1 macrophage-mediated growth inhibition is unknown, but once the macrophages are activated the growth inhibition is antigen independent (36). (viii) Analysis by gene expression profiling and Luminex multiplex cytokine analyses has revealed that the Id-specific CD4$^+$ Th1-mediated anti-tumor immune response has a striking resemblance to the characteristics of acute inflammatory responses (39). Thus, we propose that Th1-mediated inflammatory responses may protect against cancer (87).

the Matrigel/tumor, Th1 cells become re-activated by tumor-infiltrating macrophages that has endocytosed and processed myeloma protein. Th1 derived IFN-γ activates macrophages into tumoricidal M1 macrophages (34, 36, 39, 65).

UNRESOLVED ISSUES AND DIRECTIONS FOR FUTURE RESEARCH

USE OF MHC CLASS II NEGATIVE TUMOR CELL LINES IN TUMOR IMMUNOLOGY

While MHC class II positivity in tumor cells is generally to be trusted, MHC class II negativity should, for obvious reasons, be viewed with a healthy skepticism. In the case of MOPC315, many attempts by others and us have consistently failed to detect expression of MHC class II molecules *in vitro* as well as *in vivo*, even when MOPC315 cells were exposed to IFN-γ (34, 70, 82, 88). In several other models, such as the use of the erythroleukemia cell line FBL-3 (25), the UV-induced fibrosarcoma 6132A-PRO (31), and the methylcholanthrene-induced Mc51.9 (32); no MHC class II was detected on tumor cells even after IFN-γ exposure, similar to MOPC315. In the B16 melanoma model, Quezada et al. showed that the cell line used in their experiments expressed MHC class II, but only when the tumor-bearing hosts were subjected to a

combination of irradiation and adoptive transfer of Trp1-specific $CD4^+$ T cells together with anti-CTLA mAb (37). Xie et al. also reported that B16 cells express MHC class II by immunofluorescence staining of tumor biopsies, but the identity of the MHC class II^{POS} cells within the sections was not further characterized, complicating interpretation (38). In contrast, Hung et al. reports the use of B16 tumor cells that were described to be MHC II^{NEG} (30).

Peres-Diez et al. (28) reported that expression of MHC class II molecules on tumor cells was not required for rejection mediated by $CD4^+$ cells. In note, they found that: H-2^k H-Y^+ tumor cells were rejected by I–A^b-restricted, H–Y-specific $CD4^+$ T cells in an immunodeficient H-2^b mouse. An alternative approach to ensure the absence of the relevant MHC class II molecule in a tumor cell line would be to delete the corresponding MHC class II molecule genes from the tumor cells.

IS SECRETION OF TUMOR-SPECIFIC ANTIGEN BY MHC II^{NEG} TUMORS REQUIRED?

The tumor-specific antigen used in our own studies, the MOPC315 myeloma protein, is a highly secreted antigen, with serum levels reaching milligrams per milliliter levels. Concentrations of myeloma protein in tumor tissues would be expected to be even higher. Surprisingly, a non-secreting myeloma variant that only expresses an intracellularly retained mutated Id^+ L chain, but in high amounts, was not rejected (36). In the absence of sufficient tumor antigen secretion, it might be expected that either spontaneous necrosis or apoptosis of tumor cells containing such high amounts of intracellular tumor antigen could prime tumor-infiltrating APC with tumor-specific antigen. This is apparently not the case for the non-secreting variant of MOPC315. It remains to be seen whether cytotoxic drug treatment of mice with tumors caused by this particular MOPC315 variant could enhance Id priming of APCs via uptake of necrotic or apoptotic cells.

In other MHC II^{NEG} models where tumor cells is reported to be rejected by $CD4^+$ T cells (28, 31, 32), there is scarce information as to whether tumor-specific antigen is secreted or not (**Table 2**). In the case of H-Y antigen, which clearly must be transferred from the tumor cells to host APC for MHC II presentation (28), there is little information about the extent of secretion of the antigen. In yet other cases, the tumor-specific antigen is simply not known (25), precluding any analysis of secretion status. It should further be noted that in some experiments [e.g., Ref. (24, 25, 37)] it has not been rigorously excluded that non-malignant normal cells could also produce the "tumor-specific" antigen. This possibility is virtually excluded in the MOPC315 model since $CD4^+$ T cells recognize a somatically mutated tumor-specific antigen unique to MOPC315 myeloma cells. By and large, it appears that secretion of tumor-specific antigen facilitates priming of host APC and stimulation of $CD4^+$ T cells. However, it is possible that the requirement of secretion could vary for distinct tumors and tumor-specific antigens, perhaps related to differences in susceptibility for cross-presentation of antigen associated with either necrotic or apoptotic tumor cells, or secreted vesicles such as exosomes.

What about MHC II^{POS} tumors – do they also require secretion of tumor-specific antigen? For MHC II^{POS} B lymphoma, a transfectant that secretes $\lambda 2^{315}$ was rejected, while another transfectant expressing a mutated intracellularly retained $\lambda 2^{315}$ was not (26). Similarly, A20 cells expressing HA, which apparently was negligibly secreted since HA was not found in serum, was not rejected (89). The Dby minor histocompatibility antigen (H–Y) (28) and Trp1 (35, 37, 38) have both been reported to be secreted by tumor cells. A strategy to test the hypothesis that secretion of tumor-specific antigen is required for rejection of MHC II^{POS} tumors would be to transfect MOPC315.37 with CIIITA so that the tumor cells become MHC II^{POS}. If this transfectant is rejected in Id-specific TCR-transgenic mice, this would weaken the hypothesis.

BY WHICH PATHWAY IS TUMOR ANTIGEN PRESENTED BY APC IN DRAINING LYMPH NODES?

In the tumor models where it has been tested, be they MHC II^{NEG} (28, 34, 65) or MHC II^{POS} (37, 38), there was an apparent need for tumor-specific antigen to be presented by host APC to stimulate naïve (but not memory) $CD4^+$ T cells. Thus, in the case of the B16 MHC II^{POS} model, no rejection by naïve Trp1-specific $CD4^+$ T cells was obtained in hosts that lacked MHC class II molecules. By contrast, transfer of $CD4^+$ T cells that first had been primed *in vitro* could readily reject B16 tumors (37, 38). These findings indicate that MHC II^{POS} tumor cells themselves are incapable of stimulating naïve Trp1-specific $CD4^+$ T cells, and that priming by professional host APC is required. In addition, experiments reported by Xie et al. (38) using Trp1-deficient mice indicate that Trp1 derived from host tissue is redundant for priming APC and that Trp1 derived from B16 tumor cells suffice, at least for stimulation of memory $CD4^+$ T cells. It is still, however, unclear how the Trp1 antigen is transferred from tumors to host APC, and in which anatomical compartment priming of $CD4^+$ T cells take place.

The conclusions of the above experiments are supported by previous observations in the MOPC315 model, which directly demonstrate activation of Id-specific $CD4^+$ T cells in draining lymph nodes (34, 36, 85). Moreover, treatment with the sphingosine phosphate receptor modulator fingolimod that abrogates egress of T cells from lymph nodes led to a decreased number of Id-specific $CD4^+$ T cells within the tumor, resulting in failure of tumor rejection (86). Consistent with these findings, the non-secreting MOPC315.37 variant caused little activation of $CD4^+$ T cells in draining lymph nodes, and tumor rejection did not occur.

Idiotype-primed APCs are readily found in lymph nodes that drain MOPC315 tumors (Dembic and Bogen, unpublished experiments). It should therefore be possible by cell purifications and characterizations to reveal the identity of these Id-primed APCs in lymph nodes. Information from such experiments could help to define the mechanisms by which APC get primed by secreted tumor antigen. For example, if the predominant features of Id-primed APCs are that of a residential dendritic cell, this may signify priming by soluble antigen arriving to the lymph node via afferent lymphatic vessels.

ELIMINATION OF MHC II^{NEG} TUMOR CELLS

It is well documented that Th1/IFN-γ-activated M1 macrophages isolated from tumors under conditions of tumor rejection can directly inhibit the growth of MHC II^{NEG} myeloma cells *in vitro* (34, 36, 39). However, the molecular mechanisms mediating the inhibition of tumor cell growth remain to be established. Possibly,

reactive oxygen species could be of importance, since resistance against B16 cells [although in later work reported to be MHC class II^{POS} under conditions of rejection (37)] was reduced in iNOS$^{-/-}$ and NOX2$^{-/-}$ mice (30). Results of Perez-Diez et al. indicate that under some circumstances, NK cells activated by CD4$^+$ T cells are important, but the effector mechanisms employed by such NK cells have not been addressed (28).

It is also possible that CD4$^+$ T cells could themselves directly kill tumor cells, e.g., through FasL/Fas interactions, similar to what has been described for killing of MHC II^{POS} B lymphoma cells (33), or a perforin/granzyme B-dependent mechanism as described for killing of the MHC II^{POS} B16 cells (37). The efficacy of killing mechanisms of CD4$^+$ T cells could also differ for different tumors. Thus, even though Th1 cells efficiently killed transfected A20 cells *in vitro* by a FasL-dependent mechanism, the same cells could not kill MOPC315 (26, 66). Finally, it has been reported that IFN-γ produced by tumor-specific Th1 cells mediate tumor rejection by means of angiostatic effects, thus causing starvation of the tumor (32).

DO CD4$^+$ T CELL-MEDIATED IMMUNE RESPONSES AGAINST MHC II^{NEG} TUMOR CELLS CONVEY BYSTANDER KILLING OF TUMOR CELLS THAT HAVE LOST EXPRESSION OF ANTIGEN?

In theory, macrophage-mediated killing of MHC II^{NEG} tumors could be expected to indiscriminately kill surrounding cells, including tumor cells that have lost expression of antigen ("bystander killing"). If true, this would be a clinically important asset of Th1/M1 macrophage-mediated killing of tumor cells (34, 36, 39). The previously described angiostatic properties of Th1 derived IFNγ (32) would also be expected to cause bystander killing. On the other hand, direct killing of MHC II^{POS} tumor cells by cytotoxic CD4$^+$ T cells was demonstrated not to induce bystander killing (37).

WHAT CD4$^+$ T CELL PHENOTYPES SUPPORT ANTI-TUMOR IMMUNITY?

Naïve CD4$^+$ T cells in Id-specific TCR-transgenic mice, which eradicate injected MHC II^{NEG} tumor cells, develop into IFNγ-secreting Th1 TILs that induce macrophage polarization into tumoricidal M1 macrophages (33, 34, 39). Transfer of naïve Id-specific CD4$^+$ T cells could cure established MHC II^{POS} tumors (33). In the Trp1-specific TCR-transgenic model, naïve (37, 38), Th1 (35), and Th17 (35) cells have been demonstrated to eradicate MHC II^{POS} tumors. Collectively, these results indicate that the primary anti-tumor response of naïve CD4$^+$ T cells is followed by T cell differentiation into Th1 (or possibly Th17) cells that confer anti-tumor immunity irrespective of MHC class II expression on tumor cells. While Th1 cells are clearly associated with anti-tumor immunity, variable effects have been observed with other CD4$^+$ T cell subsets, reviewed in Ref. (90). Moreover, recent studies suggest that effector CD4$^+$ T cells retain some degree of functional plasticity (91, 92). The plasticity of effector Th populations may explain the differential effects of the various Th cell populations in tumor immunity. In addition, exploiting the plasticity of Th cell subsets may be utilized in immune therapy.

TOLERANCE INDUCTION OF TUMOR-SPECIFIC CD4$^+$ T CELLS

Use of TCR-transgenic mice offers the possibility of studying tolerance development by following the fate and function of tumor-reactive CD4$^+$ T cells. When Id-specific TCR-transgenic mice failed to reject high amounts of injected MHC II^{NEG} MOPC315 cells, CD4$^+$ T cells in peripheral lymphoid organs and in the tumor became deleted (93). The extent of deletion became more profound as tumor size increased. The deletion of peripheral tumor-specific CD4$^+$ T cells seen in this model for a highly secreted tumor antigen resembles that of exhaustion observed in chronic viral diseases. In addition to peripheral deletion of Id-specific CD4$^+$ T cells, progressive MOPC315 tumors also caused thymocyte deletion. It was demonstrated that circulatory myeloma protein gained access to the thymus and was presented in an MHC class II context by thymic APCs, thus causing negative selection of thymocytes (94).

In a recent paper, T cell characteristics in Trp1-specific TCR-transgenic mice developing B16 tumor recurrence following adoptive therapy were studied. Recurrence was associated with increased FoxP3$^+$ T_{reg} cell numbers, and increased expression of inhibitory ligands, including PD-1 and CTLA-4 inhibitory receptors on both T_{reg} and effector CD4$^+$ cells (95). Tumor recurrence could be prevented by concomitant depletion of T_{regs} and administration of checkpoint blockade antibodies. Collectively, these results indicate that CD4$^+$ T cells must eliminate tumor antigen-secreting tumor cells efficiently within a short timeframe. If the elimination is incomplete, T cell tolerance is induced by multiple mechanisms.

It has been shown that MHC II^{POS} A20 cells, are not rejected after i.v. injection in HA-specific TCR-transgenic mice, but induce anergy in CD4$^+$ T cells via priming of bone marrow derived APCs (89, 96). Interestingly, when presentation by bone marrow derived APCs was prevented by the use of bone marrow chimeras, anergy did not occur, and tumor cells were rejected (72). Thus, it might seem that tumor cells that poorly secrete tumor antigen could favor anergy development by induction tolerogenic APCs. The above results are consistent with previous observations that A20 cells expressing a non-secreted $\lambda 2^{315}$ were not rejected in Id-specific TCR-transgenic mice (26) (although it was not tested if anergy was induced). These results, obtained with non-secreting MHC II^{POS} A20 transfectants in two different TCR-transgenic models, are in support of the notion that tumor-specific antigen, perhaps via presentation of apoptotic or necrotic tumor cells by a special type of APC, favor induction of T cell anergy. In contrast, secretion of tumor-specific antigen and presentation (perhaps by another type of host APCs) in lymph nodes, may favor induction of potent primary anti-tumor CD4$^+$ T cell responses.

DICHOTOMOUS ROLE OF Th CELLS IN B CELL CANCERS

This review paper has focused on CD4$^+$ T cell-mediated eradication of tumor cells. However, CD4$^+$ T cells may also induce tumors. This dichotomy may especially apply to B cell tumors since B cells are known to proliferate in response to help from CD4$^+$ T cells. Extensive and prolonged B cell proliferation could indeed predispose to genetic instability and malignant transformation. In fact, B lymphoma development has been associated with continuous antigenic exposure in chronic infectious diseases caused by *Helicobacter pylori*, EBV, and hepatitis C. Moreover, chronic immune responses to self antigens in autoimmune diseases such as systemic lupus erythematosus, Sjögren's syndrome

and rheumatoid arthritis have also been linked to development of B cell lymphomas, reviewed in Ref. (97, 98). Further supporting a role for chronic antigen stimulation, diffuse large B cell lymphomas (98, 99) and follicular B cell lymphomas (98, 100, 101) are frequently infiltrated with T cells. In Ig- and TCR-transgenic mice, chronic stimulation of Id^+ B cells by Id-specific $CD4^+$ Th2 cells results in the induction of Id^+ B lymphomas (102). Moreover, two separate studies have shown that proliferation of B lymphomas (103) and MM (104) was augmented by the presence of $CD4^+$ T cells.

The MOPC315 model, reviewed herein, was used in the experiments were Id^+ lymphomas were induced. Interestingly, when such induced lymphoma cells were injected s.c into naïve Id-specific TCR-transgenic mice, the lymphoma cells were promptly rejected (102). Thus, Id^+ B lymphoma cells were eliminated by mice having naïve $CD4^+$ T cells with an identical Id-specific TCR to that of the B lymphoma-inducing Th2 cells. If naïve T cells in the protected mice differentiated into tumor-eliminating Th1 cells was not investigated. However, analogous experiments indicate that Th1 is the primary response to subcutaneously inoculated B lymphomas (34, 39). These results suggest that B lymphoma cells induced by Th2 cells are rejected by Th1 cells expressing an identical TCR. The finding has obvious implications for T cell therapy: if a B cell tumor is initiated by Th2 cells, it may be treated by Th1 cells of the same specificity (and possibly *vice versa*). The same may apply to other combinations of Th cells such as Th17/Th1 etc. Thus, re-education of T cell phenotype may become part of the tumor immunotherapy armamentarium. Given the plasticity of $CD4^+$ subsets (91, 92), such re-education may become a real possibility.

CONCLUDING REMARKS

HOW DISPARATE ARE THE MECHANISMS FOR REJECTION OF MHC II^{POS} AND MHC II^{NEG} TUMORS?

The data reviewed herein suggest that the difference between direct and indirect killing of tumors relates predominantly to the effector stage of tumor cell killing. Thus, $CD4^+$ T cells can kill MHC II^{POS} cells directly, while killing of MHC II^{NEG} occurs indirectly via macrophages or possibly NK cells, angiostatic effects, or all of these. In contrast, the primary activation of naïve tumor-specific $CD4^+$ T cells appears to be similar for the direct and indirect mechanisms, in that presentation of tumor-specific antigen by host APC seems to be required. However, the evidence for this in the context of MHC II^{POS} tumors is largely circumstantial. In an MHC II^{NEG} myeloma model, secretion of tumor-specific myeloma protein clearly facilitates priming of APC in lymph nodes and stimulation of naive $CD4^+$ T cells that subsequently infiltrate the tumor site. Thus, the nature of the antigen, by virtue of its cellular localization and accessibility to APCs, might determine the ability of the antigen to serve as an efficient tumor-specific antigen in $CD4^+$ T cell responses. A more in-depth analysis of such factors might be of value in reconciling observations made in the various TCR-transgenic models.

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Beneficial autoimmunity at body surfaces – immune surveillance and rapid type 2 immunity regulate tissue homeostasis and cancer

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Epithelial cells (ECs) line body surface tissues and provide a physicochemical barrier to the external environment. Frequent microbial and non-microbial challenges such as those imposed by mechanical disruption, injury or exposure to noxious environmental substances including chemicals, carcinogens, ultraviolet-irradiation, or toxins cause activation of ECs with release of cytokines and chemokines as well as alterations in the expression of cell-surface ligands. Such display of epithelial stress is rapidly sensed by tissue-resident immunocytes, which can directly interact with self-moieties on ECs and initiate both local and systemic immune responses. ECs are thus key drivers of immune surveillance at body surface tissues. However, ECs have a propensity to drive type 2 immunity (rather than type 1) upon non-invasive challenge or stress – a type of immunity whose regulation and function still remain enigmatic. Here, we review the induction and possible role of type 2 immunity in epithelial tissues and propose that rapid immune surveillance and type 2 immunity are key regulators of tissue homeostasis and carcinogenesis.

Keywords: immune surveillance, Type 2 immunity, epithelial cells, tissue homeostasis, carcinogenesis, IgE, intraepithelial lymphocytes, sterile stress

Epithelial cells (ECs) are the main constituent of tissues lining body surfaces like the skin, intestine, lungs, and genitourinary tract. They regulate crucial life processes such as micronutrient absorption, gaseous exchange, and thermo- and hydro-control whilst also providing a physiochemical barrier to the external environment against microbes and a plethora of non-microbial stressors. ECs are extremely dynamic and versatile cells and it is becoming increasingly clear that they are also intimately involved in the induction and regulation of local tissue- and systemic immune responses. Disruption of epithelial surfaces may therefore result in dysregulated body processes and penetrance to deeper tissues by microbes or noxious moieties. In addition, the direct response of ECs to tissue disruption strongly affects resident immunocytes and their subsequent regulation of both local and systemic innate and adaptive immunity. A growing body of evidence both from mouse models and human genetics suggest that EC dysregulation can be a primary cause of pathology in different tissues. Given the multifaceted biological actions of ECs and the multitude of challenges imposed on epithelial tissues, it is reasonable to think that ECs in conjunction with tissue-resident immunocytes possess mechanisms, both immunological and non-immunological, to maintain healthy barrier homeostasis and to minimize inflammation and cellular dysregulation. Indeed, ECs are now known to be highly immunomodulatory by virtue of the cytokines, chemokines, damage-associated molecular pattern (DAMP) molecules, and major histocompatibility (MHC) gene products they express; a repertoire that has collectively been termed the "epimmunome" (1). ECs express pattern-recognition receptors (PRRs) including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and a variety of "NK" receptor ligands, enabling them to respond to a wide variety of microbial and non-microbial (including self) moieties and disseminate the response to immunocytes. The NLR–inflammasome complex allows ECs to respond to non-microbial sterile stress elicited by toxins, irritants, and (for skin keratinocytes) ultraviolet (UV) light (2); the most pervasive environmental DNA-damaging agent (3). Thus, ECs express a suite of sensors for detecting differing insults and challenges at the body surfaces, and an armory of soluble and cell-surface molecules to direct an appropriate, restorative response. These epithelial-driven responses in health sculpt and modulate tissue homeostasis and local tissue immunity, in a manner that aids morphological tissue homeostasis, restoration of the epithelial barrier following injury, and elimination or expulsion of microbial and non-microbial insults. Here, we review how ECs drive immunity at body surfaces and how this is involved in regulating immune surveillance, tissue immune homeostasis, and cancer.

EPITHELIAL CELLS AND THEIR RESPONSE TO CHALLENGE

The vast majority of environmental challenges occur at epithelial surfaces. The repertoire of responses available to ECs to combat these daily challenges is immense. For example, EC-derived cytokines include IL-1, IL-6, IL-10, IL-18, IL-25, IL-33, TNFα, and thymic stromal lymphopoietin (TSLP). Pro-IL-1α and pro-IL-1β are constitutively produced by ECs, particularly skin keratinocytes, and are secreted following exposure to noxious stimuli or tissue damage. Corneocytes (non-nucleated skin ECs) release IL-1α in the skin in response to disruption of the outermost surface, the stratum corneum (4), while UV-irradiation induces IL-1β. In addition to agonistic effects on tissue macrophages, IL-1α induces

growth-factor expression from tissue fibroblasts, prompting a replicative burst in neighboring ECs to repair damage (5). The pro-inflammatory cytokines IL-6 and TNFα are produced in large quantities by damaged ECs; the former of which can also be used as a STAT-3-dependant autocrine growth factor, in healthy and cancerous epithelium (6). The most robustly expressed cytokines upon any EC insult however are IL-25, IL-33, and TSLP. In common, these three cytokines can drive type 2 immune responses, which thus impart a particular propensity of epithelial tissues to induce type 2 immunity. Such predisposition of stressed ECs may underlie the high frequency of allergic and atopic disease at the skin and mucosal surfaces. However, despite the intense interest in this area, the cellular and molecular linkage of type 2 immunity to barrier- and EC disruption is not clearly understood – nor is the functional role of this type of immunity to EC homeostasis or immune surveillance yet fully elucidated.

IL-25, also know as IL-17E, is a member of the IL-17 cytokine family. Despite bearing some amino acid sequence homology to the best-characterized IL-17 cytokines, IL-17A and IL-17F, IL-25 has divergent biological functions and promotes Th2 rather than Th17 responses *in vivo*. IL-25 directly amplifies expression of the Th-2 mediators IL-4, IL-5, and IL-13, and supports production of Th2 serum immunoglobulins (7). IL-25 was first reported with high steady-state mRNA expression in the kidney, and moderate to low expression in other organs and the peripheral tissue (8). Subsequently, it was found by multiple groups to be important in type 2-mediated immunity to enteric parasites, such as *Trichuris muris* (9), and is upregulated in the gut upon EC-sensing of commensal bacteria (10). The mouse gut parasite *Heligmosomoides polygyrus bakeri* elicits the EC-derived cytokine, IL-1β, which suppresses IL-25 and IL-33 and promotes pathogen chronicity by attenuating expulsive type 2 responses (11), suggesting that IL-25 is particularly important in maintaining immunity to gut pathogens. Similarly, mice and humans subjected to parenteral nutrition have impaired mucosal immunity, due to reduced gut luminal levels of antimicrobial effectors, but administration of exogenous IL-25 to parenteral nutrition-fed mice was found to be protective against enteric bacterial invasion (12). In allergic models, IL-25 expression is upregulated upon exposure to allergens both in murine or human lung EC lines and in primary murine lung ECs (13). Elevated protein levels have also been found in tissues of patients with allergic disease in the lung and skin (14). IL-25 has been found to drive tissue (airway) remodeling, and expression of the other major EC cytokines IL-33 and TSLP in a house dust mite model of allergy (15), and drive pulmonary fibrosis by inducing IL-13 expression from lung innate lymphoid cells (ILCs) in mice challenged with lung *Schistosoma mansoni* eggs (16). In addition to production by ECs, dermal dendritic cells (DCs) have been reported to be a major source of IL-25 in atopic dermatitis (AD) patients (17), while IL-25 and IL-33-activated ILC2s in mouse skin promote AD-like inflammation (18). These reports and others highlight an interesting crosstalk and autocrine regulation of EC-derived effectors, as well as a role for IL-25 in augmenting epithelial barrier immunity, or conversely promoting pathological Th2 tissue inflammation, in differing settings.

IL-33 is a multi-functional protein. The full length protein is localized in the nucleus but following cleavage the c-terminal fragment acts as a cytokine which binds the receptor ST2. IL-33 was recently discovered as an IL-1 family member with type 2-promoting functions similar to IL-25. It is expressed by ECs, macrophages, DCs, and mast cells *in vivo* and its cytokine function drives IL-4, IL-5, and IL-13 expression and differentiation of Th2 $CD4^+$ T cells (19). IL-33-induced IL-4 production appears to be mainly from innate cells and together these two cytokines will induce proliferation of B cells and amplify IgE synthesis (20). Similar to IL-25, IL-33 acts in an autocrine fashion to promote TSLP expression by ECs, particularly in response to gut nematodes, where IL-33 mRNA can be detected rapidly following colonization (21). Interestingly, the efficacy of IL-33 in this infection model (and others) seems to be highly time-dependent, with administration of exogenous IL-33 at late time points post-infection being ineffective in promoting type 2 responses that would otherwise resolve infection. IL-33 is highly expressed by intestinal ECs and inflammatory infiltrates in ulcerative colitis, with IL-33 cleavage products being detected in the serum (22). IL-33 is also rapidly released and detectable in bronchoalveolar lavage fluid following lung allergen exposure in humans, suggesting it is a rapid type 2 mediator in sites additional to the gut (23). Protective as well as immunopathological roles of EC-derived IL-33 have been described in the skin. Transgenic over-expression of IL-33 in mouse skin, driven by a keratinocyte-specific promoter, induces a spontaneous dermatitis-like disease and activates ILC2s in the dermis (24). It has also been shown in a phorbol 12-myristate 13-acetate model of skin inflammation that mice deficient for the IL-33 receptor, ST2, do not exhibit IL-33-dependant skin inflammation (25). Similarly in human inflammatory conditions, IL-33 has been reported to be upregulated in clinical psoriatic lesions and the serum of skin sclerosis patients (26). Conversely, mice treated with exogenous IL-33, following full-thickness skin wounding, demonstrate dramatically improved wound-healing, collagen deposition, and expression of extracellular matrix proteins indicative of tissue repair (27). These reports suggest a particularly rapid and acute role for IL-33 in cutaneous homeostasis and gut integrity whereas constitutive, late, or dysregulated expression may be involved in a variety of chronic inflammatory conditions. This temporally coordinated aspect fits well with current thinking of IL-33 as an "alarmin," whereby its immediate release from intranuclear stores by damaged, apoptotic, or necrotic cells rather than a classic Golgi-mediated secretion pathway (19) facilitates a rapid and restorative response to tissue damage.

TSLP is produced almost exclusively by ECs of the lung, tonsils, intestine, and skin (13), and is upregulated in response to tissue damage (28), various TLR ligands and infection, or exposure to type 2 cytokines such as IL-4, IL-13, IL25, and IL-33 (29). A protective role of TSLP in intestinal immunity to *T. muris* has been well described; mice which are knockouts for IKKβ fail to produce TSLP in response to infection, and subsequently develop chronic intestinal inflammation (30). Mechanistically, EC-derived TSLP suppresses p40 and upregulates OX40L expression in DCs, a costimulatory molecule with a propensity to license Th2 responses in $CD4^+$ T cells (31). TSLP also augments Th2 cytokine production by direct effects on $CD4^+$ T cells and has indirect, agonist effects on a variety of granulocyte populations including mast cells and basophils. Similar to IL-25 and IL-33, inappropriate

expression or dysregulation of TSLP is implicated in a number of inflammatory diseases including the triad of atopic diseases; asthma, allergic rhinitis, and AD (31). TSLP is required for allergic lung inflammation in mice exposed to inhaled antigen, and TSLP receptor knockout animals do not develop lung inflammation in this model. Interestingly, these animals do develop strong Th1 responses with high IFNγ, IL-12, and IgG2a (32), highlighting how a single epithelial-derived molecule can skew adaptive immune responses in response to tissue-challenge. In humans, AD sufferers show high TSLP expression in lesional skin (33), and mice with induced expression of TSLP in the epidermis develop spontaneous AD-like pathology (34). Production of TSLP is however critically important for resistance to skin carcinogenesis in mouse models (35, 36).

Further to cytokines and chemokines, ECs can release other proteins upon cellular stress. Of note, they can produce hedgehog morphogens, which are a family of secreted proteins that regulate a wide variety of physiological processes including tissue development during embryogenesis and tissue homeostasis as well as being implicated in carcinogenesis (37). Sonic Hedgehog h (Hh) expression was recently found to be upregulated in lung ECs in models of allergic disease, and lung resident T cells were shown to respond locally to EC-derived Hh by upregulating IL-4 (38). This demonstrates that ECs also produce non-classical immune modulators, such as tissue morphogens, which appear to contribute to the robust induction of type 2 immunity in epithelial tissues.

In addition to the secreted and soluble molecules produced by ECs, they also express a variety of cell-surface molecules enabling them to directly interact with resident and infiltrating immunocytes. For example, ECs express E-cadherin that engage CD103, which is constitutively expressed on intraepithelial lymphocytes (IELs) and tissue DCs such as the epidermal Langerhans cells (LCs). ECs also express T cell costimulatory ligands, although it remains unclear as to what extent ECs express the classical B7.1 and 2 molecules, they clearly express PD-L1 and PD-L2 (39). Some members of a novel family of B7-related molecules, the butyrophilins, appear to be preferentially expressed on ECs and have been implicated in EC-immune regulation (40) as have *Skint* family members which are exclusively expressed on ECs and have profound impact on IEL development and function (41, 42). Thus via appropriate receptor–ligand interactions ECs are capable of initiating and sculpting both local tissue immunity and further downstream systemic immunity. Under conditions of physiochemical tissue disruption or barrier perturbation (1), infection (43), genotoxic stress (44), sterile inflammation, or heavy proliferation (45), ECs respond by upregulating additional self-encoded and cell-surface markers, which are often termed as "'stress antigens" as they are indicative of a dysregulated state of the epithelium. The EC stress antigens have an important role in initiating and directing tissue immune responses during perturbations and as such these will be discussed in more detail in the Section "Immune Surveillance" below.

EPITHELIAL CELLS AND THEIR NEIGHBORS

In close association with ECs, the epithelial tissues are home to several specialized subsets of immunocytes. IELs are found in all epithelial tissues, but have most notably been studied in the intestine and skin. IELs are adaptive T cells carrying RAG-dependent rearranged T cell receptors (TCRs), nevertheless they are often MHC non-restricted cells and express many innate receptors allowing them to react to stress antigen with "innate-like" response kinetics. The IELs are a mixture of αβ and γδ T cells, which are either $CD4^{-}CD8^{-}$ or coexpress a CD8αα coreceptor. The ratio of αβ to γδ T cells depends on the anatomical site as well as the species. IEL compartments are often much less diverse than systemic T cells (for example in the mouse skin and uterus they are essentially monoclonal), implying that these cells recognize predictable antigens encountered in specific tissues – these antigens could be either pathogen encoded or self-encoded molecules that reflect a dysregulated state of the tissue they inhabit. Not many IEL TCR-specificities have yet been defined, but it seems clear that both their recognition capabilities and mode of activation are distinct from systemic T cells (46). Particularly, it has been proposed that IELs are primarily autoreactive T cells that have been agonist-selected, recognize tissue stress antigens, and have regulatory properties (47). The murine skin for example contains a specialized subset of $\gamma\delta\ (TCR)^{+}$ IELs called dendritic epidermal T cells (DETC) that exclusively carry a Vγ5Vδ1 TCR – a TCR arrangement only found on epidermal IELs (and on the progenitor fetal thymic population). The skin epithelia also contain a specialized subset of DCs, the epidermal LCs. Both LC and DETC infiltrate the epithelium very early during stratification of the skin ECs, and are long-lived and likely self-renewing immune compartments, which clearly integrate and physically interact with the ECs.

In addition to the "unconventional" T cells in the epithelium, more conventional $CD8\alpha\beta^{+}$ αβ T cells have been shown to rapidly accumulate in tissues upon infection, where they can become resident memory T cells and provide protective antigen-specific responses. This was elegantly shown in the skin following local infection with herpes simplex virus (48) or vaccinia virus (49), which induced a rapid influx of antigen-specific $CD8^{+}$ αβ T cells both into the epithelial epidermal layer and the underlying dermis. Interestingly, these infiltrating $CD8^{+}$ αβ T cells were shown to populate the entire skin and provide long-lasting protection against re-infection as a continuing tissue-resident memory T cell population. In steady state, the subepithelial layer of most tissues contains a diverse set of immunocytes that can all contribute to epithelial-immune surveillance. These include tissue-specific resident populations of myeloid cells, such as DC, macrophages and mast cells, lymphoid cells, such as $CD4^{+}$ or $CD8^{+}$ αβ T cells, γδ T cells, and ILCs, as well as stromal fibroblasts. In fact, it is becoming increasingly apparent that different tissues constitutively harbor a variety of specialized immunocytes in the subepithelial space. For example, in human skin a population of IL-22 and growth factor producing T cells (Th22) can rapidly enter the epithelium upon challenge and be involved in epidermal remodeling (50). Similarly, the gut contains a resident IL-22 producing population of NK-like cells (51). In recent years, an array of different ILCs has been discovered that are resident in the subepithelial tissue layer. Different subsets of ILCs dominate in particular tissues and their specialized functions are starting to be elucidated; many of them contribute to both homeostatic and pathophysiological conditions in the tissue they inhabit (52). The subepithelial immunocytes can respond to

epithelial cues and be recruited into the epithelium upon damage – in addition systemic immune cells can be recruited both to the subepithelial and epithelial layer. In sum, the epithelium and body surface tissues are home to an intricate array of immunocytes, which can interact and integrate activities in numerous complex ways that likely differ substantially depending on the anatomical site and the challenge encountered. Additional research is required to understand the interaction between different resident immunocytes in the tissues and how their responses may be integrated to regulate local and systemic immunity.

TYPE 2 IMMUNITY AND ITS TRIGGERS

Epithelial cells and EC-associated leukocytes such as IELs can clearly drive local and systemic type 2 immunity (53). More conventionally, however, type 2 immunity is thought to be mediated primarily by Th2 cells, IgE and IgG1 antibodies, as well as a host of innate immune cells such as mast cells, basophils, eosinophils, alternatively activated macrophages, and ILCs. The type 2 immune response *in vivo* is accordingly extremely heterogeneous and it is surprisingly poorly understood how type 2 immunity is induced, regulated – and indeed what its primary physiological function is. Type 2 immune responses are classically induced by macroparasites and conventional thinking holds that type 2 immunity has evolved to protect against infection by parasites such as helminthes and ticks. However, this is probably a too simple explanation, as it is not true that all parasites are fought by IgE and type 2 immunity. Although IgE levels are raised in people as well as mice with helminth infections, IgE is dispensable for immunity to many helminthes and much of the IgE raised is not specific to the parasite (54). Type 2 immunity is also notoriously activated in response to a broad range of different environmental challenges and antigens. Such non-infectious stimuli that trigger type 2 immunity are collectively termed allergens and form the basis of a host of allergic disorders like asthma, allergic rhinitis, food allergies, and AD. Type 2 immune responses have been explored largely in the context of helminth infections and allergic diseases. They have been thought to provide a host-beneficial role only as defense against macroparasites, whereas allergic reactions are most commonly explained as a detrimental consequence of a misdirected response mimicking parasite immunity. This paradigm is now changing and with more triggers of type 2 immunity being elucidated (**Figure 1**) it seems plausible that type 2 immunity can provide host-benefits in settings other than against parasites. In 1991, Profet published an inspired hypothesis suggesting that the physiological role of allergic responses was an immunological defense against toxins (55). This idea is resonating with recent data (56, 57) and the hypothesis has been recharged and expanded into a broader model of intentional allergic host defense not only against helminthes but also

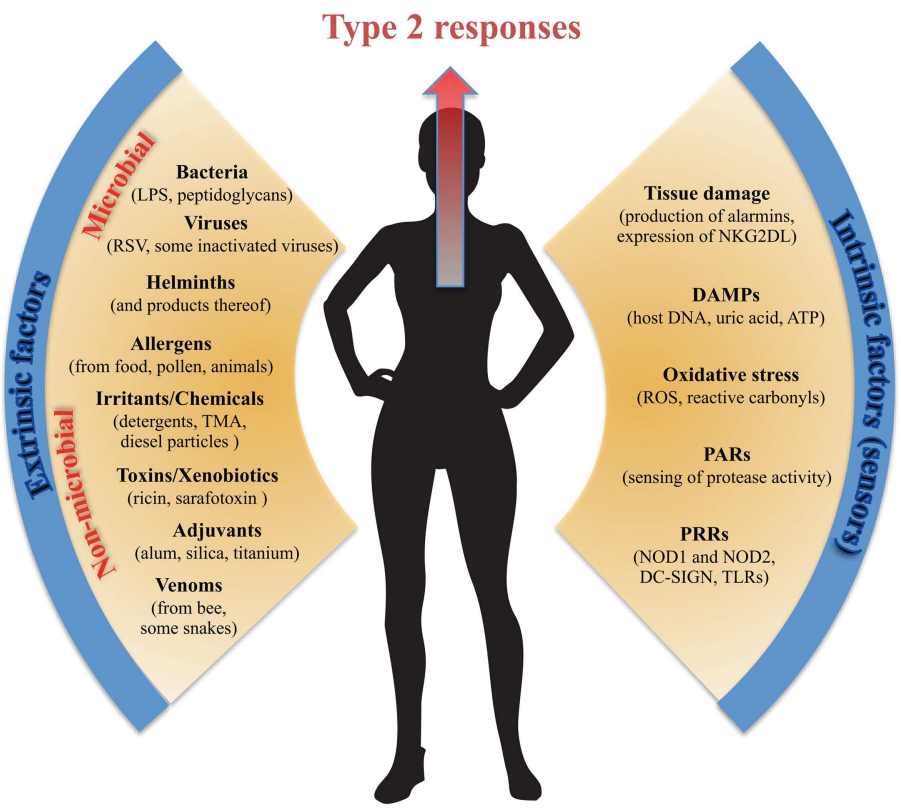

FIGURE 1 | Extrinsic and intrinsic factors promoting type 2 immunity. Type 2 immunity can be triggered by an array of diverse extrinsic stimuli from both infectious and non-infectious sources and is most potently induced at the body surface tissues. Equally, the intrinsic cellular mechanisms inducing and/or sensing type 2-triggering extrinsic stimuli are many and diverse. In common, most of the extrinsic factors promoting type 2 immunity, as well as the intrinsic factors sensing them, are founded on a breach of the protective barrier of the body and thus on tissue and cellular damage.

non-infectious environmental factors such as venoms, chemical irritants, and xenobiotics (58). Accordingly, there may be multiple pathways that lead to type 2 immunity and IgE – some more classically "adaptive" and some more "innate" (59). The route to type 2 immunity and whether protection or allergic sensitization is the outcome may depend on tissue context, allergen, dose, genetics, and species. Common for all type 2 immune responses is that their effector functions converge at the epithelial surfaces (skin and mucosa), vasculature, and smooth muscles where they promote barrier defenses and expulsion. Conspicuously, allergic disorders, unlike other immune pathologies, exclusively affect epithelial tissues that interface with the environment.

Type 2 immunity can be triggered by a bewildering array of molecules from both infectious and non-infectious sources (**Figure 1**). Much work has been done to try and identify a unifying framework for what makes a substance "an allergen" (60), but common allergens such as peanut, shellfish, pollen, nickel, bee venom, latex, house dust mite, and penicillin appear to have little in common in terms of their chemical structure or origin. In addition, type 2 immunity can be triggered by certain vaccine adjuvants [alum most notably (61)], noxious toxins (56, 58), environmental irritants and chemicals (62, 63), as well as certain infections or bacterial products (64). One commonality between both infectious and non-infectious triggers of type 2 immunity that may have been less well appreciated is that many are insults inducing some level of physical trauma that breaches the protective barrier of the body. Tissue damage, at least in the absence of strong type 1-promoting pathogen-associated molecular pattern (PAMP) signaling, appears to be a potent mechanism driving type 2 immunity. Tissue damage induces rapid release of several epithelium-derived cytokine alarmins, such as IL-33, TSLP, and IL-25 (reviewed above) – all of which can drive downstream type 2 immunity. In a macroparasite infection, the large size of the parasite and the consequent tissue damage it causes during invasion may be the most important factor in inducing type 2 immunity (65), although some parasite-derived products with direct type 2 polarizing capacity may also exist (66). The tissue-damage caused by macroparasites may be modeled by ingestion of large inert particle structures. Interestingly, it has been shown that inert silica and titanium particles induce innate type 2 immunity and can be used as adjuvants promoting Th2 responses by pathways independent of TLR4 and MyD88 (67, 68). These particles may induce cellular damage and consequently activate endogenous danger- or stress-signals. That "injury" or cellular stress alone can support induction of type 2 immunity is strongly supported by results showing that transgenic up-regulation of the NKG2D stress-ligand Rae-1 on ECs promote potent type 2 immunity and IgE to innocuous antigens (53). This rapid innate-like IgE response is also independent of MyD88 (53). Cellular damage may also explain the type 2-inducing effect of the adjuvant alum as injection of alum causes the release of DAMPs, like uric acid and host cell DNA (61, 69). Uric acid has been shown to drive type 2 immunity and again this is via pathways independent of both MyD88 and the inflammasome (69). Host DNA signaling intriguingly appears to differentially regulate IgG1 and IgE production following alum-adjuvanted immunization, where host DNA induces primary B cell responses with IgG1 through interferon response factor 3 (Irf3)-independent mechanism but more canonical Th2 responses and IgE through an Irf3-dependent mechanism (70). Furthermore, extracellular ATP, presumably released from damaged cells, binds to P2 purinergic receptors and triggers IL-33 release and innate type 2 immune responses in the lung (71). Oxidative stress, which is widespread and entwined with pathological processes, has also been shown to be involved in orchestrating type 2 immunity. For example, induction of reactive oxygen species (ROS) in ECs induces oxidation of lipids that in turn triggers TSLP release by ECs (72) and oxidative stress has been shown to induce reactive carbonyl adduction, which is reported to be a potent driver of type 2 immunity (73). DAMPs thus appear to be part of both the initiation and amplification of type 2 immunity and may as such also play an important role in allergic diseases.

Another feature that contributes to the induction of type 2 immunity by some allergens is their serine or cysteine protease activity. Allergens such as Der P1 (from house dust mite) and papain (from papaya fruit) appear to rely on their protolytic function as inactive forms of these proteins do not induce type 2 immunity (60). A cysteine protease from the parasite *Leishmania Mexicana* has also been shown to induce type 2 immunity and this could be blocked by protease inhibitors (66). The importance of controlling enzymatic activity at epithelial surfaces is dramatically demonstrated in patients with Netherton syndrome. Netherton syndrome, which is caused by hereditary mutations in the serine protease inhibitor, LEKTI, presents with severe disruption in barrier function and persistent atopy, allergic disease, and AD (74, 75). The mutation in LEKTI results in persistent activation of protease-activated receptor (PAR)-2 and induction of TSLP and type 2 immunity (76).

Other endogenous stress-signals, for example the NLR receptors, NOD-1, and NOD-2, can polarize antigen-specific immune responses toward Th2 and thus contribute to the onset of adaptive immunity (77, 78). Interestingly, NOD-1 and NOD-2 expression within the stromal compartment is necessary to prime effector $CD4^+$ Th2 responses and full Th2 induction is dependent on stromal TSLP (79). The type 2-inducing innate immune sensing is in these cases recognition of bacterial-derived products (peptidoglycans) and not self- or environmental antigens. Although the role of PAMPs and PRRs such as TLRs are usually associated with type 1 immunity, there are other examples in which TLR stimuli can induce type 2 responses. For example, low doses of lipopolysaccharides (LPS) have been proposed to promote Th2 cell responses (whereas high doses promote Th1) (80) for which stromal expression of TLR4 is critical (81). Certain microbial stimuli that signal via DC-SIGN induce Th2 biased responses and many TLR2 agonists have also been shown to suppress Th1 and promote Th2 responses (82). Furthermore, in the case of allergens, there is evidence that some can be directly sensed by PRRs; house dust mite allergens (83) as well as nickel (84) can signal via TLR4 for instance.

Given the vast array of molecules that can trigger type 2 immunity and the many innate and adaptive immune cells involved in orchestrating the response it seems reasonable that there are several routes to inducing type 2 responses and that these may yield a qualitatively different kind of type 2 immunity. The conventional mode of inducing type 2 immunity and high affinity antigen-specific IgG1 and IgE antibody has since long been described and

substantiated. Activated $CD4^+$ αβ Th cells upregulate CD40L and secrete IL-4 and IL-13, whereby they promote germ-line transcription of the γ1 and ε heavy chain to initialize class switching. This requires cognate interactions between B cell MHC II molecules and the TCR–CD3 complex. However, perhaps especially with regards to IgE, there appears to be additional non-conventional modes of inducing class switching and the requirement for T cell help may differ. In contrast to orthodox belief, mice that are deficient in αβ T cells have highly elevated levels of IgE antibodies and class switch particularly efficient to IgG1 and IgE (85, 86). Mice lacking the linker for activation of T cells (LAT) adapter protein (87) or the Tec kinase ltk also have elevated levels of IgE (88, 89), which may be regulated non-conventionally by γδ T cells. Evidence for a non-conventional route to IgE has also been demonstrated during the γδ T cell dependent "lymphoid stress-surveillance response" in the context of stressed skin epithelium (53). It has been established that the IgE produced in immunodeficient mice differ from conventional adaptive IgE not only by being MHC II-mediated T cell cognate independent but also by lacking dependence on germinal centers and thus producing IgE without significant somatic hypermutations (90). Moreover, this "natural" IgE also appears to be mainly self-reactive (85, 90). It may be that in a given circumstance a mixture of conventional adaptive routes and less-adaptive non-conventional routes to IgE are operating simultaneously. For example, infection with a helminth produces not only high affinity antigen-specific IgE but also a lot of "non-specific" IgE and similarly NKG2D-dependent induction of IgE from stressed skin produces not only antigen-specific IgE to an antigen encountered simultaneously but also "non-specific" IgE (53). Analysis of IgE repertoires and the particular requirements for development of IgE-secreting B cells is needed to further elucidate conventional (via Th2) and non-conventional routes to IgE. This may also provide invaluable information as to what actually constitutes a host-protective response (against tissue stress, toxins, parasites) versus allergic Th2 immunity.

IMMUNE SURVEILLANCE

To address the role of EC-driven type 2 immunity in tissue immune surveillance it is useful to first define "immune surveillance." Immune surveillance refers to the capacity of the immune system to sense cellular dysregulation and respond by activating a stress response to restore homeostasis. This continued "quality control" mechanism has most commonly been applied to and studied in relation to cancer. The cancer immuosurveillance hypothesis was first proposed by Ehrlich in 1909 when he predicted that the immune system could repress or destroy the outgrowth of tumors that arise spontaneously on a continued basis (91). This proposal initiated a century of debate over the immune systems role in controlling neoplasia. The idea of a natural immune response against neoplasms or pre-malignant and dysregulated cells was revisited and expanded by Burnet and Thomas in the 1950s (92, 93). They proposed that lymphocytes form the basis of a "cancer immunosurveillance" process that protects immunocompetent hosts against primary tumor development. Although the hypothesis grew in recognition with the expansion of knowledge about the immune system and tumor-antigen recognition, the architects themselves pointed to "the problem with the idea of immunosurveillance is that it cannot be shown to exist in experimental animals" (94) – and it is of course rarely appreciated in a clinical setting. By the early 1990s, little attention was paid to the idea that natural immunity could control tumor establishment *de novo*. However, by the mid-1990s and onward several observations were made that rekindled the interest in this early aspect of tumor immunity [reviewed in Ref. (95)]. In short, the physiological importance of immune surveillance was well revealed by the pathological consequences of its failure: the neutralization of IFNγ with antibodies (96) and later the use of mice lacking IFNγ responsiveness was shown to enhance tumor growth (97). Lymphocytes were unequivocally proven to play an essential role in immune surveillance by seminal observations in *rag2*$^{-/-}$ mice (98) and subsets of lymphocytes such as NK, NKT cells (99), and γδT cells (100) were shown to play prominent roles in the control of malignancy. These new data prompted a refinement of the cancer immunosurveillance concept (95) and an ongoing quest to understand the triggers and mechanistic action of this early and continuous immune response against altered self.

ELICITORS AND EFFECTORS OF IMMUNE SURVEILLANCE

Epithelial-derived cancers, called carcinomas, make up about 85% of all cancers. The epithelial barriers of our body surfaces are also where the majority of exogenous stresses and challenges occur. Both sterile and microbial insults are encountered daily at epithelial surfaces and prompt EC and immune activation. Cancer development is, however, a multifactorial and multistep process. Most solid cancers only emerge following a sequential accumulation of somatic mutations over many years, which eventually may overwhelm the barriers that normally restrain their growth and thus clonal expansion of transformed cells can occur. Cumulative mutational load, telomere dysfunction, and altered stromal milieu are all required before a solid tumor presents (101). Fortunately, numerous intrinsic and extrinsic tumor-supressor mechanisms exist to prevent the development and outgrowth of malignant cells and all cells continuously undergo these rigorous "health checks." The normal health control mechanisms can be triggered both by endogenous and exogenous stress and are executed by a cell-autonomous intrinsic surveillance system (such as delay in cell-cycle progression, repair of DNA-damage/genetic mutations, and induction of senescence or apoptosis) – and backed up by extrinsic immune surveillance mechanisms triggered by manifestations of EC dysregulation. The cell intrinsic responses to stress and the cell-extrinsic responses of the immune system are therefore intimately linked.

Damage-associated molecular patterns are mainly intracellular components of cells that are released or exposed upon physical or metabolic stress or cell death (102). For example ATP released from dying cells can act as a chemoattractant on macrophages drawing them to the stressed tissue (103). Extracellular ATP can bind to P2 purinergic receptors, which dependent on the cell engaged, can induce inflammatory (104) or anti-inflammatory (71) immune responses. Release of ROS or DNA from damaged cells can also powerfully initiate immune surveillance responses (105). Upon stress, ECs also rapidly and potently increase their synthesis of complement C3 (106), which due to its action on a multitude of innate and adaptive immune cells is likely to play a role in early

immune surveillance, although its role in cancer as well as tissue homeostasis is as yet relatively unexplored.

In addition to the release of DAMPs, complement and cytokines/chemokines ECs can in response to numerous forms of cell-dysregulation dynamically alter cell-surface antigens to engage with receptors on innate and adaptive immune cells. Ligand–receptor interactions between ECs and tissue-resident immunocytes are thus important not only for homestatic interactions but are key regulators and elicitors of immune surveillance. One of the most important and best-characterized families of stress-induced EC ligands includes Rae-1, H60, and MULT1 (mouse), MICA, MICB and ULBPs (human). These are members of the larger family of MHC class Ib molecules and are reported upregulated on ECs by stresses such as heat-shock, UV-irradiation, DNA-damage, viral and bacterial infection, and autoimmunity. These unconventional MHC molecules engage the activating lectin-type receptor NKG2D, which is constitutively expressed by tissue-resident T cells and NK, NKT cells but is also expressed on $CD8^+$ T cells and in some circumstances subsets of $CD4^+$ T cells. The NKG2D-pathway has proven important in numerous settings of cell-dysregulation, such as cancer (107), infection (108), autoimmunity (109), and transplantation [reviewed in Ref. (110)], and its key role in immune surveillance is supported by the plethora of strategies tumors and viruses have adopted to evade it (111, 112). In relation to cancer, NKG2D-ligands are expressed by most epithelial tumors and the NKG2D-pathway is strongly associated with anti-tumor responses in both humans and mice (113). NKG2D-ligands are often upregulated early upon cellular dysregulation or transformation, it has however been controversial whether immune cells could be activated by such self-moieties alone. By generating transgenic mice where an autologous NKG2D-ligand, Rae-1, could be upregulated on keratinocytes by administration of doxycycline it was shown that even in the absence of any overt microbial stress (or overt tissue/cellular dysregulation as in a tumor setting) engagement of NKG2D on the epidermal IELs (DETCs) activated these cells and caused profound changes in the local immune compartment (114). This demonstrates that resident immunocytes can recognize and act solely on alterations in autologous stress antigens and thus survey the "health-status" of a given EC, pre-malignancy. The data support the cancer immune surveillance theory as it was also shown that the tissue-resident IELs have a key role in host-protection against skin carcinogenesis (114). Afferent sensing is normally attributed to innate myeloid cells, perhaps particularly to DCs that are often viewed as the primary orchestrator of adaptive immunity. To highlight the capacity of tissue-resident T cells (as demonstrated by the epidermal γδT cells discussed above) to perform an equivalent function as sensors of dysregulation, this mode of afferent sensing has been termed "lymphoid stress-surveillance" (115, 116). Lymphoid stress-surveillance may particularly be engaged in recognition of "stressed-self" and as such confer "beneficial autoimmune" responses in our body surface tissues. It is intriguing that NKG2D is expressed primarily, perhaps exclusively, by lymphoid cells (γδT, NKT, $CD8^+$ αβT, and NK cells), suggesting that engagement of NKG2D could elicit an acute lymphocyte stress response to EC damage perhaps engaging different cells in different tissues.

In addition to the NKG2D-pathway, many other ligand–receptor pathways modulating epithelial-immune cell interactions and contributing to immune surveillance and tissue homeostasis are emerging. One such emerging family of regulators is the nectin and nectin-like (necl) proteins. Nectins are immunoglobulin-like cell–cell adhesion molecules involved in the formation of adherens junctions in ECs and fibroblast. Both nectin and the necl molecules play important roles not only in adhesion but also in migration, proliferation, and wound healing (117, 118). The group of receptors that engage these nectin molecules are therefore now being intensely studied in relation to cancer and immune surveillance (119). The major receptors that bind nectin and necl family members are DNAM-1 (CD226, PTA-1, TLiSA1), class I-restricted T cell-associated molecule (CRTAM), CD96, and TIGIT (WUCAM, VSIG9, Vstm3). All of these receptors are expressed on NK cells, γδT cells and $CD8^+$ αβT cells and can mediate effector functions in these cells upon engagement. DNAM-1 ligands are frequently upregulated on tumor cells and have been reported to be regulated through the DNA-damage response pathway (120). Activation of DNAM-1 can evoke potent cytotoxicity in both T cells and NK cells (121) and control tumor growth (122). CRTAM binds necl2, which have been shown to regulate wound healing in the skin (118) and be involved in metastasis of human tumors (123). Expression and activation of CRTAM on immunocytes is likely highly important in early control of tissue homeostasis and cancer immune surveillance. *In vitro* studies have shown CRTAM to induce IFNγ from T cells and *in vivo* ncl2 expressing tumors have been shown to be controlled by NK and $CD8^+$ T cells. Less is currently known about CD96 and TIGIT, but interestingly TIGIT appears to have an inhibitory function on NK and T cells (119).

Another example of an immune surveillance stimulator displayed by stressed ECs is the Coxsackie and adenovirus receptor (CAR). CAR is also a junctional adhesion molecule, it is upregulated on damaged ECs and potentially revealed when integrity of the tight junction is compromised. It binds junction adhesion molecule-like (JAML), which is expressed on neutrophils, tissue-resident γδT cells, and to a lesser extent on monocytes and some activated $CD8^+$ αβT cells. Resident skin and intestinal γδT cells upregulate their expression of JAML upon tissue injury and binding of JAML to CAR lead to proliferation, cytokine, and growth factor production (124). Inhibiting costimulation of resident γδT cells by blocking JAML significantly delayed wound healing, akin to the total absence of these resident T cells, suggesting that CAR-JAML interactions are important for initiation of immune surveillance and tissue homeostasis. Interestingly, it has been shown that interaction of JAML with CAR recruits the central cell signal transducer PI3K, as is known for the αβT cell costimulator CD28, further emphasizing JAMLs role as a costimulator for tissue-resident T cells with implications for immune surveillance of dysregulated ECs (125).

Similar to the role of CAR-JAML interactions between ECs and resident T cells, it has recently been shown that plexin-B2-CD100 interactions are important for regulating the activity of IELs in both the skin and intestine (126, 127). CD100 (also know as Sema4D) is a member of the large family of semaphorin proteins. These proteins interact with plexins, which were first shown to play a fundamental role in the nervous system directing axon

guidance. Intriguingly though, semaphorin–plexin interactions are also extensively involved in regulating immune responses and analysis of CD100-deficient animals have revealed a crucial role for this semaphorin in both humoral and cellular immunity (128). In relation to EC-immunocyte interactions, plexin-B2 is expressed on ECs in the epidermis and in the colon and interaction with CD100 on resident γδ IELs promotes wound repair in the skin (126) and protects against dextran sulfate sodium (DSS)-induced colitis in the intestine (127). In both tissues, $CD100^{-/-}$ mice failed to mount a proliferative EC response to tissue damage, which was attributable to the lack of activation and growth factor production by the γδ IELs required to heal the epithelium.

Tissue-resident immunocytes are in a unique position to carry out a continued maintenance function such as tissue stress-surveillance. Innate immune cells have the capacity to recognize antigens that are displayed in tissues following a variety of stressors and can respond rapidly in large numbers without requiring clonal expansion. The early stages of an immune response – the afferent phase – are therefore conventionally ascribed to myeloid cells or NK cells. However, as highlighted above tissue-resident T cells can also be afferent sensors of cellular dysregulation. The importance of a tissue-specific resident population in cancer immune surveillance has nevertheless been difficult to verify. This was addressed experimentally by taking advantage of the unique tissue location of specific γδTCR-expressing IELs in the mouse, where the epidermal population of $V\gamma5V\delta1^{+}$ IELs can be specifically knocked out (leaving all other T cell populations intact). These *vg5vd1*$^{-/-}$ mice are significantly more susceptible to cutaneous carcinogenesis than wild-type mice, demonstrating a key role for resident tissue-specific IELs in cancer immune surveillance. Consistent with the cancer immune surveillance hypothesis the γδ IEL act early and significantly suppress the development of papillomas but cannot suppress the progression from papilloma to carcinoma (114). Thus, myeloid cells, NK cells, and IELs all act as afferent sensors of dysregulation and initiators of immune surveillance in epithelial tissues. These cells can then also contribute to the downstream effector and regulatory phases of immunity. Clearly, both $CD4^{+}$ and $CD8^{+}$ αβT cells as well as B cells play important roles in cancer immune surveillance in the effector phase.

FUNCTIONS AND MECHANISMS OF IMMUNE SURVEILLANCE

The afferent phase of immune surveillance – the sensing of dysregulated self – applies to many other stresses than purely oncogenic stress. Thus the concept of immune surveillance not only pertains to cancer; accumulating evidence suggests that it can be more broadly applied to other non-malignant pathologies. For example, liver fibrosis, as a result of liver damage, is exacerbated when NK or NKT cells are depleted or the gene for perforin (required for cytotoxicity) is deleted as the stressed hepatic stellar cells cannot be controlled (129, 130). Stressed hepatic stellar cells express NKG2D-ligands upon damage, as described for ECs, facilitating their recognition by immune surveillance cells. Interestingly, natural activation of hepatic iNKT cells inhibits fibrosis whereas non-natural "over-stimulation" of iNKT cells appears to have the opposite effect and accelerate liver injury (129). Equally in the liver, tissue-resident macrophages have been shown to protect against ischemia reperfusion injury and be critical for tissue homeostasis (131).

Mice lacking normal resident IEL repertoires, such as *Tcrd*$^{-/-}$ mice, develop spontaneous chronic dermatitis, which can only be downregulated when *Tcrd*$^{-/-}$ mice are reconstituted with the tissue-specific resident IEL (the Vγ5Vδ1 TCR-expressing epidermal DETC) (132). Interestingly, these *Tcrd*$^{-/-}$ mice also show a defect in the integrity of the epidermal barrier, as measured by hydration status and transepidermal water loss (TEWL). However, the epidermal barrier defect is obvious only upon environmental challenge, consistent with the notion that the IELs survey the health-status of the ECs and promote tissue homeostasis (133). Skin IELs were also the first T cells to be implicated in promoting EC growth. Closure of full-thickness skin wounds is significantly delayed in *Tcrd*$^{-/-}$ mice, and this is attributed to the skin IELs capacity to rapidly produce EC growth factors such as insulin-like growth factor-1 (IGF-1) and keratinocyte growth factors (KGF) (134, 135). Intriguingly, it has also been shown in humans that T cells isolated from healthy or acutely wounded skin actively produce EC growth factors and participate in wound repair, whereas T cells from patients with chronic wound-healing problems are anergic and unable to produce EC growth factors (136). Similarly, *Tcrd*$^{-/-}$ mice lacking the γδ IEL population, which represents a major intestinal T cell population, are more susceptible to DSS-induced mucosal injury of the gut and demonstrate delayed tissue repair due to the lack of localized delivery of EC growth factors from the missing γδ IEL compartment (137).

The examples above clearly demonstrate that immune surveillance is not only a mechanism to control the development and outgrowth of tumors but is also a key regulator of tissue homeostasis more generally [reviewed in Ref. (138)]. The implication of immune surveillance mechanisms in the maintenance and re-establishment of tissue homeostasis thus broadens its scope and it is likely that similar cell-extrinsic immune surveillance mechanisms are important at disease-initiating (pre-disease) stages in many pathophysiological settings other than cancer.

Immune surveillance can function by many (non-exclusive) mechanisms (**Figure 2**): (1) recognize and remove damaged, stressed, senescent, and (pre)-malignant cells, (2) remove damaging substances, waste, and dead cells, (3) facilitate re-establishment of homeostasis by repair mechanisms, (4) neutralize potential harmful environmental substances, or (5) dampen detrimental inflammatory reactions.

In relation to cancer immune surveillance, the main focus has been on type 1 immunity and cytotoxic mechanisms, both of which have overwhelming experimental support for playing a role in extrinsic tumor suppression. In both genetic and carcinogen-induced tumor models, cytotoxic molecules such as perforin and TRAIL, as well as NKG2D engagement, have repeatedly been shown to be important in tumor control. Likewise mice lacking type 1 molecules such as IFNγ, IFNGR, IL-12, or type I IFN receptors are significantly more susceptible to carcinogenesis in several models. Mice lacking IFNγ, perforin, TRAIL, functioning FasL or IL-12 responsiveness can also develop spontaneous tumors of variable origin [extensively reviewed in Ref. (139)].

However, the repair functions of immune surveillance are clearly also very important in the early phases of immune

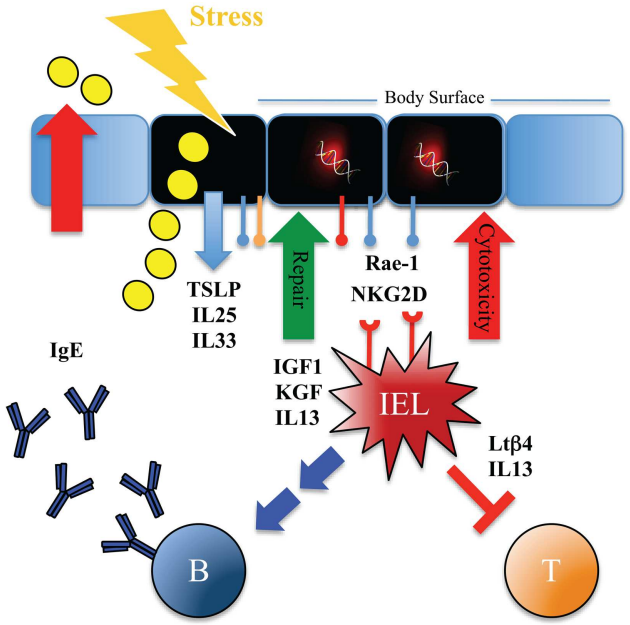

FIGURE 2 | Immune surveillance with type 2 immunity promotes tissue homeostasis and protects against carcinogenesis using numerous layers of control. Scheme illustrating possible mechanisms whereby tissue-resident IELs can provide rapid host-protective immune surveillance and re-establish tissue homeostasis at body surfaces. Tissue stress as imposed by mechanical disruption, injury, or exposure to noxious environmental substances such as chemicals, carcinogens, UV-irradiation or toxins prompts ECs to release IL-25, IL-33, and TSLP cytokines, and upregulate expression of stress-ligands such as Rae-1, CAR, and plexins. This activates the resident IELs and their responses include cytolytic effects, release of growth factors (for example IGF-1, KGF), dampening of $\alpha\beta$ T cell-mediated inflammation [for example by release of thymosin-β4 lymphoid splice variant (LTβ4)], rapid and potent production of IL-13, as well as promoting humoral IgE responses. Stress surveillance by IELs can thus recognize and remove damaged and possibly (pre-)malignant cells, promote tissue repair, and induce type 2 immunity, which in turn controls inflammation, expels or inactivates noxious substances, and promotes morphological tissue homeostasis.

surveillance. This is illustrated by the link between wounding and tumor development. It has been observed that tumors can develop at the site of chronic skin wounds or untreated mouth ulcers (140) and there are several case reports of lung metastasis at sites of accidental trauma (141). A clear illustration of the link between a defective wound-healing response and the development of cancer comes from patients with epidermolysis bullosa. These patients have mutations in genes encoding skin extracellular matrix components and suffer from chronic skin blistering and sores – and as a result of the chronic tissue damage are at increased risk of developing squamous cell carcinoma (142, 143). A diminished capacity to repair a damaged barrier can thus predispose to the development of cancer. Such associations between chronic damage/wounds and cancer as well as the histological similarities of wounds and tumors led to the often-cited phrase that "tumors are wounds that do not heal" (144).

The association between chronic wounds and development of cancer may of course not only pertain to the lack of repair *per se* but also to the onset of a detrimental chronic inflammation as a consequence. There is a close association between chronic inflammation and cancer, and once a malignant cell has escaped the early phase of immune surveillance, inflammation can exert prominent pro-carcinogenic effects (145). The tumor-promoting effects of inflammation are being intensively studied and are starting to have implications for the treatment of cancers (145, 146). An important feature of early tumor immune surveillance could thus be the release of anti-inflammatory products in the tissue. Stressed ECs promptly release many anti-inflammatory products such as IL-33, TSLP, and IL-25 – all with the propensity to drive anti-inflammatory type 2 immune responses. The role of such anti-inflammatory DAMPs and type 2 immunity in early cancer immune surveillance remains to be clarified but intriguingly when tumor-protective skin-resident IELs are activated by stressed ECs they promptly release high levels of IL-13 (53) (and Strid-J unpublished data). Interestingly, this IL-13 and a following production of IgE is dependent on engagement of NKG2D on the IELs (53), perhaps suggesting that the tumor-suppressive effect of NKG2D and skin-resident IELs may not solely be via cytotoxic/type 1-mediated immune surveillance mechanisms. The surprising association between NKG2D and anti-inflammatory type 2 immune responses (and IgE) was recently corroborated in a model of allergic pulmonary inflammation, where mice lacking NKG2D were resistant to the induction of allergic inflammation and showed reduced Th2 and IgE responses (147). The association between a stress-sensor such as NKG2D, which has been intimately linked to anti-tumor responses, and induction of type 2 immunity demands a closer look at the role of early type 2 immunity in cancer immune surveillance. The possible role of such early type 2 responses in tissue homeostasis and immune surveillance of cancer as well as its possible pro- and anti-tumor growth functions are discussed in more detail below.

ROLE OF TYPE 2 IMMUNITY IN TISSUE HOMEOSTASIS AND IMMUNE SURVEILLANCE

What is known so far of the physiological role of type 2 responses is that their host-protection properties converge in different forms of barrier defenses (58). This seems logical as epithelial surfaces have a propensity to drive type 2 immunity (rather than type 1) upon non-invasive/non-penetrating challenge or stress and type 2 immune mediators are thus well poised to play a role in early immune surveillance as well as homeostatic tissue regulation. IL-13 is the best-characterized inducer of mucus production and goblet cell hyperplasia in the respiratory and intestinal mucosa. In the skin, transgenic over-expression of IL-13 induces skin remodeling, which is primarily driven by TSLP (148). In both circumstances, hyperplasia results in improved resistance to damage and damaging substances at the body barrier either via production of mucus at the mucosal surfaces or thickening of the skin. IL-13 may also be involved in homeostatic EC differentiation/proliferation in the skin. Epidermal IELs, which are non-redundant for normal tissue homeostasis and wound-healing, are rapid and potent producers of IL-13 following skin challenge (UV-radiation, tape-stripping, NKG2D-ligand expression, and exposure to carcinogen) and mice deficient in IL-13 have delayed barrier repair following epidermal tape-stripping as measured by TEWL (Strid-J unpublished).

Removal or expulsion is another host defense strategy induced by type 2 immunity, which can directly protect against noxious toxins or parasites, as well as limiting their systemic dissemination. The removal/expulsive actions of allergic and type 2 immunity through sneezing/coughing/itching/vomiting/diarrhea are partly induced by EC-derived mediators including TSLP, which acts directly on sensory neurons in the skin triggering itching (149), and by the effect of mast cell-derived histamine on smooth muscles. Type 2 immune mediators can also confer host-protection by inactivation, neutralization, and destruction of noxious substances. This is most notably shown by the requirement for mast cells in the detoxification of snake and bee venom (150) and the evidence that mast cell proteases can specifically attack snake venom at the structures required for toxicity and thereby neutralizing it (151). Recent data strongly suggest that IgE mediates or at least contributes to protection against venoms as for example the protective responses against re-challenge with high doses of bee venom is abrogated in mice lacking B cells, FcεRI, or IgE (56, 57). It is likely that this protection is partly via the very rapid IgE-mediated degranulation of mast cells. Encapsulation and restriction is another layer of barrier defense regulated by type 2 mediators, which can help prevent the spread of noxious substances if elimination or expulsion has been insufficient. Endothelial leakage and exudate formation can be induced by mast cell-derived products and such local tissue edema may impede parasite invasion. For example it has been shown that the edema caused by IgE-mediated mast cell degranulation is important in the defense against macroparasites such as ticks (152). Another restriction mechanism, which may restrict the spread of noxious substance as well as macroparasites, involves sequestration through granuloma formation. Type 2 immune responses protect the host during infection with schistosomiasis by inducing granulomas that sequester the tissue-damaging toxins from the parasite eggs (153).

Perhaps most importantly, much of type 2 immunity seems dedicated to tissue repair and promoting tolerance to damage. Indeed, it has been hypothesized that type 2 immunity has evolved to direct innate wound repair mechanisms (154). The rationale for the induction of tissue repair as a part of type 2 immune defense is obvious. It may also explain the extreme urgency of some type 2 responses (which is not easily explained if directed only toward a slow replicating macroparasite), as damage control may well be more important than pathogen control. In evolutionary terms, it makes sense to be able to quickly expel or neutralize noxious substances as well as rapidly repair the life-essential body barrier. EC-derived cytokine alarmins and cell-ligands can activate and direct the resident tissue cells to promote repair responses; IELs rapidly sense stress and can produce growth factors locally in the absence of further inflammation and tissue-resident ILCs can amplify the type 2 response and produce amphiregulin. Indeed, depletion of ILC2 compromises lung epithelial barrier integrity (155) just as depletion of γδ IEL compromises skin and gut epithelial integrity during homeostasis as well as delaying wound healing (127, 133, 134). Almost all of the cells associated with type 2 immunity are also associated with the wound-healing response. Alternatively activated macrophages produce vascular endothelial growth factor (VEGF), arginase 1, and IGF-1; Eosinophils store preformed growth factors, matrix metalloproteinases, and lipid mediators, all of which can mediate wound healing (156). Amphiregulin is produced by mast cells following FcεRI signaling, potentially also linking IgE responses to wound repair (157). In sum, the effects of type 2 immunity at our body surface tissues play important roles in eliminating, restricting, and neutralizing noxious environmental substances as well as repairing the damage caused and minimizing inflammation – as such this type of immunity is critical for tissue homeostasis and responses to challenges that have breached the epithelial barrier.

In terms of early cancer immune surveillance, the role of type 2 immunity has been little explored. However, the effects of rapid type 2 immune responses as outlined above could indeed play a prominent role in cancer immune surveillance. It has been demonstrated that the same tissue-resident IELs act as key components of tumor resistance and potent inducers of type 2 immunity and IgE antibodies (53, 114). The humoral component of this lymphoid stress-surveillance response may limit tissue damage by targeting noxious foreign substances, such as toxins that may be the root cause of the tissue dysregulation. The IgE effector response may promote toxin expulsion and limit their systemic dissemination. Simultaneously, the cellular response can direct cytotoxicity toward dysregulated cells as well as promoting repair of the damaged tissue and dampening inflammation (**Figures 2** and **3**). To limit the likelihood of cancer, it is clearly important to repair a wound or breached barrier quickly and efficiently as is demonstrated by the close association between chronic tissue damage and cancer. Less efficient wound repair may lead to inefficient immune surveillance against (pre-)malignant cells with damaged cells being allowed to stay longer in the tissue before being replaced. Additionally, slow repair of tissue damage may lead to inflammation, which as discussed can have potent pro-carcinogenic effects.

Although one can imagine a role for type 2 immunity and its regenerative capacity in early cancer immune surveillance this may indeed be a double-edged sword in further development of cancer (**Figure 3**). Failure of the type 2 response to adequately contain or eliminate the initiating substance may lead to a chronic wound-healing response and exacerbation of inflammation. Such continued tissue damage, repair, and regeneration may ultimately result in fibrosis. Fibrotic tissue is a highly permissive environment for tumor formation and it is also well established that continuous wound-healing responses and tumorigenesis are two processes that rely on similar molecular mechanisms (158). As such it is perhaps not surprising that the literature on type 2/IgE responses and cancer is somewhat bewildering.

ROLE OF TYPE 2 IMMUNITY AND IgE IN CANCER

Both positive and negative effects of type 2 immunity on tumor growth and carcinogenesis have been reported in the literature (**Table 1**). Contrasting results are clearly in part due to the differing experimental approaches and models but most likely also reflects the divergent roles that type 2 immunity may play at different stages of carcinogenesis and in different tissues. $CD4^+$ T cell-derived IL-4 has been reported to induce granulocyte infiltration, thereby promoting tumor clearance (an action enhanced by IL-13), and conversely increase tumor cells' resistance to apoptosis by up-regulation of anti-apoptotic proteins (159). Many

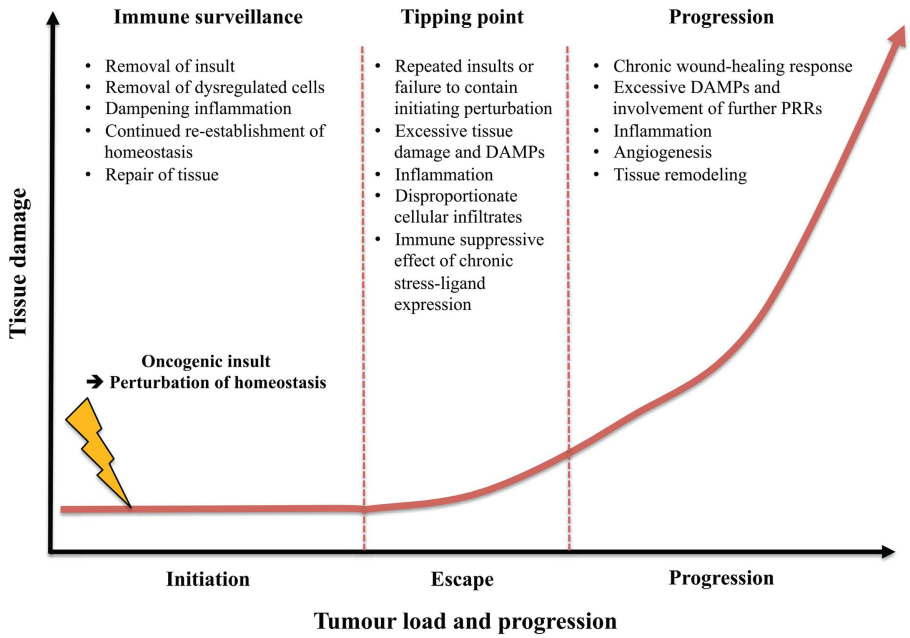

FIGURE 3 | Contrasting role of type 2 immunity in immune surveillance of early tissue dysregulation versus tumor progression. The type 2 component of immune surveillance may aid in host-protection against carcinogenesis at epithelial surfaces by removing the oncogenic insult, eliminating the dysregulated cells, dampening excessive inflammation, repairing tissue, re-establishing homeostasis, as well as improving resistance to future damage. However, following continuous perturbations, failure to eliminate the initial insult or dysregulated immune surveillance a tipping point may be reached where excessive tissue damage and DAMPs lead to inflammation, disproportionate cellular infiltrates, and escape from immune surveillance and tissue homeostasis. Once a certain level of tissue damage is reached, a perpetual type 2 response may be detrimental to the host by transitioning to aid carcinogenesis by promoting a chronic wound-healing response and fibrosis as well as supporting neo-angiogenesis.

studies carried out in the 1990s and early 2000s demonstrated that tumors or tumor cell lines engineered to produce IL-4 would exhibit increased rejection and retarded growth *in vivo*; curiously a phenotype often dependent on $CD8^+$ cytotoxic T cells (160–163). IL-10 has been reported to be both "pro-tumorigenic" by inhibiting tumor cell lysis by cytotoxic T cells and "anti-tumorigenic" by promoting NK-cell-mediated tumor clearance and inhibiting angiogenesis (164). The EC-derived cytokines IL-33 and TSLP have been shown to enhance tumorigenesis by promoting epithelial-mesenchycmal transition (EMT) in organotypic culture of *ex vivo* carcinoma-associated fibroblasts (CAFs) and squamous cell carcinoma cells (165) and by enhancing Th2 inflammation (166). On the contrary TSLP has convincingly been shown in mouse *in vivo* models to be critically important for resistance to skin carcinogenesis (35, 36) establishing TSLP as a tumor suppressor in the skin. Many epithelial cancers express receptors for type 2 mediators such as IL-4 and IL-13, allowing for a direct effect on tumor growth, death, and proliferation that is independent of their effect on immunocytes (167). This perhaps also explains the likely divergent effects of type 2 immunity in early cancer immune surveillance versus in established tumors. Human breast and renal cancer cell lines treated with exogenous IL-13 *in vitro* demonstrate reduced proliferation (168–170) – although an ovarian cancer line demonstrated enhanced invasive and enzyme (protease) activity (171) suggesting IL-13 aids primary tumor invasion and metastasis in this model. No doubt the heterogeneity of cancer cell lines and *ex vivo* tumors derived from patients and experimental animals clouds a consistent description of how potent type 2 cytokines may affect growth and immune surveillance of tumors *in vivo*. Cellular type 2 players, such as eosinophils, have an extensive description in the cancer literature, and have been shown to play a protective role against chemically induced tumors *in vivo* – and directly kill chemically induced fibrosarcomas *in vitro* (172), suggesting efficacious tissue immune surveillance. Mast cells frequently infiltrate the tumor microenvironment and are usually correlated with a poor prognosis in human cancers (173), although both tumor rejection and promotion has been attributed to them in the mouse. Interestingly, the ancient and highly conserved (174) immunoglobulin isotype IgE has been shown to play a significant role in immune surveillance of tumors. Since the 1990s IgE mAbs have been considered for cancer immunotherapy; particularly given IgE's extreme biological potency and presence of high and low affinity receptors on various effector cell types (175). Indeed, animals deficient in IgE show drastically altered susceptibility to cutaneous chemical carcinogenesis, and an altered tumor cytokine microenvironment (Dalessandri-T and Strid-J; unpublished). IgE-coated irradiated tumor cells has also been shown to generate protective, eosinophil, and T cell immunity to subsequently administered non-irradiated tumors (176). In addition, the type 2 immunoglobulin IgG1 has been reported to be potently tumoricidal when not "blocked" by competing IgG4 antibodies (177).

Table 1 | Examples of type 2 cytokines and immunoglobulins influencing tumor pathology.

Type 2 mediator	Experimental approach	Model	Tumor growth
IL-4	Tumors engineered to produce IL-4, IL-4 ↑	Primary murine renal cancer	↓ Enhanced $CD8^+$ T cell-dependent rejection (160)
		Injection of syngeneic tumor cell lines	↓ Enhanced $CD8^+$ T cell-dependent rejection (161)
			↑ Delayed primary tumor clearance, increased secondary tumor development (178)
			↑ Reduced $CD8^+$ T cell-mediated clearance (178)
		Primary murine adenocarcinoma	↓ Enhanced $CD8^+$ T cell-dependent and eosinophil-mediated rejection (162)
		Vaccination with irradiated tumor cells	↓ Enhanced $CD8^+$ T cell-dependent clearance of lung metastases (163)
	Exogenous rIL-4 treatment, IL-4 ↑	Prostate, breast and bladder cancer cell lines	↑ Enhanced resistance to apoptosis and chemotherapeutic agents (159)
IL-13	Tumors engineered to produce IL-13, IL-13 ↑	Injection of P815 mastocytoma cell line	↓ Improved rejection and development of systemic anti-tumor immunity (179)
	Exogenous IL-13 treatment, IL-13 ↑	*Ex vivo* leukemic B blasts	↓ Reduced proliferation and cell-cycle progression assessed by DNA content (168)
		Human breast cancer cell line	↓ Inhibition of estrogen-induced cell proliferation, unchanged basal proliferation (169)
		Human renal carcinoma cell line	↓ Reduced proliferation and colony formation (170)
		Ovarian cancer cell line	↑ Increased MMP and AP-1-dependant invasion and protease activity in matrigel invasion assay (171)
	Antibody-mediated IL-13 neutralization, IL-13 ↓	Hodgkin lymphoma cell line	↓ Decreased proliferation and STAT6 phosphorylation (180)
IL-33	IL-33 receptor knockout ($ST2^{-/-}$), IL-33-signaling ↓	$ST2^{-/-}$ mammary carcinoma-bearing mice	↓ Attenuated tumor growth and metastasis, increased number and cytotoxic activity of NK cells (181)
	Exogenous IL-33 treatment, IL-33 ↑	4T1 cell line tumor-bearing mice	↑ Reduced intra-tumoral tumoricidal NK cells, increased splenic MDSCs and M2 macrophages (182)
	IL-33 co-admin, with HPV DNA vaccine, IL-33 ↑	TC-1 cell line (HPV-16 E7-positive) tumor-bearing mice	↓ Improved HPV antigen-specific CD4 and CD8 T cells, increased TC-1 regression (183)
	Organotypic culture, IL-33 ↑	*Ex vivo* human carcinoma-associated fibroblasts (CAFs)	↑ CAFs promote carcinoma invasion via IL-33 signaling and EMT induction (165)
TSLP	Antibody-mediated TSLP neutralization, TSLP ↓	Murine breast tumor xenograft	↓ Inhibition of tumor development (166)
	K14-$TSLP^{Tg}$ or calcipotriol induced TSLP, TSLP ↑	DMBA/TPA chemical skin carcinogenesis	↓ Delayed tumor onset and significantly reduced tumor number and growth (35)
	TSLP receptor knockout or TSLP neutralization, TSLP-signaling ↓	Notch1/Notch2 receptor knockout	↑ Loss of TSLP-signaling in Notch-deficient epidermis leads to tumor formation (36)
IgE	IgE-loaded tumor cell vaccine, IgE ↑	Post-vaccination challenge with RMA lymphoma or MC38 adenocarcinoma	↓ Improved protective eosinophil, $CD4^+$ and $CD8^+$ T cell responses to tumor challenge (176, 184)
IgGI	Engineered tumor-antigen-specific IgG4, IgGI ↓	Human melanoma xenograft model	↑ IgG4 blocked potent IgGI-mediated anti-tumor effector functions (177)

EPIDEMIOLOGICAL AND CLINICAL ASSOCIATIONS BETWEEN TYPE 2 IMMUNITY, IgE AND CANCER

Associations between allergy history and cancer risk have been investigated in numerous epidemiological studies and their association is being defined in the nascent field of "AllergoOncology" (**Table 2**). Recent overviews of the epidemiological literature demonstrate that both potent inverse and positive associations exist, which point to complex underlying interactions as well as reflecting the heterogeneity of these diseases. Accordingly, although the relationship between cancer and allergy has intrigued researchers for decades, the biological nature of this association remains unclear.

Table 2 | Proposed hypotheses explaining associations between type 2 immunity/allergy and cancer.

Hypothesis	Predicted allergy–cancer relationship	Predicted affected tissue site	Proposed mechanisms
Antigenic stimulation or chronic inflammation (185)	Positive, causal	All sites	• Allergic inflammation and oxidative damage promote pro-tumorigenic gene mutations • Type 2-induced tissue remodeling and angiogenesis promotes tumor growth and invasion
Inappropriate Th2 skewing (186)	Positive, causal	All sites	• Diversion away from protective cytolytic type 1 responses • Non-protective IgE clonality, or poorly tumoricidal IgG4 class switching with immunosuppressive IL-10
Immune surveillance (93)	Inverse, causal	All sites	• Potent effector cells, including γδT cells, mast cells and eosinophils eradicate tumors • Tumor-specific IgE potently cytolytic via ADCC • Type 2 immunity repairs tissue damage and dampens inflammation hereby restricting tumor formation
Prophylaxis (55)	Inverse, causal	Mucosal and external surfaces	• Tissue type 2 immunity removes or neutralizes noxious and potentially carcinogenic environmental moieties before they cause genotoxicity • Type 2 immunity restricts systemic dissemination of noxious substances and enhances natural barrier defenses

Two hypotheses put forward to explain positive allergy–cancer associations are the "antigenic stimulation"/"chronic inflammation" hypothesis and the "inappropriate Th2 skewing" hypothesis (**Table 2**). The "antigenic stimulation" hypothesis was first proposed in the late 1980s (185) and has been reiterated numerous times, also termed the "chronic inflammation" hypothesis (186). This hypothesis proposes that inflammation associated with allergic disease establishes a tissue environment conducive to tumor growth. Indeed, more than 100 years ago a link between inflammation and cancer was first proposed by Virchow (146), who noted the presence of leukocytes in neoplastic tissues and suggested cancer originated at sites of chronic inflammation. Tissue damage with the release of DAMPs, chronic infection, and inflammation are all believed to contribute to the development of malignant disease. Mechanistically, cellular Th2-mediators such as macrophages promote oxidative damage through production of iNOS and hydrogen peroxide via the respiratory burst, increasing the likelihood of damage and mutation of tumor-suppressor genes or cell-cycle regulator genes. Tissue remodeling and pro-angiogenesis factors such as vasoactive mediators from tissue-resident mast cells and eosinophils, as well as VEGF, arginase and matrix metalloproteases released by macrophages, may promote local invasion of outgrowing tumors, and eventual metastasis with establishment of distal secondary loci worsening clinical outcome. Thus, the "antigenic stimulation/chronic inflammation" hypothesis predicts a positive relationship between allergic disease and cancer in any tissue site and this relationship is directly causal, i.e., inflammation secondary to or as a result of allergic disease directly promotes oncogenesis. The "inappropriate Th2 skewing" hypothesis (186) suggests that type 2 mediators – such as IL-4, IL-10, IL-13 – may redirect tissue immunity away from a potently anti-tumor and cytolytic Th1 response, toward an ineffective Th2 response, where IgE is produced and directed toward allergens and not tumor-specific or tumor-associated antigens. Additionally, with production of immunomodulatory IL-10, Th2-immunogloublin IgG4 class-switch recombination is favored over IgE, the former being far less potently tumoricidal, further attenuating anti-tumor responses. This hypothesis therefore also predicts a positive relationship between allergic disease and cancer, in any tissue site, and this relationship is directly causal – skewing to type 2 responses that are non-protective and aids oncogenesis.

Two hypotheses put forward to explain inverse allergy–cancer associations are the "immune surveillance" hypothesis, and the "prophylaxis" hypothesis (**Table 2**). Prophylaxis was first proposed by Profet (55), and suggests that the symptoms and mechanisms of allergic disease serve to repel and clear potentially mutagenic substances at the external body surfaces before mutagenesis can occur; a coopted function of type 2 immunity which also serves to expel parasites and helminths. Itch induced by type 2 mediators such as TSLP, goblet cell hypersecretion of mucus, sneezing, coughing, vomiting, and diarrhea all act as repulsive mechanisms and are particularly common allergy symptoms. In additional to physical expulsion, type 2 cellular players directly deactivate noxious xenobiotics. In mice at least, mast cells have been shown to degrade venom components through release of carboxypeptidases (150), and IgE raised to a conserved component (and allergen) of many venoms is protective against a repeat exposure (56, 57). The unpleasantness of allergy symptoms also conditions the animal to avoid potentially carcinogenic triggers. Thus, the "prophylaxis" hypothesis predicts an inverse, causal relationship between allergic disease and cancer, particularly at the exposed body barrier surfaces. This hypothesis requires that moieties encountered at the body surfaces are directly carcinogenic, or are pro-carcinogens, and predicts that individuals with allergy symptoms should present with (i) lower levels of carcinogens in their blood and (ii) restrictive or obstructive disease at mucosal

surfaces – or that treatment to reduce allergic symptoms results in greater vulnerability to cancer at those sites (187). These corollaries of the "prophylaxis" hypothesis have been poorly investigated. The "immune surveillance" hypothesis, first proposed by Burnet (93), also predicts an inverse allergy–cancer relationship and inverse associations are predicted at any body site. It suggests that allergy and atopic symptoms are indicative of an immune system that is generally hyper-responsive to challenge, and has enhanced immune surveillance capability. Potently cytolytic type 2 responses raised against tumor-associated or -specific antigens can rapidly eradicate dysregulated and proto-neoplastic cells; hence allergy symptoms are a fortuitous, albeit unpleasant, result of an individual's potent immune system, which also controls dysregulated cells and results in an inverse allergy–cancer relationship. Although the inverse allergy–cancer relationship proposed by the "immune surveillance" hypothesis was originally thought to be purely correlational, as argued in this review, type 2 immunity is also likely to play an important role in early immune surveillance in a direct casual manner by virtue of its ability to remove noxious substances, repair tissue damage and dampen initial inflammation.

In spite of many speculations and associations there is little evidence for a strong association between allergy and *overall* cancer risk (188). However, given that allergic disease occurs primarily at outer epithelial surfaces it is logical to examine the incidence of cancer at specific tissue sites, particularly those at which allergic disease is prevalent; such as the skin, respiratory, and gastrointestinal tracts. A recent large meta-analysis of more than 400 studies of relationships between allergy and cancer reported a preponderance of inverse allergy–cancer associations, and interestingly this was particularly strong for cancers of tissues that interface with the external environment, such as skin, mouth, throat, colon, and cervix (187). These results support the "prophylaxis" as well as the "immune surveillance" hypotheses. Intriguingly, while most studies investigate the link between specifically Type I allergic disease and cancer, a significant inverse association between contact (Type IV) hypersensitivity and breast and non-melanoma skin cancer has been reported, and the authors suggested these data support the "immune surveillance" hypothesis (189).

The large number of published association studies nevertheless often paints a conflicting picture, some of which may be due to methodological constraints. Most retrospective studies on allergy–cancer associations have investigated self-reported or clinician-diagnosed allergy, methodologies particularly prone to recollection and reporting bias (former) and subject selection bias (latter). Researchers have attempted to alleviate these concerns at least in part by discriminating subjects on the basis of physiological indices of allergic disease, such as serum IgE titers (190, 191) and skin-prick testing. Of course, serum total or allergen-specific IgE suffers less from human biases, but these are not a definite metric of allergic status in all individuals, all of the time; in addition this methodology potentially precludes the possibility of examining significant non-Type I hypersensitivities in the analysis. Another concern is that if variation between individual's allergy symptoms is due more to differences in individual's exposure to antigens and/or carcinogens (the environment as a confounding variable), rather than individual differences in immunity ("immune surveillance" hypothesis), then positive correlations between allergic symptoms and cancer may occur – even if the "prophylaxis" or "immune surveillance" hypotheses are true. Particularly, given that exposure to carcinogenic allergens (such as cigarette smoke or vehicle exhaust) results in increased cancer and allergic disease (187). In addition, the genetic and phenotypic heterogeneity of tumors, and the complex inflammatory niche in which they reside, may also confound epidemiological association studies.

CONCLUDING REMARKS

Epithelial cancers are products of a series of events starting with dysregulated and stressed ECs. It is now clear that challenges to ECs can trigger discrete pathways promoting the release of specific cytokines, chemokines, and expression of stress antigens on the EC surface. Together this can powerfully drive immune responses, initially from cells resident in the epithelial and subepithelial compartment. The initial response to EC challenge and damage is often a very rapid type 2 immune response. This may serve to remove or neutralize noxious challenging substances, clear waste, repair the tissue, dampen inflammation, and re-establish tissue homeostasis. It may also directly contribute to the elimination of damaged cells together with cytolytic mechanisms from resident IELs and other immunocytes. Rapid type 2 immune responses at body surfaces thus prominently contribute to immune surveillance of dysregulated ECs. Since epithelial dysregulation contributes notably to a multitude of inflammatory diseases, this may not only be important in control of (pre-)malignancy but could be important at disease-initiating stages in a variety of diseases.

Immune surveillance by its nature is mainly important in the initiation phase of tissue damage and tumor control – for maintenance of tissue homeostasis. The same effector molecules and mechanisms may play a very different role during the progression phase of tissue damage and tumor growth. Failure to eliminate the original damaging substance, damaged cells, or to repair the tissue may lead to a continual stress response with excessive release of DAMPs, persistent stress-ligand expression, inflammation, and a chronic wound-healing response and this may determine the transition point between beneficial and detrimental functions of type 2 immunity in the course of disease. Similarly, continual exposure to noxious environmental substances may eventually overwhelm the immune surveillance mechanisms keeping the damage in check and result in pathology or tumor growth. Further research is needed to study whether the balance between tumor cell growth and elimination may be tipped back upon immune manipulations aimed at enhancing naturally occurring immune surveillance.

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Opportunistic autoimmune disorders potentiated by immune-checkpoint inhibitors anti-CTLA-4 and anti-PD-1

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To improve the efficacy of immunotherapy for cancer and autoimmune diseases, recent ongoing and completed clinical trials have focused on specific targets to redirect the immune network toward eradicating a variety of tumors and ameliorating the self-destructive process. In a previous review, both systemic immunomodulators and monoclonal antibodies (mAbs), anti-CTLA-4, and anti-CD52, were discussed regarding therapeutics and autoimmune sequelae, as well as predisposing factors known to exacerbate immune-related adverse events (irAEs). This review will focus on immune-checkpoint inhibitors, and the data from most clinical trials involve blockade with anti-CTLA-4 such as ipilimumab. However, despite the mild to severe irAEs observed with ipilimumab in ~60% of patients, overall survival (OS) averaged ~22–25% at 3–5 years. To boost OS, other mAbs targeting programed death-1 and its ligand are undergoing clinical trials as monotherapy or dual therapy with anti-CTLA-4. Therapeutic combinations may generate different spectrum of opportunistic autoimmune disorders. To simulate clinical scenarios, we have applied regulatory T cell perturbation to murine models combined to examine the balance between thyroid autoimmunity and tumor-specific immunity.

Keywords: anti-CTLA-4, anti-PD-1, autoimmune disease, tumor immunity, immune-checkpoint inhibitor

INTRODUCTION

In recent decades, cancer therapy has focused on amplifying the immune system to bolster the host's anti-tumor response. Utilizing systemic immunomodulators, monoclonal antibodies (mAbs), and stem cell transplantation, progress has been rapid in prolonging survival. However, targeted immunotherapy has come with a price; altered immunoregulation provoking immune dysfunction has opened the door to opportunistic autoimmune disorders. Previously, both systemic immunomodulators and mAbs, anti-CTLA-4 and anti-CD52, were discussed in terms of therapeutic usage, autoimmune sequelae, and important predisposing factors, e.g., *HLA* class II genes and gender predilection, known to influence immune-related adverse events (irAEs) (1). Similar and additional immunotherapeutic modalities associated with autoimmunity, particularly thyroid dysfunction, were likewise highlighted by others (2, 3). In addition to CTLA-4, a number of immune-checkpoints are also being targeted in cancer immunotherapy. Thus far, the most information has come from longer and larger clinical trials with anti-CTLA-4 (ipilimumab and tremelimumab), accompanied by mild to severe irAEs (4–6). In early trials, it was hoped that irAEs could serve as a predictor of improving anti-CTLA-4 immunotherapy (7, 8). However, analysis of 139 metastatic melanoma patients given ipilimumab revealed that the frequency of irAEs after a 2–4 year follow-up was 81% with a total response rate of 17% (23 of 139) (7); of the 86 patients with irAEs, 74% (64 of 86) showed no objective improvement (1).

Thus, targeting CTLA-4, a T cell regulatory molecule, impacts on its two primary functions in the immune network: (1) Its upregulation during a T cell-mediated response serves as a negative regulator by engaging the B7 family costimulatory molecules on antigen-presenting cells with higher avidity than CD28 (9); and (2) Its constitutive expression on regulatory T cells (Tregs) is critical to the Foxp3 function in suppressing autoreactive T cell activation (10, 11). *ctla4*$^{-/-}$ mice develop severe multiorgan autoimmunity, indicative of deficiency in both these functions (12, 13). When humanized CTLA-4 mAbs were first used to treat advanced melanoma a decade ago, the major goal was to interfere with the negative signaling of an ongoing anti-tumor response discernible in many patients (4, 6). However, since a key role of CTLA-4 is to enable Treg suppression of autoreactive T cell activation at the costimulatory level of DC (10, 11), it was no surprise that opportunistic autoimmune disorders surfaced as prominent irAEs from CTLA-4 blockade. The variety stems from the ever-present autoantigens and autoreactive T cells unleashed from self tolerance regulation (1). For example, in our tolerance induction study in murine experimental autoimmune thyroiditis (EAT), a model for the prevalent Hashimoto's thyroiditis (14), co-injection of anti-CTLA-4 with the autoantigen, thyroglobulin (Tg), interfered with activation of naturally existing $CD4^+CD25^+Foxp3^+$ Tregs (nTregs); the mice developed thyroiditis, mimicking a major clinical autoimmune sequela (15).

Using revised assessment criteria including overall survival (OS) in phase II/III trials to take into account the longer survival kinetics for ipilimumab (16), recent compilation of ipilimumab phase I/II trial results showed a range of 12–36% OS at 3–5 years, with variables including the dose, patient number, prior, or adjunct treatment (4). Pooling phase II/III trials

showed irAEs approximating 60% with less severe grade 3–4 in the phase III trials, likely due to earlier recognition and management of autoimmune sequelae (4, 17). While the percentages of irAEs varied, most included skin rashes, colitis, thyroid dysfunction, hypophysitis, hepatitis, and pancreatitis (4, 17), as also reported in western Europe (5). Treatment-related deaths continued to occur and severe morbidity required stringent life-long treatment and hormonal supplementation (4, 5). The second mAb, tremelimumab, likewise underwent phase I/II trials [see Ref. (1)] and phase III trials with similar irAEs but less durable OS than with ipilimumab; in fact, survival was not much longer than after standard chemotherapy with temozolomide or dacarbazine (6). To boost OS, mAbs that blockade the function of another immune-checkpoint, programed death-1 (PD-1), or its ligand (PD-L1), have been undergoing clinical trials as monotherapy or dual therapy with anti-CTLA-4.

MODELS TO PROBE THE BALANCE BETWEEN AUTOIMMUNITY AND TUMOR IMMUNITY UPON Treg PERTURBATION

The high percentages of irAEs from anti-CTLA-4 therapy clearly show that maintenance of Treg function and self tolerance constitutes a premier CTLA-4 function. Autoimmune thyroid disease, including Hashimoto's thyroiditis and Graves' disease, represents the most prevalent autoimmune condition (18), and CTLA-4 blockade has joined other systemic immunomodulators [e.g., interferon-α, -β, interleukin (IL)-2] and leukocyte-target agents (e.g., anti-CD52) in triggering thyroid dysfunction (1, 3, 19, 20). As EAT has long served as a model to study Treg function in self tolerance to mouse Tg (14, 21, 22), we developed in recent years four murine models combining EAT and breast cancer vaccine protocols under Treg perturbation and MHC class II gene influence, using autoimmune thyroiditis as a sequela indicator (23).

The first three utilized well-established tumor models in wild type mice or mice transgenic for Her-2/neu breast cancer antigen, which harbor class II-linked, EAT-resistant haplotype ($H2^d$ or $H2^b$). In the first model, we induced EAT concurrently with anti-tumor immunity in wild type mice at the time of Treg depletion with mAb to CD25 (24). Treg depletion enhanced tumor regression and thyroiditis. Immune responses to neu and mouse Tg were greater than control groups given tumor or Tg alone, indicating that ongoing tumor regression and autoimmune response provided additional mutual stimuli. In the second model, we used rat neu-transgenic mice, which required both Treg depletion and neu DNA vaccination to develop resistance to tumor challenge and spontaneous tumorigenesis (25). Mutual stimulation during responses to neu and mouse Tg was again observed. In tumor-regressing mice, there were significant increases in interferon-γ-producing T cells and greater thyroid destruction even in the EAT-resistant strain. Lastly, in the third model, we introduced the HLA-DR3 transgene, an EAT-susceptible allele (26), and an Her-2 transgene into EAT-resistant $H2A^b$ mice to determine if anti-tumor response was independent of EAT susceptibility. (A^b-Her-2xDR3)F_1 mice expressed both A^b and DR3 and were tolerant to both Tg and Her-2 (27). After Treg depletion followed by Her-2 DNA and mouse Tg injections, tumor rejection was similar in Her-2 transgenic mice expressing either A^b or A^b/DR3, but thyroiditis was augmented only in (A^b/DR3)F_1 mice, showing that Her-2 immunity, unlike autoimmunity, was independent of DR3 expression.

In the fourth combined model, we used an EAT-susceptible CBA/J ($H2^k$) haplotype with demonstrated antigen-specific nTreg-mediated tolerance. Depleting pre-existing nTregs markedly enhanced thyroiditis development with soluble mouse Tg even without adjuvant (15). Control mice with nTregs, which had been activated/expanded after exposure to mouse Tg by either injection or physiologic release (via thyroid-stimulating hormone infusion in an osmotic pump), withstood EAT induction with mouse Tg plus adjuvant (22, 28). In contrast, nTreg-depleted mice were incapable of establishing this strong and long-lasting tolerance (14, 15, 22). For the cancer portion, CBA/J tumor was derived from a spontaneous mammary adenocarcinoma line, and resistance to lethal challenge was instilled by prior Treg depletion and vaccination with irradiated tumor cells (23). To simulate patients with MHC class II-associated predisposition to autoimmunity and subjected to immune targeting, mouse Tg was also given. Treg depletion not only augmented tumor immunity but also thyroidal infiltration (29). Furthermore, to simulate the scenario in some cancer patients with pre-existing autoimmunity and given immunotherapy, mice were pretreated with mouse Tg + low doses of IL-1 to establish a subclinical, mild thyroiditis condition. Treg depletion, tumor vaccination, and mouse Tg injections then followed. While anti-tumor immunity remained unchanged, thyroiditis was exacerbated (29). Thus, this recent model takes into account genetically predisposed patients who have no underlying thyroid dysfunction or have pre-existing, undiagnosed disease.

TARGETING CTLA-4 LED TO UNUSUAL SPECTRUM OF AUTOIMMUNE SEQUELAE

In murine EAT, tolerance induction with the known autoantigen, mouse Tg, and its blockade of nTreg activation by anti-CTLA-4 to allow thyroiditis development can be followed with timed co-administration (15). However, in cancer patients, there are multiple self antigens for which the maintenance mechanisms of self tolerance can be disrupted with anti-CTLA-4 therapy at varying doses and intervals, resulting in unpredictable manifestations of 20–60% of irAEs with grade 1–4 severity. In addition to advanced melanoma, both ipilimumab and tremelimumab have been used to treat other solid tumors and hematologic cancers and the frequent irAEs have included dermatitis, enterocolitis, hepatitis, thyroid dysfunction, and hypophysitis with widespread endocrinopathies, irrespective of cancer types (3–6, 19, 20). Although these autoimmune diseases are primarily organ-specific, T cell-mediated, and with mononuclear cell infiltration of the specific organ, plasma cells may be seen with time. Moreover, when several organs are involved with attendant proinflammatory cytokines, irAEs may become exacerbated and more widespread. Thus, different irAEs often occur throughout the treatment period and beyond.

There is agreement that the extent of severe irAEs is dose-dependent, but OS improvement generally does not correspond

linearly with dosage (5), nor correlates with severity of irAEs (1, 19). Torino et al. (19) undertook an in-depth analysis of endocrine dysfunction for 21 phase I–III trials with ipilimumab treatment of melanoma and several other solid tumors. The primary emphasis was on hypophysitis of grades 3–4, but the involvement of other endocrine dysfunctions (hypothyroidism, adrenal insufficiency) and other common irAEs (e.g., diarrhea, colitis/enterocolitis, dermatitis, hepatitis, and arthritis) was also included. A more recent report by some of the same authors, Corsello et al. (20), extended the analysis to 28 trials, subcategorizing classifiable endocrine disorders and other common irAEs, which may or may not be autoimmune in origin. As mentioned above, since irAEs generally involved up to 60% of patients, at 2–3× the OS, a direct correlation of OS with irAE would be difficult at this time (4).

As discussed earlier (1), while the increase in thyroid dysfunction could have been anticipated, given the high prevalence in the general population, the most unusual and distinct association with anti-CTLA-4 therapy is autoimmune hypophysitis, with an incidence of 3–9% in early trials far exceeding its rare occurrence (~1 in 0.5 million) (19, 30). Another early melanoma trial with ipilimumab reported 17% (8 of 46) (31). The analyses of clinical trials with ipilimumab and tremelimumab gave an average of ~4% hypophysitis, closely rivaling hypothyroidism (19, 20). While hypothyroidism is 5–6× more prevalent in females in Caucasians (18), thus far hypophysitis from anti-CTLA-4 therapy has occurred mostly in males, in part influenced by the ratio of female:male of ~1:2 in melanoma patients. Other genetic (HLA, CTLA-4 gene polymorphism, mutations) and environmental factors may also take part (1, 4, 32). The high incidence of hypophysitis, its need for early corticosteroid intervention and life-long hormonal replacement prompted the advocacy of early recognition and management, together with thyroid function tests (17, 19, 20). Searches for specific pituitary antigens for early antibody detection to replace immunofluorescence on pituitary sections are ongoing (33–35).

Although autoimmune sequelae (e.g., hypophysitis, thyroid dysfunction, and hormonal imbalance) are gaining recognition, the high incidences of colitis and dermatitis have not been investigated as to culprit antigens, which could be self, commensally microbial, or tumor-associated. A recent survey of anti-CTLA-4 therapy in 752 melanoma patients at 19 skin centers in western Europe excluded colitis and dermatitis from the usually observed 60% irAEs (4, 5), and concentrated on raising awareness to rare yet severe toxicities, some of which may be related to prior chemo- or radio-therapy. A total of 88 irAEs from 82 of 752 (11%) patients were tabulated, 12 with hypophysitis (5). With melanoma patients, the side effects of vitiligo and uveitis were often noted because of shared melanocyte antigens, often aggravated by vaccination (1, 5). The 15 rarer cases included gastric problems, ischemia and bowel perforations, fatal hepatitis, hypophysitis with brain edema, and inflammation of the central nervous system. There were also respiratory and cardiac problems. Thus, the untoward autoimmune disorders are opportunistic and unpredictable, as well as far-reaching.

SPECIFIC ANTIGENS TO BOOST TUMOR IMMUNITY AND MONITOR Treg ACTIVITY

As seen above, the weakly immunogenic, tumor-associated antigens, which can be overexpressed self, altered self, or neoantigens, are further interwoven with unexpected pathogenic autoantigens during CTLA-4 blockade. Examination of tumor infiltrates after tremelimumab therapy revealed an increase in activated $CD8^+$ T cells in many melanoma patients; while not Tregs, this non-specific increase was observed in a greater proportion of patients than patients deriving any clinical benefit and showed no correlation with either tumor progression or regression (36). In phase II trials of melanoma patients with ipilimumab, a peripheral increase in the $\%CD4^+$ and $\%CD8^+$ activated T cells, but not those with a Treg phenotype, was noted (37). Of interest was the greater serologic reactivity to several melanoma antigens and a cancer-testis antigen NY-ESO-1. Because NY-ESO-1 is expressed in a number of solid tumors and 30–40% of advanced melanoma patients, it has been examined as a specific biomarker for increased T and B cell reactivity after CTLA-4 blockade, and if such reactivities could be associated with positive clinical outcome (37–40). While an increase in anti-NY-ESO-1 has often been detected, its association with positive benefit is still controversial (38–40). However, it appeared that clinical benefit could be associated with NY-ESO-1 reactivity if both antibody and $CD8^+$ T cells were considered in concert (10 of 13 or 77%) (39). More such studies will be needed to determine if specific reactivity to NY-ESO-1 as well as other melanoma peptides has a predictive value. Meanwhile, to increase the efficacy of CTLA-4 blockade and detect changes in tumor-specific effector T cells, therapeutic vaccines are being incorporated. In a large phase III trial (41), a gp100 peptide vaccine was given with ipilimumab (403 patients), compared to ipilimumab (137) or gp100 (136) alone. However, there was no significant increase in OS provided by including gp100 vaccination, compared to ipilimumab alone.

Detecting antigen-specific effector T cells is complicated by the presence of Tregs. In the tumor microenvironment, induced Tregs (iTregs) represent a major component of suppressor cell subsets and, as opposed to nTregs, usually arise or are converted from naive T cells in response to antigenic stimulus plus local TGF-β and IL-10 production (42). Intratumoral iTregs often exist in greater percentages than in the periphery. Although in murine melanoma studies Fc-dependent ADCC-like depletion of Tregs was demonstrable upon anti-CTLA treatment (43), the frequent detection of intratumoral iTregs in the human suggests that this kind of depletion might be an adjunct to improved clinical benefit. But the challenge remains to identify whether the effect was on tumor-specific iTregs, so as to monitor and inhibit their suppressor role (44). Following therapeutic vaccination, multiple peptides have been used to monitor iTreg activity. In one phase II trial in renal cell cancer patients, cyclophosphamide, known to deplete Tregs (22), was given before the IMA901 vaccine, consisting of multiple tumor-associated peptides used to demonstrate heightened T cell responses (45). In another study, synthetic peptides were used to compare the repertoire of tumor-specific iTregs and effector T cells in colorectal carcinoma patients (46). While

differences were noted between the two populations, the suppressor activity was shown to stem from iTregs that shared the same repertoire as effector T cells. Additional studies have made use of class II tetramers to monitor antigen specificity. After vaccination of melanoma patients with HLA-A2-restricted Melan-A peptide, monitoring with HLA-DQ6-restricted Melan-A peptide multimers revealed a downshift in specific Tregs with increased effector T cell responses (47). In breast cancer patients, tetramers from HLA-DR4-, DR7-restricted mammaglobin peptides were used to detect iTregs in the periphery (48). After their depletion *in vitro*, effector T cell responses to several mammaglobin peptides increased. Thus, antigenic specificity, if known, could aid assessment of immunotherapeutic efficacy.

PD-1 AND PD-L1 BLOCKADE TO AUGMENT TUMOR IMMUNITY

Recent reviews have discussed in depth the phenomenon of T cell exhaustion, where activated T cells highly expressing PD-1, as exemplified by virus-specific $CD8^+$ cells, exhibited exhaustion phenotypes and failed to combat chronic infections (49–51). In the tumor microenvironment, PD-1 expression was found on impaired infiltrating lymphocytes (52) and Tregs (53). Its major ligand, PD-L1, has been found on multiple epithelial carcinomas, compared to PD-L2 on lymphoid tumors associated with its expression pattern (49–51). There is no clear consensus on whether PD-L1 expression by tumors is associated with greater objective response from PD-1 blockade; more specific staining reagents have led to reports of positive association (54, 55). PD-1 is also expressed on activated B, NK, and NKT cells and is involved in down-regulating autoimmunity. PD-1-deficient C57BL/6 mice exhibited lupus-like arthritis and glomerulonephritis (56), and PD-1-deficient BALB/c mice developed cardiomyopathy from an autoantibody to cardiac troponin 1 (57). Thus, these autoimmune syndromes have a strong pathogenic autoantibody component. While preclinical tumor models have shown clinical benefit with PD-1 blockade, it is uncertain if only activated $CD8^+$ cells participated in the OS (50).

Two phase I, ongoing dose-escalation trials have been reported with two IgG4 mAbs to PD-1. Lambrolizumab (MK-3475), which has recently been designated by the FDA as a "breakthrough therapy" drug to treat advanced melanoma (58), was used to treat 135 melanoma patients, who were evaluated for up to 70 weeks (59). The confirmed response rate was 38% (44 of 117), irrespective of prior ipilimumab treatment, and appeared dose-dependent. Biopsies of regressing lesions revealed densely infiltrated $CD8^+$ T cells. irAEs were 79% (107 of 135); 17 of the 107 had grade 3–4. Beside skin and gastrointestinal problems, hypothyroidism was 8%. The second phase I trial with nivolumab (BMS-936558) included 296 patients with melanoma, non-small cell lung cancer, prostate cancer, renal cell, or colorectal cancer. The first report in 2012 was after ~1 year and the objective response averaged ~20–25% (55). Grade 3–4 irAEs were observed in 14% (41 of 296), of which 11% were considered serious; 15 patients discontinued the study and 3 deaths (1%) were attributed to pneumonitis. The spectrum of irAEs was mostly similar to lambrolizumab therapy (59). But with twice the number of patients, the variety of irAEs resembled those seen with ipilimumab, albeit at lower overall percentages. The spectrum was dose-dependent and included rash, pruritus, and diarrhea (all at ~27%), with hyperthyroidism and hypothyroidism at 3–7% (55). An exception to the ipilimumab treatment was the prominent pulmonary toxicities seen in 2–4% of patients (55, 59). In addition to fatalities (55), the 2–4% in phase I trials were of grade 3–4 (55, 59). A recent report about ongoing phase II/III trials also listed pneumonitis as a notable side effect (60). Whether it is autoimmune in origin is undetermined. An updated analysis on 107 melanoma patients treated with nivolumab showed 40% OS at 3 years (61).

Another multicenter phase I trial enrolled 207 patients without prior experience with CTLA-4, PD-1, or PD-L1 mAb, who were given anti-PD-L1 (IgG4, BMS-936559) for a number of solid tumors for up to 2 years (62). The durable response rate was 6–17%. Treatment-related adverse events were noted in 61% (126 of 207), and 39% (81 of 207) were considered irAEs; these patients have a somewhat different spectrum and a lower percentage of irAEs compared to patients in the anti-PD-1 trials. Because these investigators were also conducting the phase I trial with nivolumab (55), their initial clinical impression was that anti-PD-L1 blockade was inferior to anti-PD-1 in achieving objective responses (62).

Interestingly, pneumonitis was not a noted side effect in the anti-PD-L1 phase I trials (60, 62), but the 39% irAEs showed a distinct autoimmune-related trend: rash, hypothyroidism, hepatitis, plus isolated cases of diabetes mellitus, and myasthenia gravis, all mostly of grade 1–2 (62). PD-1 binds to both PD-L1 (broad tissue distribution) and PD-L2 (limited primarily to DCs) (63, 64). Since lung tissues express PD-L1 and contain activated alveolar macrophages, it is possible that anti-PD-1 blockade removes the inhibitory signals that control tissue proliferation and cytokine production more so in the lung, resulting in pneumonitis, whereas anti-PD-L1 blockade does not block the immune-checkpoint between PD-1 and PD-L2. Another possibility is that, since self tolerance is maintained by the continuing interaction between PD-1 and PD-L1 to prevent TCR-driven signaling (65), upon anti-PD-L1 blockade, autoreactive T cells could become activated resulting in the autoimmune syndromes reported.

COMBINING IMMUNE-CHECKPOINT INHIBITORS COULD POTENTIATE AUTOIMMUNE SEQUELAE

There are multiple national clinical trials planned or ongoing with ipilimumab plus chemo- or radio-therapeutic agents, cytokines (e.g., GM-CSF, IL-2, IL-21), and other systemic immunomodulators, most with the goal of stimulating the effector T cell arm with some targeting dendritic and B cells also [see Ref. (51)]. Because anti-CTLA inhibited Treg function and potentiated irAEs, the use of low dose cyclophosphamide could further target nTregs (22, 45) and increase autoimmune sequelae, similar to Treg-depleting anti-CD52 (1). In murine models of B16 melanoma tumors (66) and CT26 colon and ID8-VEGF ovarian carcinomas (67), dual blockade of CTLA-4 and PD-1 enhanced greater tumor rejection than each alone. It should be noted that, unlike in clinical trials, these tumors were manipulated to express GM-CSF, as was

Table 1 | Function of CTLA-4 and PD-1 in the immune network and the impact of immune-checkpoint inhibitors anti-CTLA-4 and/or anti-PD-1 on examples of tumor immunity and autoimmunity.

Functions	Consequences	Examples of anti-CTLA-4 and/or anti-PD-1 blockade on	
		Tumor immunity	Autoimmunity
CTLA-4 upregulation on APCs/peptide-stimulated Tregs downmodulates B7-1/B7-2 on APCs	Suppresses priming of naive/autoreactive T cells and maintains peripheral tolerance		Morris et al. (15): EAT Read et al. (70): colitis Ansari et al. (72): type I diabetes
CTLA-4 binding to B7-1 and B7-2 causes reverse signaling through B7-1/B7-2	Activation of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase inhibits T cell priming and proliferation	Holmgaard et al. (68): melanoma	Kwidzinski et al. (73): EAE
CTLA-4 signaling stimulates production of cytokines TGF-β and/or IL-10 by Tregs	Inhibits function of APCs and T cells		Liu et al. (75): colitis
CTLA-4 upregulation on activated T cells binds to B7-1/B7-2 at high affinity	Negative feedback signaling inhibits continued T cell proliferation	Leach et al. (69): colon carcinoma Hurwitz et al. (71): prostate cancer	Oaks and Hallett (76): AITD Hurwitz et al. (71): prostatitis Choi et al. (77): CIA Hurwitz et al. (78): EAE Torino et al. (19): clinical hypophysitis
	Fc-dependent depletion of iTregs	Simpson et al. (43) melanoma	
CTLA-4 signaling alters motility and inhibits T cell receptor-mediated "stop" signal	Reduces efficiency of effector T cell killing and APC interaction	Ruocco et al. (74): breast cancer	
PD-1 signaling enhances Treg function	Inhibits T cell priming and maintains self tolerance		Ansari et al. (72): type I diabetes
PD-1 signaling inhibits motility and T cell receptor-mediated "stop" signal	Inhibits autoreactive T cell activation and reduces effector T cell function	Holmgaard et al. (68): melanoma	Fife et al. (65): type I diabetes
PD-1 upregulation on activated T cells	Inhibits effector T cell function (anergy or exhaustion)	Holmgaard et al. (68): melanoma Ahmadzadeh et al. (52): melanoma patients Wang et al. (53): melanoma patients	Ansari et al. (72) type I diabetes
Dual blockade		Duraiswamy et al. (67): colon and ovarian cancers Curran et al. (66): melanoma	

AITD, human autoimmune thyroid disease; APC, antigen-presenting cell; CIA, collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; EAT, experimental autoimmune thyroiditis; iTreg, induced regulatory T cell; Treg, regulatory T cell.

whole cell vaccination included in some experiments. The presence of GM-CSF could influence the expression pattern reported for tumor-infiltrating lymphocytes (67). **Table 1** lists the important functions of CTLA-4 and PD-1 molecules in maintaining homeostasis of the immune network, and provides examples of the impact of anti-CTLA-4 and/or anti-PD-1 blockade on tumor immunity and autoimmunity.

Since CTLA-4 blockade interferes with peripheral tolerance induction/maintenance and affects primarily the early stages of the immune response, and PD-1 blockade acts toward the late stages at the tissue sites (79) and each can augment OS, dual therapy could further enhance OS, provided that the irAEs are not unreasonably additive. In melanoma patients, a phase I trial combining ipilimumab and nivolumab has begun (80). The need to test different regimens resulted in small patient sizes of 33–53. While the spectrum of irAEs was essentially similar to monotherapy, OS was higher at 24 weeks, based on previous experience.

Longer follow-ups and additional trials will be necessary to assess various parameters affecting irAEs: HLA genotype, environmental and gender influences, and antigen specificities for both Tregs and effector T cells. As seen in our EAT-anti-tumor

models, subclinical autoimmune conditions (29), and mutual stimulation arising from anti-tumor and autoimmune inflammation also contribute to the overall response enhancing tumor immunity and autoimmunity (24, 25). Importantly, there appears to be different pathogenic pathways to autoimmune manifestations, with PD-1-deficiency favoring a pathogenic autoantibody profile and CTLA-4 blockade favoring T cell-mediated organ damage.

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The progression of cell death affects the rejection of allogeneic tumors in immune-competent mice – implications for cancer therapy

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Large amounts of dead and dying cells are produced during cancer therapy and allograft rejection. Depending on the death pathway and stimuli involved, dying cells exhibit diverse features, resulting in defined physiological consequences for the host. It is not fully understood how dying and dead cells modulate the immune response of the host. To address this problem, different death stimuli were studied in B16F10 melanoma cells by regulated inducible transgene expression of the pro-apoptotic active forms of caspase-3 (revCasp-3), Bid (tBid), and the *Mycobacterium tuberculosis*-necrosis inducing toxin ($CpnT_{CTD}$). The immune outcome elicited for each death stimulus was assessed by evaluating the allograft rejection of melanoma tumors implanted subcutaneously in BALB/c mice immunized with dying cells. Expression of all proteins efficiently killed cells *in vitro* (>90%) and displayed distinctive morphological and physiological features as assessed by multiparametric flow cytometry analysis. BALB/c mice immunized with allogeneic dying melanoma cells expressing revCasp-3 or $CpnT_{CTD}$ showed strong rejection of the allogeneic challenge. In contrast, mice immunized with cells dying either after expression of tBid or irradiation with UVB did not, suggesting an immunologically silent cell death. Surprisingly, immunogenic cell death induced by expression of revCasp-3 or $CpnT_{CTD}$ correlated with elevated intracellular reactive oxygen species (ROS) levels at the time point of immunization. Conversely, early mitochondrial dysfunction induced by tBid expression or UVB irradiation accounted for the absence of intracellular ROS accumulation at the time point of immunization. Although ROS inhibition *in vitro* was not sufficient to abrogate the immunogenicity in our allo-immunization model, we suggest that the point of ROS generation and its intracellular accumulation may be an important factor for its role as damage associated molecular pattern in the development of allogeneic responses.

Keywords: immunogenicity, apoptosis, cancer, ROS, caspase-3, tBid, necrosis, DAMPs

INTRODUCTION

The appearance of cell death during disease therapy is a two-pronged sword. On one hand, cell death is desirable during cancer treatment in order to control malignant cell growth in the patient. On the other hand, excessive cell death should be avoided during transplantation to allow the grafted cells to survive in a foreign host. In the latter case, a considerable number of stresses are involved and several kinds of injuries may additionally compromise tissue viability, leading to progressive graft dysfunction and, eventually, also to graft loss (1, 2).

Furthermore, the mechanism and type of cell death might profoundly affect the reaction of the host toward surviving cells, making the situation more complex. Apoptosis, necrosis, autophagy, necroptosis, and other processes have been reported as common cell death mechanisms observed *in vivo* during therapies. However, how these types of cell death modulate interactions of the dying and dead cells with the immune system remains elusive. Depending on the immune response elicited, it is possible to distinguish between cases of cell death able to induce immunogenicity (immunogenic cell death) and those inducing immune tolerance or unresponsiveness (tolerogenic/silent cell death) (3, 4). Dying cells can exhibit completely different characteristics and immunological features. To understand these differences, an accurate

characterization of the features, types, and phases of cell death is required.

The latter has become especially important in the context of diseases like cancer where conventional treatments (e.g., radiation and chemotherapy) are based on the massive induction of tumor cell death. In such cases, the immune system is prone to be decisive for tumor fate. Because the guidelines for drug screening in antineoplastic therapies require evaluation of human tumors xenotransplanted into immune-compromised mice (5), the role of the immune system has been neglected (6), making studies focused on the interplay between immune system and dying cells necessary. Modern anti-cancer therapies aim at inducing immunogenic cancer cell death. However, there are a plethora of factors involved in this process that have to be revisited and reassessed carefully. These include intrinsic cell immunogenicity, the nature of the initial death stimulus, the type of damage associated molecular patterns (DAMPs) released, the clearance capacity of the affected tissue for dying and dead cells, and the respective death pathway. Considering the large number of cytotoxic drugs currently used in the treatment of neoplastic diseases, much information is missing to predict the anti-tumor response of the host reliably.

In this study, we showed how different mechanisms and types of cell death, induced by different stimuli, affect the outcome of allogeneic tumor transplants in BALB/c immune-competent mice. Additionally, a morpho-physiological characterization of dying and dead cells, based on a multiparametric flow cytometry analysis, was assessed. A murine allograft model allowed evaluation of the immune response *in vivo*. The results of this work may have important implications for both cancer therapy and procedures for experimental allotransplantation.

MATERIALS AND METHODS

REAGENTS AND MOLECULAR PROBES

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), G418, penicillin–streptomycin, and glutamine were from Gibco-Invitrogen. Recombinant chicken annexin A5 (AxA5) was purchased from Responsif. The FluoroTag fluorescein isothiocyanate (FITC) conjugation kit was from Sigma-Aldrich, the 1,10,3,3,30,30-hexamethylindodicarbocyanine iodide dye [DiIC1(5)], Hoechst 33342, Lipofectamine™ 2000, and puromycin dihydrochloride were from Invitrogen. Propidium iodide (PI) was obtained from Amersham Biosciences. Doxycycline hydrochloride and trypsin–EDTA solution were purchased from Sigma-Aldrich. Ringer's solution was from Delta Select. The caspase3 inhibitor z-DEVD-fmk, the caspase-9 inhibitor Ac-LEHD-cmk, and the general caspase inhibitor z-VAD-fmk were purchased from Bachem.

CELL LINES AND CULTURE CONDITIONS

The C57BL/6 mouse-derived melanoma cell line B16F10 bearing the haplotype H2b was purchased from ATCC (#CRL-6475) and propagated in DMEM supplemented with 10% FBS and penicillin–streptomycin (D10) at 37°C in a 5% CO_2 atmosphere.

For the morpho-physiological characterization by flow cytometry, B16F10 cells were cultured in 24-well plates at 100,000 cells/2 ml D10 and harvested at the time points indicated. Harvesting was performed as follows: supernatants containing dead cells were harvested into polypropylene tubes. A trypsin–EDTA solution was added to the wells for 10–15 min at room temperature to detach the remaining adherent cells. Thus, detached cells were collected by adding D10 and combined with their corresponding supernatant fraction. Finally, cells were centrifuged at 300 g for 5 min, resuspended in 500 µl D10 medium.

GENERATION OF STABLE Tet-CONTROLLED SUICIDE CELL LINES

The cell lines B16F10-644, B16F10-tBid, and B16F10-revCasp-3 have been described (7). The parental cell line B16F10-644 was transfected with the *Ahd*I-linearized plasmid pWHE655TREtight-$CpnT_{CTD}$, carrying the C-terminal domain of the channel protein with necrosis inducing toxin ($CpnT_{CTD}$) characterized in *Mycobacterium tuberculosis* (8) (**Figures 1A–C**), and stable transfectants were selected by limited dilution in the presence of 1500 µg/ml G418. Individual subclones were cultured in 48-well plates and tested for cell death with AxA5/PI staining by FACS after 24 h of doxycycline (1 µg/ml) addition. One out of several positive clones was chosen for further experiments and named B16F10-$CpnT_{CTD}$.

MULTI-PARAMETER CLASSIFICATION OF CELL DEATH BY FLOW CYTOMETRY

The cell death characterization method analyzing size, granularity, PS exposure, plasma membrane integrity, mitochondrial membrane potential, and DNA content in a one-tube-measurement has been thoroughly described elsewhere (9). This method classifies eight different phases of cell death. Briefly, the harvested cells were incubated for 30 min at room temperature with 400 µl of freshly prepared 4-color staining solution [1.8 µg/ml AxA5-FITC, 100 ng/ml PI, 10 nM DiIC1(5), 1 ng/ml Hoechst 33342] in Ringer's solution and subsequently analyzed. Flow cytometry was performed with a Gallios cytofluorometer (Beckman Coulter, Fullerton, CA, USA). Excitation of FITC and PI was at 488 nm, the FITC fluorescence was detected with the FL1 sensor (525/38 nm BP), the PI fluorescence with the FL3 sensor (620/30 nm BP), the DiIC1(5) fluorescence was excited at 638 nm and detected with the FL6 sensor (675/20 nm BP), and the Hoechst 33342 fluorescence was excited at 405 nm and detected with the FL9 sensor (430/40 nm BP). Electronic compensation was applied to reduce bleed-through fluorescence. Data analysis was performed with Kaluza software version 2.0 (Beckman Coulter, Fullerton, CA, USA). Cells were classified according to their location in the forward scatter (FSc; size) vs. side scatter (SSc; granularity) dot plot and their staining patterns in the FL1 vs. FL3 and FL6 vs. FL9 dot plots (**Figure 2A**).

CELL DEATH INDUCTION

Cell death was induced by (1) doxycycline *in vitro* (5–10 µg/ml) for expression of the cell death inducing proteins tBid, revCasp-3, and $CpnT_{CTD}$. (2) Irradiation with ultraviolet light type B (UVB) at 1.5 mJ/cm^2/s. (3) By heat shock (56°C for 30 min).

MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES

Intracellular reactive oxygen species (ROS) levels were assessed using the redox-sensitive dye 2,7-dichlorofluorescein diacetate (C-DCFH-DA). At the time points indicated, dying B16F10 cells were harvested, incubated for 30 min with C-DCFH-DA (10 µM) at 37°C in protein-free medium (D0) in the dark. Cells were then

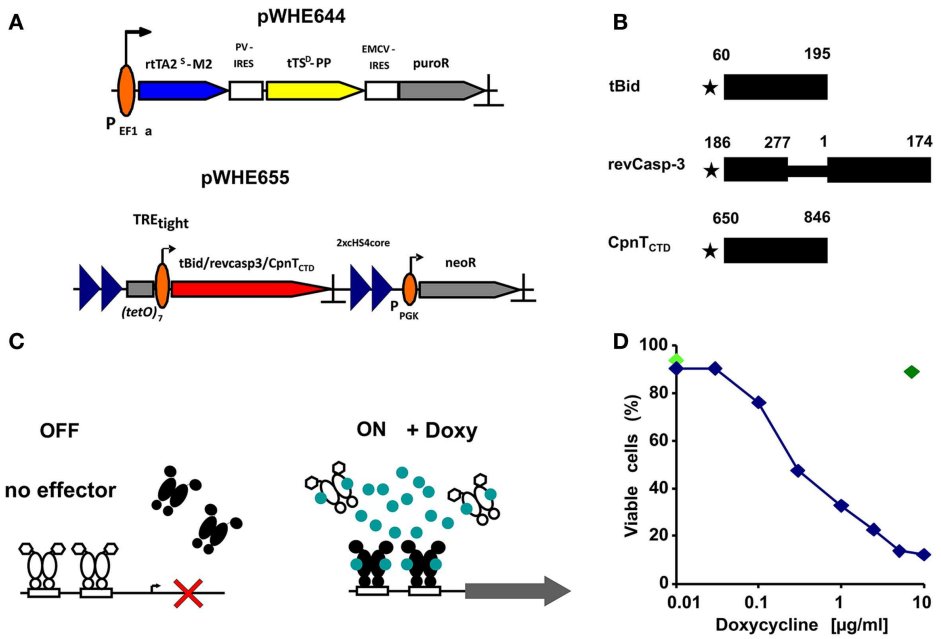

FIGURE 1 | Conditional expression of death inducing proteins.
(A) Schematic overview of the constructs used to establish the regulatory system. The vector pWHE644 represents the regulator construct. A human EF1α promoter constitutively transcribes a tricistronic mRNA. This mRNA contains the reverse transactivator rtTA2^{S}-M2 (blue arrow), the transsilencer tTS^{D}-PP (yellow arrow), and a selection marker (puromycin resistance; gray arrow). Translation of the latter two genes is mediated by internal ribosome entry sites (IRES; open boxes) from polio-virus (PV) and encephalomyocarditis-virus (EMCV). The vector pWHE655 contains the response unit used for stable transfections. It features the target gene (red arrow) driven by the Tet-responsive promoter TRE$_{tight}$ (open box, broken arrow) and flanked by two repeats each of a 250 bp sequence from the chicken HS4 insulator (blue triangles). A murine phosphoglycerate kinase 1 promoter (PGK; broken arrow) drives expression of a gene mediating G418-resistance. PolyA sites in all vectors are marked by a "⊥." **(B)** Schematic representation of the cytotoxic test proteins. The residues that border the active domains expressed in the experiment are indicated above their respective closed box. A methionine added to allow translation is represented by a star. **(C)** Schematic overview of the regulatory system. In the OFF-State, a transsilencer (white) binds to the minimal promoter (open boxes, broken arrow) and actively suppresses transcription (cross). In the ON-State, doxycycline (blue circles) binds to both transsilencer and reverse transactivator (black). The former dissociates from, the latter binds to the minimal promoter and activates transcription (gray arrow). **(D)** Response of the regulatory system to different doxycycline concentrations. The B16F10-tBid transfected cell line was incubated for 24 h with various concentrations of doxycycline and mortality was measured, shown for one representative experiment out of three performed. Concentrations between 5 and 10 μg/ml showed the highest extent of cell death. An additional control at 10 μg/ml Doxy with the parental stably transfected cell line B16F10-644 was included to discard doxycycline toxicity at higher concentrations as cause of cell death (dark green diamond). Cell viability at time point "0" is shown as light green diamond.

washed with D10 by centrifugation, co-stained with PI, and analyzed by flow cytometry. Only PI-negative cells were analyzed and ROS levels were presented as the mean fluorescence intensity in the FL1 channel (MFI-FL1). The anti-oxidants *N*-acetylcysteine (NAC) and 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (mitoTEMPO) were used at 100 μM as indicated.

ALLOGENEIC TUMOR GROWTH MODEL AND IMMUNIZATIONS

The allogeneic tumor growth model consisted of the host mouse (BALB/c, MHC haplotype H2d) and B16F10 melanoma cells derived from C57BL/6 mice (MHC haplotype H2b). Specified amounts of viable cells or dead/dying cells were subcutaneously (s.c.) implanted in 500 μl Ringer's solution in the right flank using a syringe with a 25 G needle. The growth of solid melanoma tumors was registered by direct measurement of width, height, and depth of the black subcutaneous protuberance with a caliper for up to a maximum of 40 days. In accordance with the guidelines for the welfare of animals in experimental neoplasia, the animal was sacrificed if the mouse tumor volume exceeded more than 10% of the host's body weight.

In order to evaluate the immune response against implanted cells, mice were challenged s.c. with 2 million viable cells in the opposite flank, after immunization as indicated. The use of the syngeneic host (C57BL/6) as recipient of B16F10 melanoma cell lines was ruled out because of the aggressiveness of the B16F10 cells, which may cause discomfort and premature death of the animal. Mice were purchased from Charles River Laboratories International, Inc., and kept on a standard diet with drinking water available *ad libitum*. Experiments were conducted according to the European principles and local guidelines for care and use of laboratory animals at the Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv (10–12).

STATISTICAL ANALYSIS

The software package GraphPad Prism 5.0 was used for graphics and statistical tests. For comparisons between control and experimental groups, Mann–Whitney *U* test or two way ANOVA tests

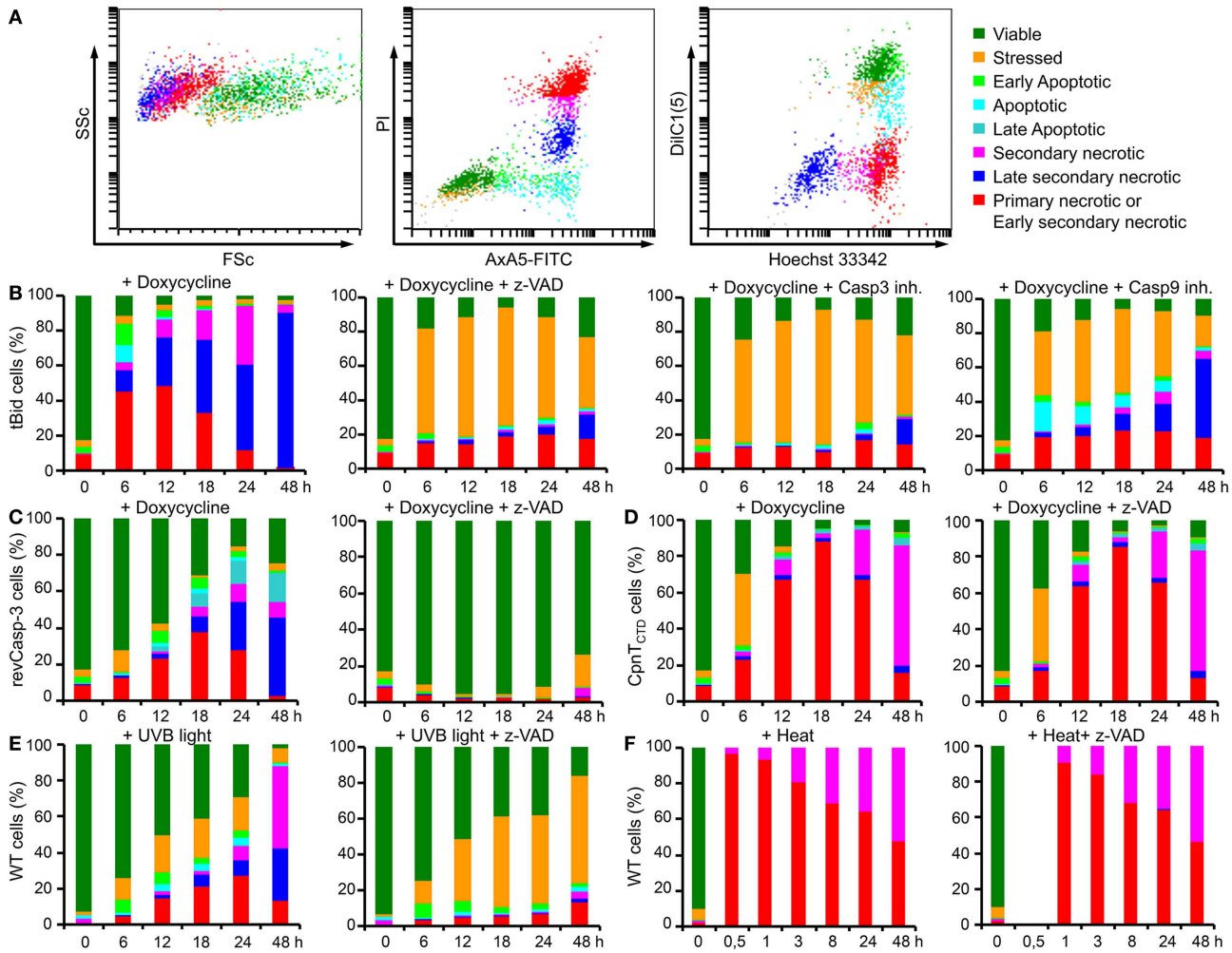

FIGURE 2 | Six parameter classification by flow cytometry of the cell death phenotype of dying and dead B16F10 cells. Cell death analysis is based on morphological features (FSc and SSc), on the exposure of PS (annexin A5-FITC) and plasma membrane ion selectivity (PI), on the mitochondrial membrane potential [DiIC1(5)] and on nuclear DNA content (Hoechst 33342) detected by flow cytometry. Note: after proper gating, up to eight physiologically different subpopulations can be recorded. Dot plots exemplarily show B16F10-revCasp-3 cells after 18 h of doxycycline (5 μg/ml) treatment **(A)**. Rapid cell death occurred after 6 h in tBid-expressing cells and more than 95% cell death was observed after 24 h. In the presence of various caspase inhibitors [z-VAD-fmk, z-DEVD-fmk (caspase-3 inhibitor) and Ac-LEHD-cmk (caspase-9 inhibitor); all 50 μM], a significant increase in the stressed cell fraction displaying low-mitochondrial potential was observed **(B)**. Expression of revCasp-3 in B16F10 cells induced cell death after 24 h in more than 80% of the cells. z-VAD-fmk (50 μM) completely inhibited doxycycline-driven apoptosis. Note: stressed cells do not arise in this type of cell death induction **(C)**. Expression of CpnT$_{CTD}$ induced cell death in more than 90% of the cells after 18 h. Note: primary necrosis was the most common type of cell death observed and death occurred independently of caspase activity (50 μM z-VAD-fmk) **(D)**. Lethal UVB irradiation (240 mJ/cm^2) of parental B16F10 cells causes a rather slow progressing kind of cell death. Note: in the presence of z-VAD-fmk (50 μM), a significant increase of the stressed cell fraction displaying low-mitochondrial potential was observed **(E)**. Heat shock (56°C, 30 min) caused immediate necrosis in 100% of cells independent of caspase activity **(F)**. Displayed are the mean values from three independent experiments of relative percentages of each cell phenotype during 48 h of culture **(B–F)**.

were employed as appropriate. Statistical significance was assumed if $p < 0.05$.

RESULTS

STABLE Tet-CONTROLLED MOUSE MELANOMA B16F10 SUICIDE CELL LINES

Subcloned transfected mouse melanoma B16F10 cell lines were tested for the response of the regulatory system to different doxycycline (doxy) concentrations after 24 h. Mortality rates were calculated by annexin A5/PI staining and flow cytometry. Concentrations around 10 μg/ml doxy showed the highest degree of cell death (**Figure 1D**).

CELL DEATH INDUCED BY EXPRESSION OF CYTOTOXIC PROTEINS IN B16F10 CELLS AND MORPHO-PHYSIOLOGICAL CLASSIFICATION OF DEAD AND DYING TUMOR CELLS BY FLOW CYTOMETRY

Since dying cells may exhibit various biological features that modulate the immune response, it is necessary to accurately

characterize the phenotypes and phases of cell death. Employing a six parameter protocol to characterize the morphological and physiological features of dying and dead cells, we identified eight different states of cell death in B16F10 melanoma cells: (1) viable; (2) stressed; (3) early apoptosis; (4) apoptosis; (5) late apoptosis; (6) secondary necrosis; (7) late secondary necrosis; and (8) primary necrosis or early secondary necrosis (**Figure 2A**) (9, 13). This method allows us to closely describe biological features of death in cell lines expressing cytotoxic proteins. In our test system, doxycycline relieves active repression of the promoter by the tetracycline-dependent transsilencer (tTS) and simultaneously induces binding of the reverse tetracycline-dependent transactivator (rtTA) leading to transgene expression of each death-inducing-protein (**Figure 1C**). Additionally, the classical death stimuli UVB irradiation and heat shock were also employed.

Activation of the suicide switch in B16F10-tBid cells with 5 μg/ml doxy was followed by very fast and efficient killing. After 6 h, up to 90% of the cells were dead. Approximately 30% of the cells displayed features typical for initial phases of apoptosis and the rest of the cells showed a necrotic phenotype, predominantly early secondary necrosis (**Figure 2B**). The role of caspases in the cell death induced by tBid in B16F10 cells was studied by adding the pan-caspase inhibitor z-VAD-fmk (50 μM). Considering the physiological stringency of this method, treatment with z-VAD-fmk did not change the fraction of viable cells treated with doxy. Interestingly, a significant increase in the fraction of stressed cells was observed for all times points. Stressed cells are defined as cells with conserved membrane asymmetry, membrane integrity, and cell morphology, but very low-mitochondrial membrane potential [DiIC1(5) low] (13). This fraction reached its maximum at 18 h post-induction with up to 80% of the cells displaying a stressed phenotype (**Figure 2B**). In this case, stressed cells can be considered to be in a "pre-mortal" state, induced by the expression of tBid and concomitant inhibition of caspases. Stressed cells are detected efficiently by the six parameter method described above. tBid death kinetics in the presence of the more specific caspase-3 and caspase-9 inhibitors were similar to those observed in the presence of z-VAD-fmk, and showed comparable amounts of dead cells (**Figure 2B**).

Expression of revCasp-3 in B16F10 cells induced cell death after 24 h, albeit more slowly than tBid, with more than 80% of the cells displaying dead phenotypes (**Figure 2C**). Early stages of apoptosis were observed at 12 and 18 h, while late apoptotic and secondary necrotic stages were more predominant at 24 and 48 h. As expected, cell death by expression of revCasp-3 was completely inhibited in the presence of z-VAD-fmk (**Figure 2C**).

Expression of the necrosis inducing protein $CpnT_{CTD}$ in B16F10 cells led to cell death after 18 h with more than 80% of the cells displaying features of primary necrosis (**Figure 2D**). Early stages of apoptosis were not detected by the expression of this protein. In contrast to pro-apoptotic proteins, z-VAD-fmk did not affect the death phenotype induced by $CpnT_{CTD}$, suggesting a caspase-independent type of cell death (**Figure 2D**).

It is important to note that cell death induced by doxycycline-controlled expression of the proteins tBid, revCasp-3, and $CpnT_{CTD}$ did not kill all cells – some cells may have failed to respond to doxy. In agreement with this assumption, long-term culture of doxy-treated cells resulted in confluent growth of the surviving non-responder cells.

The parental cell line B16F10-644 was lethally irradiated with UVB (240 mJ/cm^2). After 6 h, some stressed and early apoptotic cells were observed. After 12 h, stressed, apoptotic, and some forms of necrotic cells were present. Secondary necrotic cells increasingly appeared from 24 to 48 h. The presence of z-VAD-fmk produced an important increase in the fraction of stressed cells preserving the fraction of viable cells, as was also observed for cells expressing tBid (**Figure 2E**). UVB irradiation caused much slower death in comparison to that induced by the expression of tBid, revCasp-3, or $CpnT_{CTD}$. Importantly, no surviving cells were observed even when the plates were cultured for 7 days after irradiation.

Cell death by heat shock was studied in the parental cell line B16F10-644. Cells were incubated at 56°C for 30 min in a water bath to induce abrupt membrane disruption. More than 98% of the cells became primary necrotic. No differences were observed by the addition of z-VAD-fmk (**Figure 2F**). In summary, the suicide switch system efficiently induced morpho-physiologically different forms of tumor cell death.

REACTIVE OXYGEN SPECIES PRODUCTION IN DYING TUMOR CELLS

In order to further characterize the aforementioned forms of cell death, we measured the ability of the dying cells to produce free radicals. After addition of doxy to B16F10-revCasp-3 and B16F10-$CpnT_{CTD}$ cells, a significant, five to sixfold, increase in the production of ROS was observed after 6 h and reached its maximum at 9 h in both cell lines. Interestingly, dying B16F10-$CpnT_{CTD}$ cells showed the highest accumulation of ROS (12-fold) before disruption of the plasma membranes occurred (**Figure 3**). This response was compromised by the addition of the anti-oxidants *N*-acetylcysteine (NAC) and 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (mitoTEMPO). In contrast, B16F10-tBid expressing and UVB-irradiated dying cells did not produce significant amounts of ROS up to 12 h after death induction.

GROWTH OF B16F10 CELLS IN THE ALLOGENEIC BALB/C HOST AND CONCOMITANT IMMUNITY

Tumor growth of the B16F10 cells was studied in BALB/c mice by implanting four million viable parental B16F10-644 cells s.c. into their right flanks (viability >90%). Five days later, black solid tumors were observed beneath the shaved skin. These tumors reached their maximum size between days 12 and 15 and spontaneously regressed thereafter (**Figure 4A**). In most cases, small tumors (5–10 mm^3) persisted subcutaneously in the animal showing no further change in size. Tumor recurrence was verified by keeping mice under observation for more than 2 months; no further tumor growth was seen during this time period. Since the tumors are growing in allogeneic hosts, we regard this as allograft rejection. Metastases were never observed in any organ.

B16F10 is a poorly immunogenic cell line, not able to generate concomitant immunity in its syngeneic host. Accordingly, C57BL/6 mice bearing a progressive tumor are not able to elicit a protective immune response (14). In order to evaluate the immunity in this allogeneic model, mice were implanted s.c. as follows:

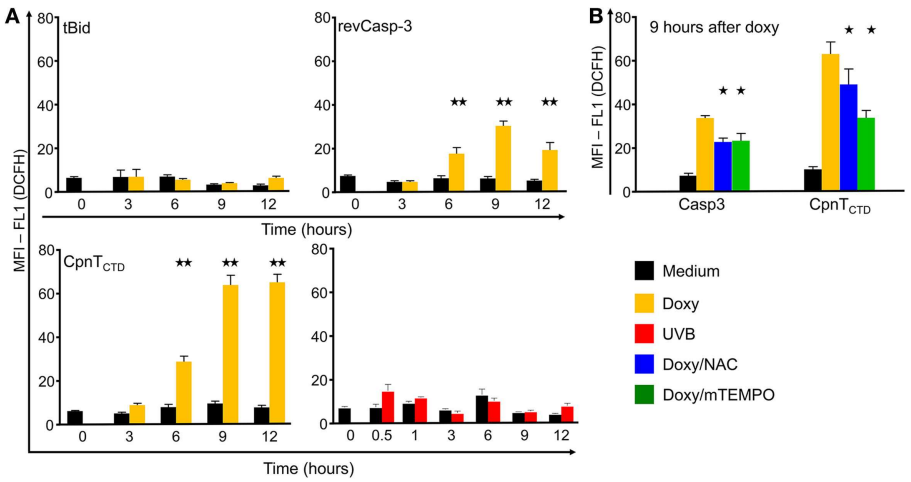

FIGURE 3 | Reactive oxygen species (ROS) production by dying B16F10 melanoma cells. Cells were induced to die by conditional expression of the death proteins tBid, revCasp-3, and CpnT$_{CTD}$ or by UVB irradiation, stained with the ROS sensor DCFH and with PI and analyzed by flow cytometry **(A)**. Inhibition of ROS production was performed by treatment with *N*-acetyl-cysteine (NAC) or mitoTEMPO, 100 μM, respectively, and recorded at 9 h after death induction **(B)**. Mean and SEM values of the mean fluorescence intensities of FL1 in viable cells (PI-negative) are displayed for different time points. At least three independent experiments were performed (Two and one stars indicate statistical significance at the $p < 0.001$ and $p < 0.05$ levels, respectively).

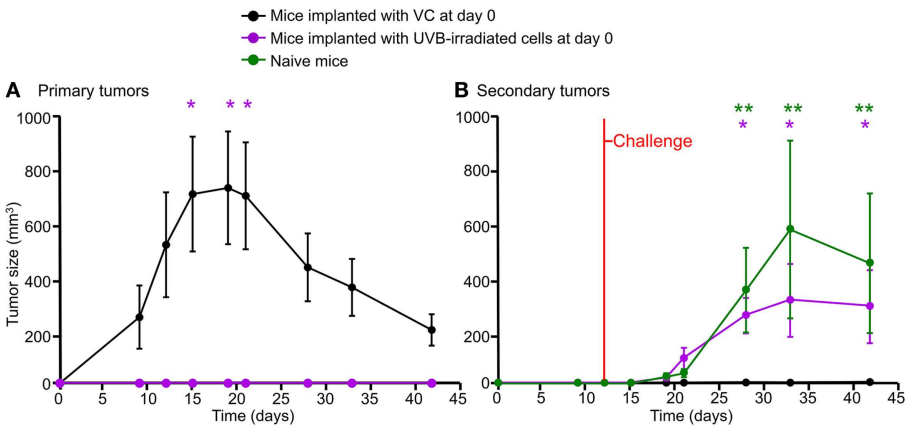

FIGURE 4 | Growth of B16F10 melanoma cells in the allogeneic host and concomitant immunity. Four million viable B16F10 cells (VC) were implanted s.c. in the right flank of BALB/c mice. Mice developed tumors reaching their maximum size after 2–3 weeks, followed by rejection [**(A)**, black line]. Mice implanted with 4 million UVB-irradiated cells did not develop primary tumors [**(A)**, purple line]. After challenge with 2 million viable cells s.c. on the left flank, those mice bearing primary tumors did not develop secondary tumors [**(B)**, black line], while mice primarily inoculated with irradiated cells developed tumors similar to those of the naïve group [**(B)**, purple and green lines, respectively]. Mean values ($n = 8$) and the SEM are displayed. Time points showing statistical significance when compared to the group of mice implanted with VC are highlighted. Two stars and one star indicate statistical significance at the $p < 0.01$ and $p < 0.05$ levels, respectively. The two way ANOVA test corrected by Bonferroni was applied in this experiment.

one group was implanted with 4 million viable cells, the second group with lethally UVB-irradiated cells. A third group was injected with Ringer solution (naïve group). After 12 days, all groups were challenged s.c. with 2 million viable cells in the opposite flank. The black line in **Figure 4A** shows growth and rejection of primary tumors derived from 4 million viable cells. Mice implanted with UVB-irradiated cells showed no growth of primary tumors (**Figure 4A**, purple line $p < 0.05$ compared to the black line). After challenge, all mice carrying primary tumors rejected secondary tumors (**Figure 4B**, black line). In contrast, mice that had received UVB-irradiated cells supported the growth of secondary tumors, as the naïve group did (**Figure 4B**, purple $p < 0.05$ and green $p < 0.01$ lines compared to the black line). This observation suggests the existence of tumor related immunity in the allogeneic model. We propose to use this system for the evaluation of host immunity after immunization with dead and dying cells.

ALLOGENEIC RESPONSE ELICITED BY DYING AND DEAD CELLS

The allogeneic implantation of dead and dying tumor cells in an immune-competent host allows us to evaluate whether

the mechanism of cell death induces a silent, a tolerogenic, or an immunogenic cell death by recording the response after a standardized challenge. A silent type of cell death would not affect the growth of the allotumor, a tolerogenic type of cell death would overcome the expected allograft rejection, while an immunogenic cell death would favor the rejection of the allotumor. The host response against each previously characterized form of cell death was assessed in 9–11-week-old female BALB/c (WT) mice immunized with dying/dead B16F10 cells induced to die by the following stimuli: (1) UVB irradiation; (2) doxycycline-controlled expression of the death proteins tBid, revCasp-3, and $CpnT_{CTD}$; and (3) heat shock. Stably transfected B16F10 cells were induced to die by adding 10 μg/ml doxy to the tissue culture dishes (150 cm^2) at 37°C and 5% CO_2 for 5 h. Afterward, UVB irradiation (240 mJ/cm^2) was performed in order to kill the remaining non-responsive cells. Previous experiments had demonstrated that the additional UVB irradiation at this time point neither altered the expression level of the death-inducing proteins nor the kinetics of the specific death phenotype (Figure S1 in Supplementary Material). Sixteen million dying/dead cells were injected s.c. in the right flank as single immunization dose at day 0. After 10 days, mice were challenged in the left flank with 2 million viable cells of the parental cell line B16F10-644. Tumor growth was recorded at days 5, 8, 11, 14, 20, and 30 after challenge (**Figure 5A**). Viable cells implanted in naïve BALB/c mice generated tumors that reached their maximal size after 2 weeks followed by regression, as expected (**Figure 5B**, black lines). This group was used as reference cohort.

Mice immunized with UVB-irradiated cells and tBid-expressing cells developed tumors with sizes similar to that of the naïve group. This points to a state of unresponsiveness in these mice (**Figure 5B**). In contrast, and especially at the days 8, 11, and 14, mice immunized with cells dying because of the expression of revCasp-3 or $CpnT_{CTD}$ displayed significantly smaller tumors than naïve mice (**Figure 5B**; $p < 0.05$).

In order to estimate the total tumor mass generated for each immunization cohort, an analysis based on the cumulative area under the curve (integral) was performed. **Figure 5C** shows the total amount of tumor mass developed in the allogeneic host for each cohort. Mice immunized with UVB-irradiated cells or with cells that express tBid developed similar tumor masses. Conversely, mice immunized with dying/dead cells because of revCasp-3 and $CpnT_{CTD}$ expression developed significantly smaller tumor masses (**Figure 5C**). Based on these observations, we considered UVB irradiation and over-expression of tBid as silent or tolerogenic forms of cell death. Accordingly, over-expression of revCasp-3 and $CpnT_{CTD}$ induced immunogenic cell death. At best, only a weak immunogenic response was elicited by cells treated with heat shock reflecting that the rapid induction of necrosis by heat shock has the inevitable disadvantage of diffusion of danger signals to the medium. One major advantage of doxy-controlled induction of cell death, in particular necrotic cell death, is the achievement of a necrotic phenotype without physical interaction with the cells.

Interestingly, those forms of death showing significant ROS production upon *in vitro* stimulation died in an immunogenic fashion (**Figure 5D**). To more closely address the role of ROS production in the development of allo-responses, we treated ROS-producing $CpnT_{CTD}$-expressing dead and dying cells with the anti-oxidant NAC before immunization. This treatment resulted in the amelioration of the allo-response against the challenge; however, it was not statistically significant (Figure S2 in Supplementary Material).

DISCUSSION

Conventional studies characterizing cell death are based on PS exposure and ion selectivity of the plasma membrane (AxA5 and PI staining) (15–17). These methods have traditionally classified cells as viable, apoptotic, or necrotic. However, because of the important role of the mitochondria as modulator of cell death, a concomitant analysis that monitors changes in the mitochondrial membrane potential is required. On the one hand, mitochondria play a role during apoptosis through the release of several apoptogenic proteins located in the inter-membrane space and, consequently, in apoptosome formation (18). On the other hand, mitochondria determine the outcome of many ATP-dependent cell physiological processes and, thus, are important for necrotic cell death. In this work, we used a modified staining and cytofluorometry protocol for a more detailed analysis of tumor cell death induced by various stimuli. Its major advantage is the possibility to classify at least eight different stages of cell death (viable, stressed, early/medium/late apoptosis, early/late secondary necrosis, primary necrosis) in a fast and reliable one-tube assay. A similar method employing a four-color staining for evaluation of PS exposure (AxA5-FITC), plasma membrane integrity (PI), mitochondrial membrane potential (JC-1), and nuclear DNA content (Hoechst 33342) was reported (19). However, a clear classification and identification of different types and stages of cell death was missing in that report.

Usually, standard methods are employed to generate dead cells for immunization experiments, e.g., *in vitro* induction of apoptosis/necrosis in cells and their subsequent injection into the mouse. This approach allowed the dissection of important modulatory effects of apoptotic cells in living multicellular organisms, as well as the employment of adjuvants in cell-based immunization models (20–22). However, the manipulation of dying and dead cells *ex vivo* has the disadvantage of their rapid progression from apoptosis to secondary necrosis and the concomitant decay and dilution of labile and short-range active immunomodulatory signals, respectively. In order to avoid the aforementioned limitations, we have established a conditional doxycycline-dependent expression system able to trigger various types of tumor cell death. Vectors harboring cDNAs encoding for tBid, revCasp-3, and $CpnT_{CTD}$ were stably transfected into B16F10 melanoma cells.

tBid is a major pro-apoptotic protein activated in both extrinsic and intrinsic pathways and is an important connector between these canonical apoptosis pathways. tBid translocates to the mitochondria promoting mitochondrial outer membrane permeabilization (MOMP), a process involving self-assembly of activated BAX and BAK into transmembrane pores, which can be inhibited by anti-apoptotic BCL2 family proteins (23–25). Moreover, tBid accumulation determines the timing of MOMP (26). In addition to cytochrome c, tBid induces the release of further mitochondrial death effectors that promote caspase-independent apoptosis and induce mitochondrial remodeling. All these processes result in loss of the mitochondrial membrane potential, blocking of ATP synthesis and, consequently, loss of function of ATP-dependent transporters.

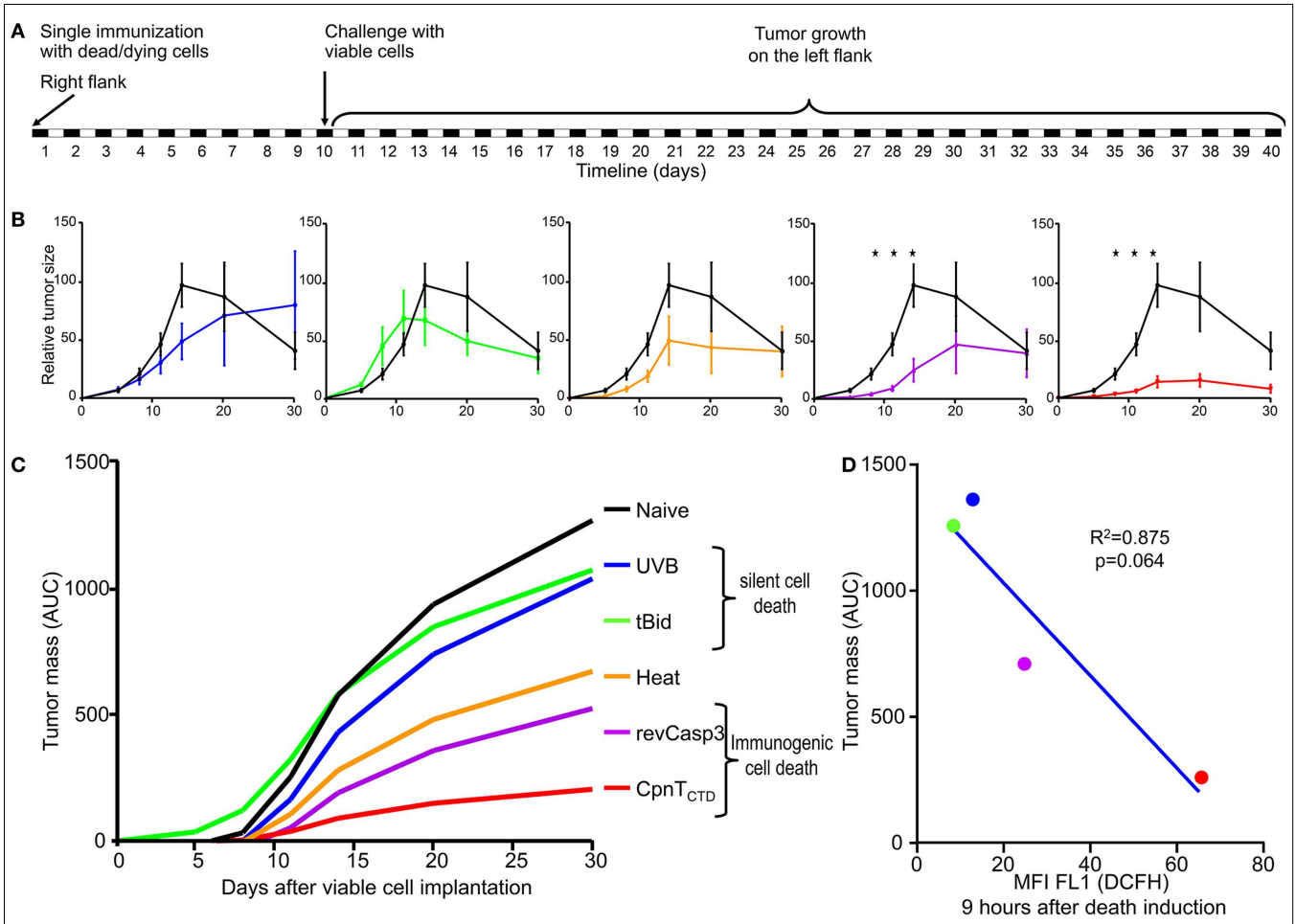

FIGURE 5 | Immune response against dead or dying allogeneic tumor cells. BALB/c mice ($n = 5$) were immunized in the right flank s.c. (single dose) with B16F10 dying/dead cells. After 10 days, mice were challenged s.c. in the left flank with 2 million viable cells of the parental cell line B16F10-644. Tumor growth was monitored for 30 further days **(A)**. Cell death was induced by UVB irradiation; heat shock; doxycycline-controlled expression of death proteins tBid, revCasp-3, and $CpnT_{CTD}$. Displayed are the mean values ($n = 5$) of relative tumor volumes and SEM [**(B)**, $*p < 0.05$ after Mann–Whitney U test] and the integral of tumor size [**(C)**, total tumor mass]. Inverse association between ROS production and total tumor mass developed in the allogeneic host **(D)**.

Over-expression of tBid can be considered to represent a harsh stimulus that quickly drives cells toward advanced stages of the apoptotic process with metabolic collapse leading to an early loss of plasma membrane integrity and rupture. We have defined this particular state of early loss of plasma membrane integrity as early secondary necrosis. It clearly differs from primary necrosis because of its susceptibility to inhibition by caspase inhibitors. Similar stimuli have already been reported to induce necrosis (27–29). This new cell death state may have particular relevance under clinical conditions like treatment with chemotherapy in cancer. Our cell death classification method allowed the identification of a further new stage in the process of dying, which we propose to refer to as *stressed* cells. The latter were observed especially when the over-expression of tBid was induced in the presence of z-VAD-fmk. Stressed cells show low-mitochondrial potential, suggesting the presence of MOMP and severe damage to the mitochondrial membranes in the absence of any further signs of apoptosis. The pan-caspase inhibitor z-VAD-fmk efficiently blocks certain types of apoptosis. In consequence, the common hallmarks of the execution phase of apoptosis, like PS exposure, cell shrinkage, and DNA fragmentation are inhibited. The cells appear viable although they are actually dead because of severe damage of the mitochondrial membranes after over-expression of tBid, which directly activates the mitochondrial pathway.

Compared to the expression of tBid, melanoma cells dying after the expression of revCasp-3 showed slower cell death kinetics. Twenty four hours of expression of revCasp-3 were required to reach a similar degree of cell death. Caspase-3 is the major caspase activated during the execution phase of apoptosis (30). However, expression of revCasp-3 does not directly act on the mitochondria to induce MOMP. Caspase-3 needs to cleave sufficient MOMP-inducing substrates like Bid to tBid before MOMP and cell death can occur by a feed-forward amplification loop (31). Additionally, caspase-3 can then enter the mitochondria and cleave specific substrates of the electron transfer chain, which will ultimately result in mitochondrial uncoupling and loss of membrane potential.

Addition of z-VAD-fmk to revCasp-3 expressing cells inhibited up to 95% of the cell death occurring (more than 90% viable cells). These results demonstrate that, after blocking caspases, damage to mitochondria and to cell morphology and physiology was effectively inhibited, as confirmed by the lack of appearance of large numbers of stressed cells in **Figure 2C**.

Induction of apoptosis by UVB irradiation has been recognized to be a complex process involving a variety of independent pathways. There are at least two major mechanisms involved in apoptosis induced by UVB: (1) DNA damage; UVB induces two types of lesions in chromosomal DNA, photoproducts, and cyclobutane pyrimidine dimers (CPD), the latter being the predominant ones (32). (2) Cell death receptor activation; UVB is able to directly activate cell surface receptors (i.e., CD95/Fas and TNF receptor-1) by inducing receptor trimerization and clustering without ligand interaction (33–35). In our study, B16F10 melanoma cells were lethally irradiated with 240 mJ/cm^2 UVB applied as a single dose. Lower doses failed to completely kill the cells (data not shown). In the 24 h period following irradiation, we observed slow cell death kinetics mainly characterized by the presence of stressed (mitochondrial damage) and early secondary necrotic cells (membrane disruption and DNA preservation). Substantial amounts of late apoptotic and late secondary necrotic cells were only observed after 48 h.

Cells dying by UVB irradiation in the presence of z-VAD-fmk also showed a significant increase in the fraction of stressed cells, indicating a blockade of the apoptotic execution phase, which may be responsible for the preservation of the cell's morphology. In the presence of z-VAD-fmk, the percentage of viable cells did not change significantly, showing that inhibition of caspases cannot rescue cells from death. This observation challenges previous own and foreign reports presenting effective inhibition of UVB-induced apoptosis by z-VAD-fmk (36–38). Employing our multiparametric analysis, we here put forward a more detailed characterization of cell death phenotypes induced by several stimuli. We conclude that UVB mainly acts in B16F10 cells by triggering the intrinsic mitochondrial death pathway.

Necrosis is a cellular state that follows acute injuries, sudden anoxia, or extreme stimuli (heat, irradiation, toxins, mechanical, or oxidative stress) and, according to that, it can be viewed as a violent kind of cell death. Conventional methods used to induce necrosis in immunological studies applied heat shock at 56°C or alternating cycles of freeze–thawing. In this work, we present an alternative approach expressing a necrosis inducing protein ($CpnT_{CTD}$) via a doxy-dependent inducible expression system. CpnT is a novel outer membrane protein of *M. tuberculosis* containing a C-terminal domain that is cytotoxic when expressed in eukaryotic cells (8). It has been suggested to be required for the escape of *M. tuberculosis* from macrophages allowing subsequent bacterial dissemination. However, how $CpnT_{CTD}$ induces necrosis is still mechanistically elusive. Six hours after the addition of doxy, a significant fraction of stressed cells was observed. This suggests loss of the mitochondrial membrane potential and mitochondrial damage. Twelve and 18 h after the addition of doxy, more than 50 and 80% of the cells were primary necrotic, respectively. Only after 48 h post-doxy addition, was DNA degradation observed. The decreased DNA content of the latter mimics some features of secondary necrotic death. However, DNA degradation occurred in cells that had already lost their membrane integrity. The cells should, therefore, be referred to as post-necrotic and not as post-apoptotic. The passive influx of DNA degrading enzymes and/or Ca^{2+} most likely causes the chromatin degradation observed in necrotic cells. Expression of the $CpnT_{CTD}$ protein in the presence of z-VAD-fmk showed the same phenotype as those not treated with the inhibitor, arguing for a caspase-independent type of cell death. A classical method to induce necrosis is a heat shock at 56°C for 30 min. As expected, cells became necrotic immediately after heating. This process was also not affected by the presence of z-VAD-fmk, suggesting that caspases were not required for death induced by this kind of heat shock.

Most anti-cancer therapies, like chemotherapy and radiotherapy, aim to induce cancer cell death. However, a central problem is to understand how the immune system determines whether cell death elicits immunogenic, tolerogenic, or silent responses. It has been reported that type and/or phase of cell death affect the immune response (3, 39). For example, cells treated with antracyclines, oxaliplatin, or UVC light develop a kind of endoplasmic reticulum (ER) stress response that involves recruitment of several actors of the apoptotic pathway and contributes to the exposure of calreticulin, which represents an important determinant of immunogenic cell death (40–42).

In order to evaluate whether a certain type of cell death is immunogenic or tolerogenic/silent, an allogeneic murine graft rejection model was employed. The s.c. tumor progression in BALB/c mice was monitored after immunizing the host with various kinds of dying/dead cells. We hypothesized that immunization with tolerogenic or silent forms of cell death would overcome or not affect the natural host immunity allowing tumor development in BALB/c mice, respectively. In contrast, immunogenic cell death would result in faster and more efficient rejection and persistent immunity (3, 43–47). Syngeneic implantation of viable B16F10 cells in C57/Bl6 mice reportedly resulted in high mortality because of the potent carcinogenicity making it difficult to evaluate the immune response against various kinds of cell death (48, 49). The allogeneic model, instead, allows us to compare the immune modulatory effects of cell death forms without compromising the welfare of the host (10–12).

Immune responses elicited by dying/dead cells in BALB/c mice were dependent on the death stimulus applied to the vaccine. Mice implanted with viable melanoma cells transiently produced primary tumors, but never developed secondary tumors after re-challenge (100% rejection). In contrast, mice immunized with irradiated cells supported the growth of secondary tumors similar to the naïve cohort. In order to explore this effect thoroughly, mice were challenged after having been immunized with dying/dead cells killed by various mechanisms.

Expression of the necrosis inducing protein domain $CpnT_{CTD}$ in a B16F10 cell-based vaccine resulted in the most immunogenic type of cell death. Unexpectedly, expression of the pro-apoptotic constitutively active form of caspase-3 (revCasp-3) also induced a strongly immunogenic type of cell death. Massive apoptosis induced by the regulated expression of revCasp-3 in already implanted syngeneic tumors has been reported to be immunogenic (50). Expression of caspase-3 induced a high percentage of necrotic

cell death (early and late secondary necrosis). Heat-necrotized cells, which display membrane disruption already before injection, exhibited poor immunogenicity. This points to short-lived necrotic cell-derived signaling molecules, which would not be active anymore in the vaccine that had been necrotized before injection. Summarizing, in our model the time of appearance of membrane disruption in B16F10 cells did not correlate with the type of immune response the cells elicited. Noteworthy, UVB-irradiated cells showed the lowest percentage of necrotic cells and failed to trigger immunity. tBid-expressing cells and UVB-irradiated cells used for immunization clearly behaved silently and allowed the growth of allogeneic tumors, despite the presence of high levels of necrotic cells, as in naïve animals.

The unresponsiveness observed in our system cannot be explained solely by the exposure of PS. Similarly, DAMPs released or expressed after disruption of the plasma membrane are also not sufficient to explain the immunogenicity observed in our model. Therefore, we looked for common features of the two "silent" types of cell death, over-expression of tBid and UVB irradiation, and of the two immunogenic types of cell death, over-expression of revCasp-3 and $CpnT_{CTD}$. The common denominator in both cases is the prominent role of the mitochondria in the execution of death (39).

In our system, activation of the intrinsic pathway either by the recombinant expression of cleaved active Bid or by UVB irradiation may have caused death without relevant ROS production during the time frame the cells were employed for immunization (3–9 h after doxy treatment). Although some caspase-3 activity is to be expected in both tBid- and UVB-induced death, this was not enough to secondarily generate ROS. In contrast, downstream expression of revCasp-3 and $CpnT_{CTD}$ may have caused death by activation of terminal effector mechanisms excluding the involvement of mitochondria. Therefore, we assume that in the revCasp-3 and $CpnT_{CTD}$ cases, execution of cell death starts before mitochondrial function is severely compromised. After over-expression of revCasp-3, MOMP formation is induced allowing access of caspase-3 to the mitochondrial inter-membrane space, cleaving specific substrates in the complex I of the electron transport chain (like p75 NDUSF1) (51, 52). As a consequence, the electron transport is disrupted, and ROS is generated. In contrast, tBid expression causes severe damage to the mitochondria measured as a fast loss of the mitochondrial potential, suggesting fast MOMP formation thereby destroying electron transport abruptly. We propose that ROS can be produced in cells overexpressing revCasp-3 and possibly $CpnT_{CTD}$ because mitochondrial activity is not severely affected by an intrinsic death stimulus. Ongoing cell death in the presence of functioning mitochondria would be important for intracellular ROS accumulation. Free radicals generated in this context may induce considerable stress in the ER, which has been associated with immunogenicity of the dying cells (3).

We suggest that intracellular accumulation of ROS intermediates may play a critical role in the generation of DAMPs affecting the ongoing allogeneic immune response. For example, oxidative damage to DNA enhances its immune-stimulatory capabilities once processed by the immune system (53). Avoiding intracellular accumulation of ROS by a selective manipulation of mitochondrial functionality before killing cells is a possible option to down-regulate the subsequent immune response. Reversely, conserving mitochondrial function as long as possible during cancer cell killing may increase the immunogenicity, if this is wanted.

Although NAC significantly inhibited ROS production in $CpnT_{CTD}$-expressing cells *in vitro* (**Figure 3**), the employment of this anti-oxidant during cell death induced by the expression of $CpnT_{CTD}$ was not sufficient to abrogate their immunogenicity in our allo-immunization model. Interestingly, the addition of the specific mitochondrial ROS scavenger mitoTEMPO induced significant inhibition of ROS production in B16F10-revCasp-3 and B16F10-$CpnT_{CTD}$ dying cells *in vitro* arguing for an important role of the mitochondria in ROS production. Since inhibition *in vitro* by NAC or mitoTEMPO was partial, ROS may be additionally produced in other cellular compartments like the ER. Noteworthy, it was demonstrated in yeasts that mitochondrial dysfunction indirectly induces ROS production in the ER by a novel mechanism mediated by suppression of the endoplasmic reticulum-associated degradation (ERAD) pathway (54, 55). In line with this, ER stress may result in increased cytoplasmic Ca^{2+} concentrations and immunogenic cell death (56). Further investigation addressing the source and localization of ROS in these cells is needed to elucidate the exact role of ROS production during cell death and its immunological consequences, especially in *in vivo* models.

We speculate that some of the mechanisms observed in this manuscript might also work in patients with tumors, during tumor therapy and in some syngeneic tumor models. However, the application of tumor vaccines in human beings has, so far, been less successful. The association between intracellular ROS accumulation in dying cells and allogeneic tumor rejection may have the potential to intentionally shape anti-tumor immunity.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/Journal/10.3389/fimmu.2014.00560/abstract

Figure S1 | UVB irradiation of B16F10 cells after doxycycline treatment. In order to rule out that an additional irradiation step interferes with the type of cell death induced by specific expression of the respective cytotoxic protein in the cell lines B16F10-tBid, B16F10-revCasp-3, and B16F10-$CpnT_{CTD}$, cells were induced to die with doxycyline (5 μg/ml) and irradiated with a single dose of 240 mJ/cm^2 UVB at different time points ($t = 4$ h, $t = 5$ h, $t = 6$ h, and $t = 7$ h). Eighteen hours after doxycycline addition (t_0), the cells were harvested by

trypsinization and analyzed by FACS for PI staining. Cells killed solely by UVB or doxycycline were used as controls. Blue bars show death induced by irradiation alone. Red bars show death by doxycycline-regulated expression of each cytotoxic protein alone. Green bars show the combined effect of cytotoxic protein expression plus irradiation at different time points. Note that cells irradiated 4, 5, 6, and 7 h after doxycycline addition (green bars) died to the same extent as cells killed by doxycycline alone (red bars).These results suggest that after 4 h of incubation with doxy, the additional irradiation step did not significantly affect the degree of doxycycline-induced cell death despite of the additional damage caused by the irradiation. We, therefore, assumed that after 4 h the cytotoxic protein expression is sufficient to induce cell death as it would happen without irradiation. Irradiation at earlier time points significantly impaired doxycycline-regulated cell death (data not shown). This procedure ensured killing of the doxycycline-resistant cells in primary grafts in order to consequently avoid complications caused by proliferative signals emitted from the dying cells acting on the surviving tumor cells.

Figure S2 | Immune response against allogeneic dead and dying cells in the presence of the ROS inhibitor NAC. BALB/c mice were immunized in the right flank s.c. (single dose) with dying B16F10 cells expressing $CpnT_{CTD}$ (red lines/bars) or dying B16F10 cells expressing $CpnT_{CTD}$ in the presence of the ROS inhibitor *n*-Acetylcysteine (NAC, green lines/bars). Displayed are the mean values ($n = 7$) of the relative tumor volumes and their SEMs and the respective integral of tumor size (total tumor mass) that was obtained in mice challenged s.c. in the left flank with 2 million VC of the parental cell line B16F10-644 10 days after immunization. The Mann–Whitney *U* test was applied to compare groups.

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Tumor immunotherapy: lessons from autoimmunity

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The sequel to the landmark article "The Hallmarks of Cancer" adds two emerging hallmarks and two enabling characteristics to the six original hallmarks (1). One emerging hallmark is the property of cancer cells to escape the immune system. Clinically apparent tumors arise as winners in a complex, hard-fought duel between cancer cell survival and eradication by the immune system.

Immunoediting, a term used for describing interactions between tumor and immune system, only occurs when, during the process of malignant transformation, cells develop features recognized by the immune system (2). The contribution of the immune system to recognition and elimination of malignant cells has been and still is being discussed controversially: some studies support the concept of immunosurveillance (3, 4), whereas others only observed small effects of the immune system in the prevention of cancer (5, 6). Recent studies suggest that, while there is evidence for immunosurveillance, not all aspects of the interaction between malignant cells and the immune system can be explained by immunoediting alone (7): some tumors never show properties making them targets of the immune system, whereas other tumors are recognized, but not eliminated due to immune suppression induced by the tumor.

However, if tumor cells are recognized as "altered cells," their perpetual confrontation with the immune system evokes strong selection conditions favoring tumor cells that (I) lose properties making them targets of the immune system and (II) gain properties making them appear non-dangerous (8). If the tumor succeeds in decreasing its immunogenicity, it will reach a stage when the immune system does not consider those cells to be "altered-self" anymore. The tumor is now perceived as "self" and non-dangerous, with all privileges of normal healthy tissues.

When we think about therapies that elicit anti-tumor responses at this stage, we actually have to think about re-creating and enforcing tumor recognition, because, malignant tissues, although having been infiltrated by T-effector lymphocytes and, thus, being recognized by the immune system, frequently do not show remission. This correlates with reports that recruitment of T-effector lymphocytes to the site of the tumor is not necessarily sufficient for its eradication and that tumor immunity heavily depends on breaking tumor tolerance, i.e., by depletion of T-regulatory lymphocytes or by shielding T-effector lymphocytes from immune-suppressive molecules like PD-L1 (9). We propose that the need for inducing immunity and breaking of tolerance might be akin to activating some kind of tumor-specific (auto)-immunity.

The ideal tumor therapy results in local control of the primary tumor, systemic control of potential metastases and triggers an anti-tumor immune response ultimately leading to the elimination of all malignant cells. To achieve this, tumor therapy needs to deal with the problem that the immune system does not consider the tumor being dangerous anymore – it has been adopted as "self-organ." Consequently, tumor therapy should focus on making the immune system aware of this hidden danger.

This concept was first put into practice by William Coley, who injected a cocktail of dead bacteria into tumors in the late 1800s, achieving cures in ≈30% of his patients with sarcoma and lymphoma (10, 11). The mechanism responsible for this seems to be LPS-induced IL-12 secretion triggering a robust bystander T_h1-response against the tumor cells (12). Likewise, an attenuated *Salmonella* vaccine can induce a shift in the tumor milieu from an immune-suppressive to an immunogenic microenvironment (13). The most successful application derived from Coley's work is treatment of bladder cancer with the Bacillus Calmette–Guerin vaccine: it has become the standard therapy for superficial bladder cancer, eradicating existing tumors, reducing the frequency of tumor recurrence, delaying stage progression, and increasing survival (14). The advantage of such strategies is their lack of specificity. The immune response is not restricted to a single and, most likely, highly specific and selectable "tumor-antigen," but the presence of danger signals at the site of the tumor "uncloaks" the cancer cells, turning them into broad range immune targets. At this point, we can exploit a mechanism, which causes a break in self-tolerance in autoimmune diseases: transient autoimmunity accompanying any inflammatory process can, in the context of steady exposure to auto-antigens and danger signals, develop into stable autoimmunity. Following Polly Matzinger's ideas, the key to success of danger-based tumor vaccination strategies rests on repeated administration of the vaccine (15). Repeated immunization should help overcome transient tumor immunity and establish persistent protection.

One danger-based tumor vaccination approach conducts the immunization with dying tumor cells (16, 17). Certain kinds of dying or dead cells can trigger immune responses under the right conditions. The potential of dying/dead cells to induce autoimmunity can be seen in "systemic lupus erythematosus" (SLE), a chronic inflammatory disease, in which defective clearance of apoptotic cells leads to the accumulation of secondary necrotic cells, the release of danger signals, the presentation of auto-antigens and, finally, a chronic break in self-tolerance (18–20). Based on these observations, one can assume that, under the appropriate conditions, entities once considered to be non-dangerous can become re-considered dangerous. We propose that one can learn from the processes which cause breaks of self-tolerance in patients with SLE and try to harness them to induce tumor (auto-) immunity.

In the context of tumor immunology, cell death is a double-edged sword. Tumor cells often modulate apoptotic pathways rendering them less responsive to death stimuli. Down-regulation of Fas expression or resistance to Fas-mediated apoptosis are common strategies of tumor cells to escape immunosurveillance (21) and are associated with resistance to therapy, metastatic capacity, and poor prognosis. For example, c-Jun and Stat-3 act as oncogenes by cooperatively repressing the transcription of *Fas*, rendering tumor cells insensitive to FasL-induced apoptosis (22). A complete loss of Fas expression is less common, possibly to low-level expression of Fas supporting tumor growth (23). Many other mechanisms to evade elimination by apoptosis, i.e., suppression of caspase-8 activity by CDK1/CYCLIN B1 dependent phosphorylation (24), *bcl-2* amplification (25), and loss of pro-apoptotic proteins like BAX (26) and PUMA (27), have been reported for a large variety of cancer types (28).

These findings are hard to reconcile with the observation that a high rate of tumor cell apoptosis is accompanied by poor prognosis in some types of cancer (29–31). It is known that cancer cells show many different changes to the apoptotic machinery (28, 32); but does this mean they have lost all capability to execute apoptosis? Apoptosis is necessary for tissue homeostasis, contributes to the maintenance of peripheral tolerance and might even play a role in the induction of the latter (33, 34). The fact that most chemotherapeutics at least initially induce tumor apoptosis confirms that cancer cells frequently retain their ability to execute apoptosis (35, 36). It is reasonable to assume that those parts of the apoptotic machinery involved in the induction of extrinsic apoptosis by the immune system preferentially experience negative selection. If other parts of the apoptotic pathway would also be a potential source of harm, why do they, in defiance of the exceptional adaptability of cancer cells, still function properly? We suggest that, in contrast to the oversimplified illustration, cancer cells do not completely lose their capability to undergo apoptosis, but that their apoptotic machinery can instead be "hijacked" in a way that not only sustains their existence, but also accelerates tumor formation (37–39): an "altruistic" death of limited amounts of cancer cells is a possible way to support the survival of the tumor on the whole.

Over the years, the tumor-supportive effects of apoptotic tumor cells have received greater recognition, and it is now assumed that apoptotic tumor cells and the corresponding phagocytes participate in forming and shaping the tumor microenvironment (40). Apoptotic cells release a diverse spectrum of molecules, which act as "keep-out," "find-me," "eat-me," and "tolerate-me" signals and ensure that the clearance of apoptotic cells is facilitated by defined groups of phagocytes, in particular by macrophages (41).

Of particular interest are lipid mediators, which are released from cells undergoing apoptosis: (I) lysophosphatidylcholine is a potent chemoattractant for macrophages and is released from cells executing apoptosis (42). (II) Upon proteolytic activation of sphingosine kinase 2, sphingosine-1-phosphate (S1P) is released from apoptotic cells (43). In addition to its role as a chemoattractant (44), S1P polarizes macrophages toward a non-inflammatory phenotype (M2), characterized by decreased secretion of TNF-α and IL-12-p70 and increased formation of IL-8 and Il-10 (45).

The engulfment of apoptotic cells by macrophages induces their polarization toward the M2-phenotype (**Figure 1A**). These alternatively activated macrophages tune down inflammation and promote angiogenesis, tissue remodeling, and repair (46, 47). Furthermore, phagocytosis of apoptotic cells by M1-macrophages also triggers a shift toward alternative activation (48). Fittingly, a large number of macrophages at the site of the tumor are associated with a poor prognosis and these tumor-associated macrophages share many characteristics with M2-macrophages (49, 50). Their presence at the site of a tumor supports Dvorak's concept that tumors are "wounds that do not heal" (51).

In line with these findings is the observation that inhibiting the clearance of apoptotic tumor cells by administration of Annexin-A5 retards tumor growth in a colorectal carcinoma model and greatly enhances the effect of immunization with irradiated lymphoma cells in a lymphoma model (52, 53). The data presented suggests that this is due to the fact that the non-inflammatory clearance of apoptotic cells by macrophages is blocked so that the apoptotic cells get secondarily necrotic. The concomitant loss of membrane integrity is accompanied by the release of danger-associated molecular patterns (DAMP), which act as natural adjuvants. Phagocytosis of secondary necrotic cells by macrophages (**Figure 1B**) leads to an increased expression of TNF-α and IL-1β. In addition, several DAMPs released from secondary necrotic cells, like HMGB1 and HMGN1, are potent stimuli for dendritic cell maturation (54).

The close interaction between tumors, the immune system and cell death gives rise to new therapeutic approaches. Some aspects of this interaction may be exploited to support conventional cancer therapies. Systemic administration of Annexin-A5 or other phosphatidylserine ligands may help slow down tumor progression by blocking the tumor-supportive properties of apoptotic cells. In combination with radio- or chemotherapy, Annexin-A5 could be used as a natural adjuvant, which increases the immunogenicity of dying tumor cells and, thus, helps elicit an anti-tumor immune response (55). This may be especially helpful in targeting cancer cells, which have

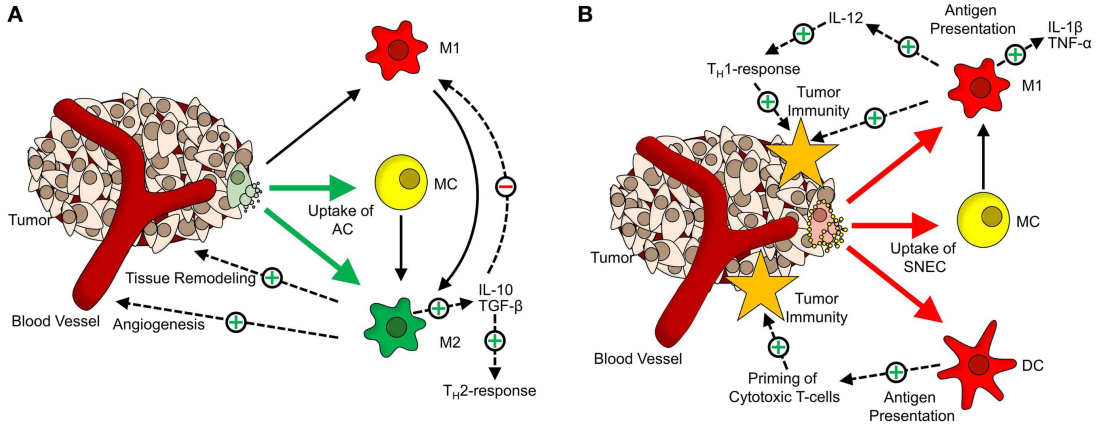

FIGURE 1 | The dual role of cell death in tumor tolerance/immunity. **(A)** Role of apoptotic cells in formation of the tumor microenvironment. Apoptotic cells (AC) are mainly taken up by monocytes (MC; yellow) and alternatively activated macrophages (M2; green). Upon phagocytosis of ACs, MCs, and classically activated macrophages (M1, red) get polarized toward an M2-phenotype. M2-macrophages participate in tissue remodeling and angiogenesis and via secretion of anti-inflammatory cytokines (TGF-β, IL-10), inhibit M1-activation of macrophages and shift T_H1-responses toward the T_H2-phenotype. **(B)** Tumor-supportive effects of apoptotic cells are abrogated by Annexin-A5. Annexin-A5 (yellow circles on secondary necrotic cells) inhibits swift clearance of apoptotic cells, leading to progression of ACs into secondary necrosis. Secondary necrotic cells (SNEC) are mainly taken up by MCs, classically activated macrophages and dendritic cells (DC; red). Upon phagocytosis of SNEC, MCs get polarized toward the M1-phenotype. Phagocytosis of SNEC by DCs leads to antigen presentation and priming of T cells. Classically activated macrophages secrete inflammatory cytokines (TNF-α, IL-1β) and induce T_H1-responses via IL-12.

resisted therapy and would possibly lead to a relapse.

Until recently, cell death was either characterized as programed and apoptotic, or accidental and necrotic. This paradigm has been undermined by the discovery of several other forms of cell death, ranging from immunogenic apoptosis (56) or necroptosis (57) to pyroptosis (58, 59). So, in addition to manipulating cell death induced by radio- or chemotherapy in a way to increase its immunogenicity, the direct induction of immunogenic tumor cell death pathways might become a promising approach in cancer therapy (17, 54, 60), especially, since our means of controlling the manner of cell death have greatly increased during recent years (61–63).

Surgical removal of malignant tissue plays an important role in modern cancer therapy. The cancer cells obtained in this process may be used as a vaccine to establish anti-tumor immunity, if treated and administered properly. The focus must be on cancer cells dying by immunostimulatory forms of cell death leading to necrotic cell corpses, whose deployment would activate antigen-presenting-cells. This way, the specific autologous tumor cells can serve as reservoirs of tumor antigens, which, upon phagocytosis by inflammatory macrophages and dendritic cells, are effectively (cross-)presented. The impact of the vaccine could be optimized by repeated administration of the dying cells. However, we have to be very careful, since a recent study indicates that excessive immune responses against cancer can result in an increased risk of developing the autoimmune disease scleroderma (64), pointing out several parallels between the induction of autoimmunity and immunosurveillance. While this study actually supports the idea that mechanisms inducing autoimmunity can also be used to elicit tumor immunity, it also suggests that any agents used to recruit anti-tumor responses must be well-balanced. After all, nobody wants to escape cancer's fire by jumping into the frying pan of autoimmunity.

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Modulation of antigen-specific T-cells as immune therapy for chronic infectious diseases and cancer

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T-cell responses are induced by antigen presenting cells (APC) and signals from the microenvironment. Antigen persistence and inflammatory microenvironments in chronic infections and cancer can induce a tolerant state in T-cells resulting in hyporesponsiveness, loss of effector function, and weak biochemical signaling patterns in response to antigen stimulation. Although the mechanisms of T-cell tolerance induced in chronic infection and cancer may differ from those involved in tolerance to self-antigen, the impaired proliferation and production of IL-2 in response to antigen stimulation are hallmarks of all tolerant T cells. In this review, we will summarize the evidence that the immune responses change from non-self to "self"-like in chronic infection and cancer, and will provide an overview of strategies for re-balancing the immune response of antigen-specific T cells in chronic infection and cancer without affecting the homeostasis of the immune system.

Keywords: tolerance induction, antigen-specific T cells, bystander T-cells, nanoAPC, reverse tolerance

INTRODUCTION

T cells are essential for robust adaptive immune responses against pathogen invasion, as well as maintaining immune tolerance to self-antigens. In the tolerant state, T cells generally fail to proliferate and produce IL-2 in response to antigen stimulation (1, 2). Anergy and immune regulation are two interconnected mechanisms that maintain peripheral tolerance to self-antigens *in vivo*. In contrast to the biochemical events induced during effective responses to pathogenic antigens, in anergy the biochemical signaling pathways in T cells are only partially activated. Activation of the calcium/calcineurin/nuclear factor of activated T cells (NFAT) pathway, but not AP1 and NFκB pathways (1–3), in anergic conditions results in the expression of tolerance associated genes such as E3-ligases (1–3). This partial TCR signaling is largely due to the lack of additional signals such as costimulatory signals and activating cytokines such as IL-2, or due to direct regulation by Treg (2). Therefore, altered expression of costimulatory signals and/or activating cytokines, or defective Treg function, results in full activation of TCR signals in response to self-antigens and may induce autoimmune responses. Recent studies have uncovered hyporesponsive phenotypes with partial activation of biochemical events in virus specific T cells in chronic infectious diseases (4, 5) and models mimicking chronic infectious conditions (5, 6). These findings indicate that during chronic infection the T-cells switch from mounting robust non-self responses to a state similar to self-tolerance due to antigen persistence and/or changes in the microenvironment. Similar to the immunological milieu of chronic infection, the tumor microenvironment contains a multitude of suppressive mechanisms that allow tumors to escape immune surveillance (4, 7). Immune hyporesponsive states have been studied in many different models *in vitro* and *in vivo* and have been categorized based on the phenotypes discovered in each tolerant state (8).

This review will briefly summarize the extracellular signals that affect self-tolerance or effector function of antigen-specific T cells. We will describe the application of these signals in therapeutic intervention and focus on the recently developed nano-technologies that can reverse the tolerant state of viral specific T cells by delivering costimulatory or cytokine signals to antigen-specific T cells.

ALTERED T-CELL RESPONSES DURING CHRONIC VIRUS INFECTION AND CANCER

Chronic virus infections are associated with impaired anti-viral immunity, particularly in the infections caused by highly replicative viruses such as HIV, HBV, and HCV. In chronic infection, persistent viral antigen, and often chronic inflammation, renders T-cells dysfunctional. The mechanisms underlying dysfunctional immune responses in patients are largely unknown. Based on experimental systems studied *in vitro* and *in vivo*, different states of T-cell dysfunction have been discovered and are classified as exhaustion, tolerance, anergy, senescence, deletion, induced Treg, and ignorance based on the phenotypes, production of inhibitory cytokines such as IL-10 and TGFβ, impairment of T-cell receptor signaling molecules, and apoptosis of the T-cells in these models (**Figure 1**) (4–6, 8–10). These findings have been extensively reviewed (4–6, 8–10). Despite the differences in dysfunctional T-cells characterized in different model systems, the common feature is proliferative hyporesponsiveness, and impaired production of IL-2 following antigen stimulation *in vivo* or *in vitro* (4–6, 8–10). The chronic LCMV infection model resembles the observations from patients with chronic virus infections more closely than other models in terms of induction of dysfunctional T cells (4, 5). The phenotype of exhaustion of CD8 T cells in the chronic LCMV model is well-characterized, with hierarchical loss of effector cytokine production, including IL-2, TNFα, and IFNγ and

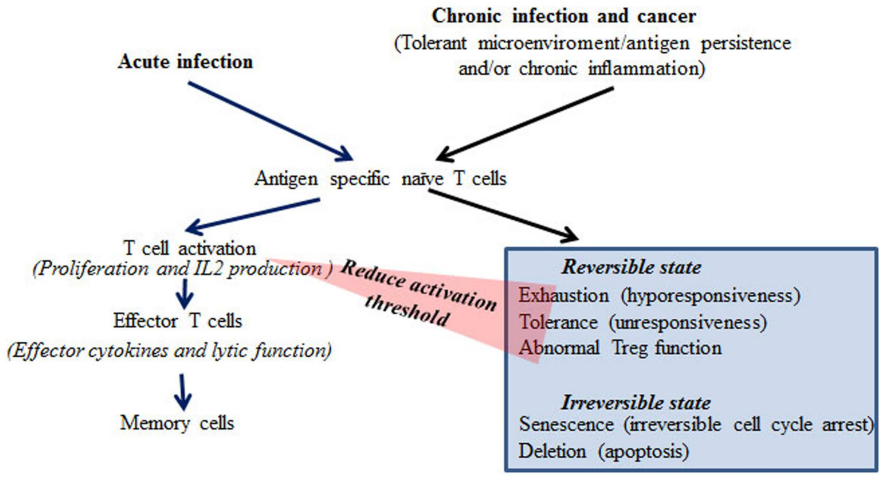

FIGURE 1 | Differential responses of T cells during acute and chronic infection or cancer.

impaired proliferation in response to antigen receptor stimulation *in vitro* (4, 5). In addition to this hyporesponsive phenotype, increased expression of the inhibitory costimulatory molecule PD-1 and production of the repressive cytokine IL-10 are also found in T cells from chronic LCMV infected mice (9, 10). Notably, similar phenotypes have been found in T cells from HIV, HBV, and HCV patients (11–14). Under chronic infectious conditions, viral specific CD8 T cells often lose cytotoxic function (15, 16). At the late stages of exhaustion, viral specific CD8 T cells may be deleted (5, 6). However, in contrast to CD8 T cells, viral specific CD4 T cells can persist under chronic infectious conditions, but in a hyporesponsive state (17). Therefore, there is the potential to restore CD4 responses, which may thereafter help CD8 function. It has been reported that Treg cells are increased or induced in chronic infection (18, 19). The increased Treg cells can reduce chronic inflammation from persistent viral antigen stimulation, but may also contribute to the establishment of immune tolerance toward the virus (18, 19).

Comparable to chronic infection, high levels of tumor antigens and chronic inflammation can establish an immunosuppressive microenvironment. Tumor reactive T cells have been shown to respond to tumor antigens in a similar fashion to viral specific T cells in chronic infection with expression of high levels of inhibitory costimulatory molecules such as PD-1, CTLA-4, and LAG-3 and impaired production of effector cytokines including IFNg, TNFa, and IL-2 (7, 20–22). It has been shown that advanced tumors with high loads of tumor antigens cause functional exhaustion and rapid elimination of tumor reactive T cells (23). However, in contrast to chronic viral infections, tumor antigens are generally poorly antigenic. Therefore, the frequency and avidity of tumor reactive T cells are low.

IMPAIRED TCR SIGNALING DURING CHRONIC VIRUS INFECTION

We have found that antigen persistence can impair TCR signaling resulting in hyporesponsiveness (24). This hyporesponsiveness is gradually induced during antigen persistence with reduction of NFkB and AP1 activation (2, 24). This characteristic phenotype of T-cell tolerance is similar to that observed in chronic HBV infection (25). Down-regulation of TCR proximal signaling molecules has been found in CD8 T cells from chronic HBV patients (25). The impaired TCR signaling in CD8 T cells from chronic HBV patients is partly due to the down-regulation of CD3ζ (25). The reduced expression of CD3ζ is associated with up-regulation of PD-1 and impaired production of IL-2, suggesting that it is part of the mechanism leading to exhaustion (25). Viral protein Nef of HIV and E2 and core protein of HCV directly modulate TCR signaling (26). HIV Nef protein interacts with a number of TCR signaling molecules including Lyn, Hck, and Lck (27). The interaction stimulates the TCR signaling pathways in the absence of antigens leading to maintenance of viral replication (26, 27). The altered TCR signaling induced by Nef negatively affects antigen-mediated TCR signaling (28). Similarly, viral proteins from HCV also modulate TCR signaling (29). E2 protein of HCV binds CD81 and promotes TCR signaling while the core protein inhibits JNK signaling and IL-2 expression (29). However, HCV does not infect T cells. Therefore, the altered TCR responses during chronic HCV infection are largely due to the persistence of viral antigens. Whether the persistent, but abnormal, TCR signaling induced by viral proteins causes the development of T-cell exhaustion is yet to be investigated.

It has been found that the tumor microenvironment impairs the formation of T-cell immunological synapses; supramolecular structures that assemble at the T cell-APC interface (30). Dysregulated synapse formation is associated with impaired activation of Rho-GTPases and can lead to partial activation or anergy of T cells.

INDUCTION OF NEGATIVE COSTIMULATORY MOLECULES

One of the important changes to the phenotype of CD8 T cells in chronic LCMV infection is the increased expression of the negative costimulatory molecules PD-1, 2B4, CTLA-4, and LAG-3 (5). A similar phenotype of increased negative costimulatory molecules has been discovered in CD4 and CD8 T cells from

chronic HBV and HIV patients (5, 15, 31–35). The function of these negative costimulatory molecules is important in the maintenance of immune tolerance toward self-antigens. Although the mechanisms underlying the induction of negative costimulatory molecules in T cells during chronic infection are not clear, it may be part of a physiological protection mechanism to reduce immunopathology induced by viral persistence and chronic inflammation. These negative costimulatory molecules are transiently up-regulated in activated effector T cells in the early stages of acute infection. However, the sustained expression of PD-1 on virus-specific CD8 T cells is associated with chronic infection, both in LCMV mouse models and in HBV patients (5). Co-expression of multiple inhibitory molecules correlates with increased functional deficits in anti-virus responses and decreased control of viral loads. Similarly, increased expression of PD-1 and CTLA-4 has been found on tumor infiltrating T cells (TIL), which can be associated with E3-ligase expression and increased Treg cells (7). Thus, the overexpression of inhibitory molecules results in shifting the balance of the immune responses from effective anti-virus or -cancer responses toward tolerance.

ALTERED CYTOKINE PRODUCTION IN T CELLS

One of the most pronounced changes in T cells in chronic infectious conditions is the altered production of cytokines (4–6). In contrast to acute infection, antigen-specific T cells from chronic infectious diseases fail to produce IL-2 and TNFα, but express the regulatory cytokine IL-10 (4–6). We have discovered that antigen-specific CD4 T cells gradually alter their cytokine profile in response to antigen persistence *in vivo* (24). Initial antigen stimulation effectively induces IL-2 production in antigen-specific CD4 T cells *in vivo*, while repeated exposure to the same antigen yields CD4 T cells that produce both IL-2 and IL-10 (24). Antigen persistence can finally switch off the expression of IL-2 in T cells, but these cells still produce high levels of IL-10 (24). This altered cytokine profile is associated with impaired proliferative responses and reduced AP1 and NFκB activation in response to antigen stimulation *in vivo* (24). Impaired production of effector cytokines such as IL-2, TNFα, and IFNγ is also associated with the defective activation of TCR signaling pathways and effector function of viral-specific CD4 and CD8 T cells in chronic HBV, HCV, and HIV infections (5, 6, 25). The up-regulation of inhibitory molecules, especially PD-1, is closely associated with the production of IL-10 and/or TGFβ (5, 6). Thus, virus persistence skews the T-cell response from activation and differentiation into effector cells toward antigen-specific immune tolerance. However, the mechanisms whereby IL-10 and/or TGFβ result in tolerance in chronic infections are still undefined. In the LCMV model, the lack of IL-10 or a defect in IL-10 signaling improves CD8 T-cell responses and drastically enhances the control of the infection (36, 37). TIL also display an altered cytokine profile, which is similar to that seen in chronic infections. High levels of IL-10 producing Treg cells have been found in TILs, which is associated with impaired production of IL-2, TNFa and IFNg (7, 38).

THERAPEUTIC INTERVENTIONS TO REVERSE IMMUNE TOLERANCE IN CHRONIC INFECTION AND CANCER

Therapeutic interventions for chronic viral infection and cancer aim to counter the effects of the immunosuppressive microenvironment and skew responses toward antigenic determinants that are highly immunogenic. Various approaches have been tried to increase antigen presentation quality via immunization with selected antigenic peptides, using methods such as recombinant vaccinia vaccines, DNA vaccines, peptide vaccines, and DC vaccines, to boost the anti-viral and -tumor responses (39). So far these therapeutic vaccines have not been successful. One of the possible explanations is that the hyporesponsiveness of T cells is not due to the lack of antigens, but to aspects of the chronic disease such as antigen persistence and chronic inflammation, which increase the activation threshold of T cells in response to antigen. Therefore, to overcome the high activation threshold of antigen-specific T cells in these conditions, immune therapy has to consider the antagonizing tolerogenic environment. Thus, therapeutic vaccines in combination with targeted immune modulation have been proposed as a more effective strategy to reverse the hyporesponsive state of T cells in chronic infections and cancer. In ovarian cancer, improved anti-tumor immune responses were observed after blockade of PD-1 (40). Similarly, in the LCMV model, immunization with LCMV GP33 encoding vaccinia virus coupled with administration of anti-PD-L1 blocking antibody significantly improved viral-specific CD8 T-cell responses and reduced viral load (41). Moreover, in chronic LCMV, combined therapy with a DNA vaccine and IL-10 neutralizing antibody effectively reversed viral specific CD8 T-cell tolerance (42). Immune tolerance induced by virus persistence is due to a network with multiple suppressive components. Blockade of multiple inhibitory receptors including PD-1, LAG-3, and CTLA-4 or combined blockade of inhibitory receptors and immunosuppressive cytokines achieves greater efficacy than blockade of a single inhibitory molecule in chronic LCMV models (43, 44). Although the increased T-cell function and concomitant decrease in viral load in these interventions are transient, these data support the hypothesis that reversing immune tolerance to the virus or tumor is the key for successful immunotherapy. While blockade of PD-1 and IL-10 resulted in restoration of viral specific CD8 T-cell function in a mouse model (45), the mechanisms underlying this recovery of effector function are still unknown. As many of these interventions do not specifically target the virus- or tumor-specific T cells and these pathways are important for maintenance of peripheral tolerance, it is essential to control the balance between restoration of anti-viral or -tumor responses and prevention of autoimmune diseases (5, 7). The ideal intervention will be to specifically reverse the tolerance of viral or tumor specific T cells, while maintaining the overall self-tolerance of the immune system.

RESTORING NON-SELF-RESPONSES OF VIRAL SPECIFIC T CELLS, WHILE MAINTAINING THE SELF-TOLERANCE OF BYSTANDER T CELLS IN CHRONIC INFECTION

The differential responses of antigen-specific T cells result from biochemical signals induced in T cells following interaction with antigen-MHC complexes, costimulatory molecules, and cytokines. When the mitogenic biochemical signals break the activation threshold, the T cell will enter into the cell cycle and produce growth cytokines such as IL-2 to promote clonal expansion. Due to the persistence of viral antigen, the chronic inflammatory environment and the increased production of inhibitory molecules,

the activation threshold of viral specific T cells is increased and the T cells are unable to enter the cell cycle following antigen stimulation (5, 24). However, chronic infection normally does not induce tolerance in T cells responding to antigens other than those derived from the virus itself. Therefore, systemic intervention may reverse the tolerance of viral-specific T cells, but also break the self-tolerance of bystander T cells potentially resulting in autoimmunity (5). Therefore, the ideal strategy is to reverse tolerance via modulations that increase positive and/or dampen negative costimulatory signals thereby breaking the activation threshold and driving clonal expansion of virus responding T cells, but importantly, without affecting bystander T cells. Cytokine modified and viral antigen pulsed DCs have been used to deliver antigen and positive costimulatory signals to viral specific T cells in chronic infection (46–48). However, DCs are unstable and very heterogeneous in terms of population and function. It is therefore difficult to target and deliver additional positive signals to antigen-specific T cells (49). We found that an increase in the amount of antigen presented by activated dendritic cells (DC) cannot reverse tolerance (50, 51). Although exogenous IL-2 can effectively overcome tolerance and restore the full activation of tolerant T cells in response to antigen stimulation in animal models and HBV specific CD8 T cells, systemic administration of high doses of IL-2 not only induces severe side effects, such as cardiovascular, pulmonary, hematological, hepatic, neurological, endocrine, renal, and/or dermatological complications (52), but may also promote Treg function, which can further increase the activation threshold of antigen-specific T cells (53).

In order to use IL-2 and/or anti-PD1 to overcome the hyporesponsiveness of viral specific T cells induced in chronic HBV infection while avoiding the side effects of systemic administration, we have developed a novel therapeutic vaccine (nanoAPC). These nanoAPC are derived from an APC line; the human B cell line 721.221. This cell line is MHC deficient, but expresses high levels of costimulatory molecules (51). The nanoAPC are prepared from the endoplasmic reticulum (ER) membranes of 721.221 cells (51), that are genetically engineered to express ER retained MHC class I alleles and membrane-bound IL-2. Therefore, MHC and IL-2 are synthesized physiologically in 721.221 human B cells and immobilized on ER-membranes (**Figure 2**) (51). After assembly with HBV antigenic peptide *in vitro*, the nanoAPC contain peptide-MHC complexes, costimulatory molecules, and IL-2. Unlike therapeutic DCs, the nanoAPC are homogeneous, stable, and can be stored at −80°C (50, 51). Equipped with defined viral-peptide-MHC complexes, the administered nanoAPC can directly interact with antigen-specific T cells *in vivo* (51). Due to the native structure of their membranes, nanoAPC effectively induce immune synapses and expression of the high affinity IL-2 receptor on T cells (**Figure 2**) (51). The IL-2 delivered by nanoAPC enhanced antigen-specific T-cell responses and effector function, but did not affect bystander T cells or Treg cells. When assembled with a pool of HLA A2 associated HBV peptides and HBV peptides associated with HLA DR and DP, IL-2-nanoAPC induced strong CD4 and CD8 T-cell responses in peripheral lymphocytes from chronic HBV patients (51). We demonstrated that IL-2 on nanoAPC is able to enhance TCR signaling and downregulate PD-1 expression on virus responding CD8 T cells from chronic HBV patients, which could effectively reverse tolerance as demonstrated by induction of IFNγ producing CD8 T cells in lymphocytes from chronic HBV patients (51). In

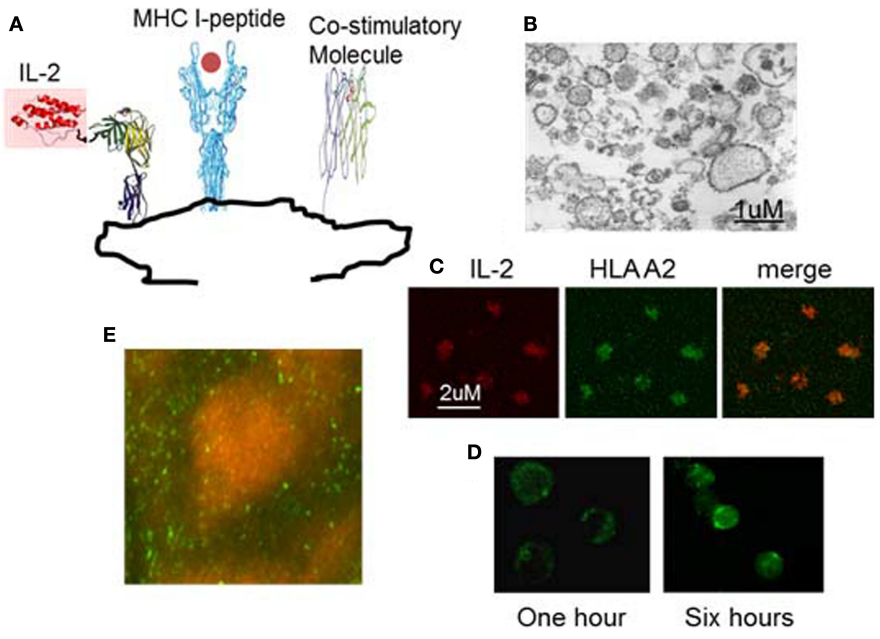

FIGURE 2 | Example of nanoAPC designed to deliver peptide-MHC complexes and IL-2 to their receptors on antigen-specific T lymphocytes. **(A)** Graphical representation of nanoAPC. **(B)** Electron microscopy shows purified nanoAPC from IL-2 engineered 721.221 B cells. **(C)** Confocal microscopy shows nanoAPC stained with anti-IL-2 (red) and anti-HLA A2 (green). **(D)** Confocal microscopy shows interaction of nanoAPC (green) with T lymphocytes after 1 and 6 h *in vitro*. After 6 h, nanoAPCs are internalized into T cells. **(E)** Distribution of nanoAPC (green) prepared from mouse dendritic cells in T lymphocyte areas of mouse lymph node 48 h after i.v. injection. B cells were stained by B220 (orange).

addition to TCR signaling, MAPK activation can result directly from IL-2R signaling (53). It has been found that the activation of MAPK and PI3K through Shc recruited by the IL-2R is independent of STAT5 signaling in effector T cells, which differs from that in Treg cells, and is important for the expansion of activated CD8 T cells (54). We have demonstrated that nanoAPC can induce CD25 expression and immune synapse formation, which not only enables the induction of T-cell activation but also brings engineered bio-adjuvants such as IL-2 stably into signalsomes of effector T cells (51). The increased expression of CD25 on CMV antigen-specific CD8 T cells by IL-2-CMV_{nlv}A2-nanoAPC is consistent with the well-known observation that IL-2 can induce CD25 expression on pre-activated CD8 T cells (51). Thus, together with peptide-MHC complexes and costimulatory molecules, the selective delivery of IL-2 is important in inducing activation of HBV responding T cells in chronic HBV patients. As the overall pharmacological dose remains low, the IL-2-nanoAPC do not activate Treg cells indicating that this approach can be adapted for use with other bio-adjuvants. Our results demonstrate that IL-2-nanoAPC, which deliver both antigen and IL-2 to antigen-responding T cells, can significantly increase functional anti-viral responses, thereby overcoming the immune tolerance induced by persistent viral load.

Nano-particles prepared from synthetic materials or genetically engineered microbes have been used to deliver antigens to DC for induction of anti-viral or -cancer immune responses (55). In contrast to these particles, nanoAPC are prepared from the ER-membranes of bio-engineered APC. Therefore, they are not only more biocompatible than synthetic nano-particles or microbes, but also deliver therapeutic molecules that are physiologically synthesized by APC seed cells. Thus, the IL-2 on IL-2-nanoAPC is more stable than free IL-2 *in vivo*, and maintains its physiological conformation allowing optimal interaction with the IL-2 receptor (data not shown). Unlike other nano-particle based vaccines, we have demonstrated that nanoAPC can directly activate T cells. NanoAPC are derived from APC cells and contain high levels of costimulatory molecules (51). Therefore, the nanoAPC mimic live DC to induce lipid raft clustering on T cells and formation of an immunological synapse, which is essential for T-cell activation. Furthermore, using HLA I negative 721.221 cells as seed cells allows us to specifically express selected HLA alleles allowing construction of HLA allele matched nanoAPC for individual patient populations.

Previously, we observed nanoAPC homing to T-cell areas of peripheral lymphoid organs, largely due to the expression of homing receptors by the cells from which the nanoAPC are derived (50). We have now further demonstrated that nanoAPC are not efficiently endocytosed by DC *in vivo* (50, 51). This is important as it allows the nanoAPC to remain as free-particles in peripheral lymphoid organs. The absence of endocytosis may be due to the lack of molecules on nanoAPC recognized by DC pattern recognition molecules (56). Thus, nanoAPC effectively target viral specific T cells and deliver immune modulation to reverse their tolerant state.

SUMMARY

In chronic infection and cancer, T cells are continuously confronted with moderate to high levels of antigens, which, in combination with the induced immunosuppressive microenvironment resulting from high antigen load and dysregulated immune responses, leads to increased activation thresholds and, subsequently, a reduction in effector function resulting in a tolerant state. This tolerant state can be reversed by positive regulatory molecules such as IL-2, IL-7, and/or blockade of PD-1 and CTLA-4. However, systemic administration of positive regulatory cytokines, or blocking antibodies, may cause autoimmunity. Therefore, one of the major challenges for immunotherapy against chronic infectious diseases and cancer is to reverse the tolerance of antigen-specific T cells, without affecting bystander T cells, thereby maintaining immune homeostasis to self-antigens. The development of delivery vehicles targeting antigen-specific T cells allows the provision of not only antigen but also engineered bio-adjuvant(s), which can restore effector function.

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Immunotherapy for prostate cancer: lessons from responses to tumor-associated antigens

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Prostate cancer (PCa) is the most common cancer in men and the second most common cause of cancer-related death in men. In recent years, novel therapeutic options for PCa have been developed and studied extensively in clinical trials. Sipuleucel-T is the first cell-based immunotherapeutic vaccine for treatment of cancer. This vaccine consists of autologous mononuclear cells stimulated and loaded with an immunostimulatory fusion protein containing the prostate tumor antigen prostate acid posphatase. The choice of antigen might be key for the efficiency of cell-based immunotherapy. Depending on the treatment strategy, target antigens should be immunogenic, abundantly expressed by tumor cells, and preferably functionally important for the tumor to prevent loss of antigen expression. Autoimmune responses have been reported against several antigens expressed in the prostate, indicating that PCa is a suitable target for immunotherapy. In this review, we will discuss PCa antigens that exhibit immunogenic features and/or have been targeted in immunotherapeutic settings with promising results, and we highlight the hurdles and opportunities for cancer immunotherapy.

Keywords: immunotherapy of cancer, prostate cancer, tumor-associated antigens, CRPC, immunotherapy

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed non-cutaneous cancer among men in the United States and is the second leading cause of death from cancer in men (1). In Europe, PCa is also the cancer type with the highest incidence in men apart from skin cancer, while it is the third most common type of cancer after lung cancer and colorectal cancer (2). PCa is usually diagnosed in men above 65 years of age. Depending on the severity of the disease, current treatment options for PCa consist of active surveillance, prostatectomy, radiation therapy, hormonal therapy, or chemotherapy. Up to one-third of patients with a localized tumor eventually fails on local therapy and progress to advanced-stage or metastatic PCa within 10 years. For advanced PCa, androgen deprivation therapy is the standard of care. Although the majority of patients initially respond, most tumors become resistant to primary hormonal therapy within 14–30 months (3). For men with metastatic castration-resistant prostate cancer (mCRPC), the median survival in phase III studies range from 15 to 19 months. For several years, the chemotherapeutic drug docetaxel was the only treatment option for mCRPC, resulting in a median overall survival benefit of 2–3 months compared with the previous treatment regimes mitoxantrone and prednisone (4–6). However, new agents targeting the androgen signaling pathway, immunotherapeutic options, radium-223 treatment, and the new chemotherapeutic treatment modality taxane cabazitaxel are emerging therapies with the ability to improve both the survival and the quality of life.

In 2010, the first cellular immunotherapy was approved as a treatment for mCRPC by the US Food and Drug Administration (FDA). More recently, cancer immunotherapy hit a new peak, Science Magazine elected cancer immunotherapy the breakthrough of 2013 (7). Especially, modulation of T-cell checkpoints via immune checkpoint inhibiting [anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) monoclonal antibodies and anti-programed death (ligand) 1 (PD-(L)1] monoclonal antibodies has been successful. Instead of tacking of the brake of the immune system, as is the case with checkpoint inhibitors, another challenge is out there: enhancement of immune responses to tumor-specific antigens. In this review, we discuss tumor antigens expressed by PCa, how they can be used to combat PCa via immunotherapy, and which hurdles need to be addressed and overcome. Other new treatment modalities are beyond the scope of this study.

INFLAMMATORY RESPONSES IN THE PROSTATE

Inflammation is an innate response to harmful stimuli, such as infections, tissue damage, or tissue malfunction (8, 9). The main goal with the inflammatory process is to clear the potential threat and restore tissue homeostasis. This normally occurs in two phases – the recognition and elimination phase and the resolution and repair phase (8, 10). If the acute response fails in eliminating the inflammatory agents, the inflammation shifts toward a chronic state. Instead of initiating the resolution phase, additional macrophages and lymphocytes are recruited and, depending on the inflammatory inducer, act to remodel the local microenvironment to adapt to an altered tissue homeostasis.

Cancer has been described as a wound that refuses to heal (11), and today many cancers have been tightly correlated with preceding inflammatory responses (12, 13). Several lines of evidence support the theory that inflammation also precedes PCa (9). Proliferative inflammatory atrophy lesions are areas in the prostate with an increased infiltration of inflammatory cells. These regions can merge with prostatic intraepithelial neoplasia, which is considered to be a risk factor for the development of PCa (14, 15). Also, a correlation with regular intake of non-steroidal anti-inflammatory drugs and reduced PCa risk has been observed (16–18).

AUTOIMMUNITY AND PCa

Inflammatory response inducers in prostate vary from infections to life style factors, such as diet or smoking (19). Symptomatic prostatitis caused by bacterial infection has been correlated with an increased risk of PCa development (20, 21). However, the causing agents of the majority of symptomatic and asymptomatic prostatitis are not well characterized and are probably multifaceted events (22).

Several studies have reported autoimmune responses against both seminal proteins (23, 24) and prostate antigens causing prostatitis to become chronic (25–27). These finding are additionally verified in animal models, where a cytotoxic cellular response seems to be driving the autoimmune reaction (28). Androgen ablation in patients with PCa is shown to induce high levels of T-cell infiltration into both benign and cancerous prostate sites, indicating that autoimmune responses against prostate antigens might be hormonally regulated (29).

ANTIGEN-BASED CANCER IMMUNOTHERAPY

The increased knowledge of how specific immune responses are evoked and the development of tools to manipulate the immune system have enabled implementation of novel immune-based cancer therapies. The rationale of these immunotherapies is to induce anti-tumor immune responses, decrease tumor-load, and change the course of the disease. Recognition of target antigens by the immune system is crucial. Several types of immunotherapeutics have been developed, such as peptide vaccines, DNA/RNA vaccines, cell-based vaccines, and T-cell modulators. Although improving overall survival is the primary endpoint of most clinical studies, a better understanding of induced T-cell responses, boosting pre-existing immune responses, and the effect of the tumor microenvironment on the T cells is needed to further improve PCa immunotherapy.

Tumor-associated antigens in PCa can be proteins that are present on prostate cells and on their malignant counterparts. Examples are prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), and the cancer/testis antigens (CTAs). In a steady state, these antigens are not provoking strong immune responses. Immunosuppressive mechanisms in the prostate microenvironment, such as transforming growth factor (TGF)-β, regulatory T cells (Tregs), or myeloid-derived suppressor cells, will maintain prostate infiltrating lymphocytes in an inactive state (30–32). In addition, PCa cells exploit several mechanisms to enhance immune tolerance (33). Despite the immunosuppressive microenvironment, several immunotherapeutic approaches are able to induce or enhance tumor-specific immune responses. In the following section, potential tumor antigens and their application as immunotherapeutic targets will be discussed. **Table 1** provides an overview of the antigens discussed and clinical results of antigen-based immunotherapy trials.

PROSTATE CANCER ANTIGENS

PROSTATE-SPECIFIC ANTIGEN

Prostate-specific antigen is a serine protease produced primarily in the epithelial cells lining the acini and ducts of the prostate gland (51–53). Physiologically, PSA is present at high concentrations in the seminal fluid. Its function is to cleave high molecular weight proteins into smaller peptides, which results in liquification of these peptides. This allows the spermatozoa to swim freely (51). Membrane-bound PSA is expressed by most PCa cells. Upon disruption of the prostate gland tissue by cancerous growth, PSA is released into the circulation. There, PSA can interact with several inflammatory cells, including fibroblasts and macrophages, which might cause chronic inflammation (9, 54, 55). PSA serum levels correlate with the extent of disease and are therefore a useful tumor marker, accurately reflecting tumor status and prognostic for clinical outcome. In case of relapse, PSA levels correlate with tumor recurrence (51, 56). Transcription of the PSA gene is positively regulated by the androgen receptor, which can partly explain the decline in PSA levels in response to androgen deprivation therapy (52). However, high PSA levels are also observed in patients with CRPC, due to the acquired ability of the tumor cells to maintain the androgen receptor function even in the androgen-ablated environment (57).

PSA as tumor antigen

Cellular autoimmune responses against PSA have been detected in both healthy men and patients suffering from chronic prostatitis (26, 27, 58), suggesting that PSA has immunogenic properties. It has been used as a target antigen in several immunotherapeutic constructs. Hodge et al. used a vector designated TRICOM, containing three co-stimulatory molecules B7-1, ICAM-1 and LFA-3, and a PSA peptide, for T-cell stimulation (59). Using a similar approach, Kantoff et al. studied a combination of PSA-expressing recombinant viral vectors, where treatment with a vaccinia-based priming vector was followed by six booster injections of a fowlpox-based vector (PROSTVAC-VF). In the phase II, randomized controlled trial in patients with mCRPC, no significant difference in progression-free survival was detected between control group and the vaccinated group. However, vaccinated patients had a longer median overall survival, and a better 3-year survival (60). These clinically meaningful results have to be confirmed in an ongoing phase III trial (**Table 2**).

Other PSA-expressing vectors have been tested in phase I trials in patients with PCa with rising PSA levels. Vaccinations with vaccinia-based vectors expressing PSA resulted in stabilization of serum PSA levels and PSA-specific T-cell responses were observed (34). PSA-specific T cells were also detected after vaccination with a liposome-based PSA vaccine and a dendritic cell (DC)-based vaccine (35, 36). Treatment with a PSA encoding poxviral vector vaccine in combination with radiotherapy not only showed PSA-specific T-cell activation, but also T-cell responses against prostate-associated antigens not encoded by the vaccine. This is

Table 1 | Antigens and their immunogenicity in prostate cancer.

Antigen	Function	Immunogenicity in PCa	Human clinical trials in PCa	Number of patients with PCa
PSA	Serine protease which cleaves high molecular weight proteins into smaller peptides, resulting in the necessary liquification for spermatozoa to swim freely	Stimulates CTLs *in vivo*	Poxviral vaccine PROSTVAC-VF/PSA-TRICOM showed a longer median overall survival when compared to placebo (34)	82 vs. 40 controls
			A phase I trial with a recombinant vaccinia virus expressing PSA (rV-PSA) showed a stable PSA level for at least 6 months in 14 patients (35)	33
		Production of immunosuppressive cytokines	A study with JBT 1001, a recombinant PSA vaccine, showed a T-cell response in eight patients (36)	10
			A study reported a PSA decrease between 6 and 39% compared to baseline in 11 of the treated patients with PSA-loaded DCs (37)	24
PAP	Protein tyrosine phosphatase which enhances the mobility of sperm	Stimulates CTLs *in vivo*	A phase I/II study reported PAP-specific T-cell responses and an increased PSA doubling time for the plasmid DNA accine pTVG-HP PAP when compared to placebo (38)	22
		Elevated in both prostatic hyperplasia and PCa	Three phase III RCTs, of which two showed a significant increase in overall survival (39, 40), and one (41) showed a trend to increase in overall survival for sipuleucel-T compared with placebo	341 vs. 171 placebo (39) 82 vs. 45 placebo (40) 65 vs. 33 placebo (41)
PSMA	Folate hydrolase activity	Presented at the cell surface and in the endothelial lumen, the latter promotes integrin signaling	A phase I trial reported a 50% PSA reduction in four patients treated with ^{177}lutetium-labeled J591, a radiolabeled monoclonal antibody against PMSA (42)	35
		Highly overexpressed in PCa	A study using an HLA-A2 restricted PMSA peptide (LLHETDSAV) showed neither clinical nor immune responses. The authors concluded that the used PSMA epitope was poorly immunogenic compared with other HLA-A2-presented peptides (43)	12
			A phase II trial with DCs pulsed with PMSA peptides showed a 50% reduction of PSA in nine patients (44)	33
PSCA	Unknown, overexpressed by most PCas	T-cell activation and proliferation	Two vaccination studies in humans with DCs loaded with PSCA alone or in combination with PAP, PSMA, and/or PSA reported that the vaccine was well tolerated and increased both the PSA doubling time and median overall survival of the patients (45, 46)	12 (45) 6 (46)
MUC-1	Limiting the activation of inflammatory responses	T-cell proliferation	A phase I/II trial with DCs loaded with MUC-1 glycopeptide and KLH showed a reduction of PSA rise in six patients. Immune responses to KLH (6/7) and Tn-MUC-1 (5/7) have been detected (47)	7
			Radioimmunotherapy was combined with or without low-dose paclitaxel in patients with mCRPC and breast cancer. In two patients with mCRPC who received m170 (MUC-1 monoclonal antibody) linked to indium-111, a 50% decline in PSA level was shown which lasted 2 months, and two patients described a decrease in bone pain (48)	9

(Continued)

Table 1 | Continued

Antigen	Function	Immunogenicity in PCa	Human clinical trials in PCa	Number of patients with PCa
NY-ESO-1	Unknown, expressed in a variety of tumors	CTLs and antibody-mediated responses	In patients with mCRPC, NY-ESO-1 peptides vaccines were tolerable. Among nine patients, vaccinations appeared to slow PSA doubling time, and yielded antigen-specific T-cell responses in six patients (49)	14
			Immunoactivation following an NY-ESO-1 protein-based vaccine combined with CpG showed humoral and cellular immune responses specific for NY-ESO-1 in 12 and 9 of the vaccinated patients, respectively (50)	13
MAGE-A genes	Down-regulates p53 function through histone deacetylase recruitment	Stimulates CTLs *in vivo*	No human clinical trial performed in PCa	
AKAP-4	Binding protein involved in cytoskeletal regulation and organization by affecting cyclic AMP-dependent protein kinase-A	Stimulated CTLs *in vitro*	No human clinical trial performed in PCa	

indicative for tumor cell killing and subsequent epitope spreading (37). Hence, PSA-targeted immunotherapy can boost conventional treatment strategies to induce stronger and broader effects. This was also shown in a recent study combining PSA-TRICOM treatment with the T-cell checkpoint inhibitor ipilimumab, where the majority of chemo-naive patients displayed a decline in serum PSA levels (61).

Despite the fact that PSA-based immunotherapeutic approaches can stimulate cytotoxic T lymphocytes (CTLs) both *in vitro* and *in vivo*, untreated patients with PCa often fail to induce a potent immune response against this antigen (62–64). Several factors might contribute to this phenomenon: (i) PSA activates TGF-β, which can suppress immune responses in the tumor microenvironment (65, 66); (ii) PSA has a negative effect on lymphocyte proliferation and differentiation (63, 64); (iii) PSA can inhibit the maturation, function, and survival of DCs (63, 64).

In summary, the serine protease PSA is expressed at high levels by most PCa. Targeting PSA might not only elicit a tumor-specific immune response, but also counteract the negative effect of PSA on both T cells and DCs. Therefore, PSA poses as a promising target antigen in immunotherapy, and this is underscored by the results of phase II trials using PSA in vector-based peptide vaccines (60, 67). The ongoing phase III clinical trial (NCT01322490) might provide more evidence on the clinical relevance of PSA-TRICOM/PROSTVAC-VF vaccinations (**Table 2**).

PROSTATE ACID POSPHATASE

Human prostate acid posphatase (PAP) is a secreted glycoprotein enzyme synthesized in the prostate epithelium (68). Only a few substrates have so far been identified for PAP, including adenosine monophosphate, phosphotyrosine, phosphocholine, phosphocreatine, and ErbB-2 (69, 70) Since PAP can act as a protein tyrosine phosphatase, many other yet to be identified substrates might be involved in the signal transduction of this protein. PAP is secreted by the prostate gland following puberty and its expression is correlated with testosterone. It is reported to enhance the mobility of sperm (71). Serum PAP levels are low in healthy individuals and increased levels are associated with PCa. For example, it is shown that PAP is aberrantly expressed in high Gleason score PCa (72, 73). Ozu et al. showed that serum PAP levels, like serum PSA, are significantly increased within the escalating PCa disease stages. PAP is also elevated in patients with bone metastasis, compared to those without bone metastasis (74). Elevation of PAP is associated with significantly shortened survival, while its decrease is correlated with responsiveness to therapy (75–77).

PAP as tumor antigen

Due to its elevated expression in PCa, PAP has been investigated as a possible target antigen for immunotherapeutic approaches. PAP-specific cytotoxic T cells (CTLs) can be found in blood of healthy donors and in patients with chronic prostatitis (26, 78, 79). In addition, patients with PCa vaccinated with DCs loaded with murine PAP showed responses against human PAP coinciding with significant clinical anti-tumor responses (80). Specific CTLs can also be generated by culturing with antigen presenting cells pulsed with a PAP-derived HLA-A2 binding peptide. The obtained CTLs can lyse peptide-loaded target cells in an antigen-specific manner, as

Table 2 | Ongoing trials encompassing antigen-based immunotherapy.

Antigen	Study design	Trial identifier	Immunologic endpoints
PSA	Phase II trial of PROSTVAC-VF/PSA-TRICOM with docetaxel and prednisone vs. docetaxel and prednisone alone in patients with mCRPC	NCT01145508 (the study is ongoing but not recruiting new patients anymore)	Immune responses before and after docetaxel and PSA-specific immune responses Primary endpoint: overall survival
	Phase II trial with enzalutamide with or without PROSTVAC-VF/PSA-TRICOM in patients with mCRPC	NCT01867333 (ongoing and recruiting trial, estimated completion date June 2016)	Immune response (not further specified) Primary endpoint: to show increase in time to progression
	Phase III study of PROSTVAC-VF/PSA-TRICOM with or without GM-CSF in patients with mCRPC	NCT01322490 (ongoing and recruiting trial, estimated completion date August 2016)	No immunologic endpoints Primary endpoint: overall survival
PAP	Phase II trial of sipuleucel-T with a pTVG-HP DNA vaccine in patients with mCRPC	NCT01706458 (ongoing and recruiting trial, estimated completion date June 2015)	Primary endpoint: immune responses following treatment with sipuleucel-T
	Phase II trial of sipuleucel-T with concurrent or sequential abiraterone acetate plus prednisone in patients with mCRPC	NCT01487863 (active study, not recruiting, estimated completion date June 2015)	Primary endpoint: sipuleucel-T CD54 upregulation
	Phase II trial of sipuleucel-T and ipilimumab given immediately sequential vs. delayed sequential in patients with mCRPC	NCT01804465 (active study, not recruiting, estimated completion date August 2015)	Primary endpoints: safety of both treatment arms and induction of antibody responses by sipuleucel-T, the proportion of patients on each study arm who achieve an immune response to PAP and/or PA2024
	Phase I study of sipuleucel-T and ipilimumab in patients with mCRPC	NCT01832870 (ongoing and recruiting trial, estimated completion date December 2015)	Primary endpoint: antigen-specific memory T-cell response, antigen-specific proliferation and antibody responses against PAP, PA2024 and PHA
	Phase II trial of sipuleucel-T with or without anti-PD-1 monoclonal antibodies and cyclophosphamide	NCT01420965 (ongoing and recruiting trial, estimated completion date December 2017)	Primary endpoints: feasibility and the immune efficacy of sipuleucel-T alone vs. sipuleucel-T plus cyclophosphamide and anti-PD-1 monoclonal antibodies (CT011) on the change in specific immune response
PSMA	Phase I trial of adoptive T-cell transfer targeted to PSMA in patients with mCRPC	NCT01140373 (ongoing and recruiting trial, estimated completion date June 2014)	No immunologic endpoints Primary endpoint: progression-free survival
	Phase II trial of PSMA antibody drug conjugate in patients with mCRPC	NCT01695044 (ongoing and recruiting trial, estimated completion date January 2015)	Primary endpoints: changes in tumor assessments, serum PSA and circulating tumor cells
	Phase II study of prodrug chemotherapy (G-202) which is activated *in situ* by PSMA of PCa cells or within cancer blood vessels of patients with mCRPC	NCT01734681 (study is not yet open for recruitment, estimated completion date January 2015)	Changes in circulating tumor cells and humoral and cell-mediated immunity to PSMA and other known PCa antigens and to track the persistence, accumulation, and migration of genetically retargeted anti-PSMA autologous T cells Primary endpoint: safety and tolerability of immunotherapy

(Continued)

Table 2 | Continued

Antigen	Study design	Trial identifier	Immunologic endpoints
	Phase I trial of anti-PSMA designer T cells after non-myeloablative conditioning in patients with mCRPC	NCT00664196 (ongoing and recruiting trial, estimated completion date July 2016)	Pharmacokinetics and pharmacodynamics of the anti-PSMA designer T cells Primary endpoint: the safety of using modified T cells
PSCA	No active or recruiting clinical trials in patients with PCa		
NY-ESO-1	Phase I trial of IMF-001 (CHP-NY-ESO-1 complex) vaccine in NY-ESO-1 expressing malignities	NCT01234012 (active study, not recruiting, estimated completion date December 2013)	NY-ESO-1 specific cellular (specific CD4 and CD8+ T cells) and humoral immunity (NY-ESO-1 antibody titer) Primary endpoint: safety and tolerability of the vaccine
	Phase I trial of DEC-205-NY-ESO-1 fusion protein vaccine in NY-ESO-1 expressing solid tumors	NCT01522820 (ongoing and recruiting trial, estimated completion date September 2014)	NY-ESO-1 specific cellular and humoral immunity Primary endpoint: safety of the vaccine
MAGE-A genes	No active or recruiting clinical trials in patients with PCa		
AKAP-4	No active or recruiting clinical trials in patients with PCa		
MUC-1	Phase I/II study of autologous DCs loaded with Tn-MUC-1 peptide in patients with CRPC	NCT00852007 (active study, not recruiting, estimated completion date March 2014)	Induction of CD4/CD8 responses measured by CFSE or ICS assay and/or induction of humoral response measured by specific antibodies or antibody isotype switching Primary endpoint: time to radiographic progression
	Phase I study of MUC-1 vaccine in conjunction with poly-ICLC in patients with recurrent or advanced PCa	NCT00374049 (active study, not recruiting, estimated completion date July 2014)	Primary endpoint: to evaluate the efficacy of poly-ICLC in boosting the immunologic response of a MUC-1 vaccine
	Phase II study of L-BLP25 (Stimuvax) in combination with androgen deprivation therapy and radiation therapy in patients with high-risk PCa. L-BLP25 vaccination is thought to work via killing of MUC-1 overexpressing cancer cells	NCT01496131 (ongoing and recruiting trial, estimated completion date January 2016)	Change in the ELISPOT level of Mucin-1-specific T cells after radiation therapy

well as HLA-A2 positive prostate tumor cells *in vitro* (78). PAP-specific cytolytic T-cell responses have additionally been identified in HLA-A2 transgenic mice immunized with the PAP encoding DNA vaccine pTVG-HP (81). Moreover, PAP peptides with the ability to bind additional HLA-A alleles has also been described (82, 83). Also, small clinical studies using a PAP-derived peptide for different HLA-subclasses show promising results in patients with PCa (84, 85). Naturally occurring PAP-specific CD4+ T cells are only found in 7–11% of patients with PCa, but this can be augmented by immunotherapy. Overall, these data suggest that PAP-specific T-cell responses can be initiated, and that PAP is an interesting candidate to use in cancer immunotherapy (81, 83, 84).

DNA-based PAP vaccine

In a PAP-based DNA vaccine, patients with CRPC received six vaccinations with granulocyte-macrophage colony-stimulating factor (GM-CSF) biweekly. Both humoral and cellular immune responses were detected in 3 of the 22 patients, with an at least threefold increase in PAP-specific IFN-gamma secreting CD8+ T cells. Nine of 22 patients showed PAP-specific CD4+ and/or CD8+ T-cell responses, but no antibody responses were detected. Also, an increase in the PSA doubling time was observed (86). The results of two ongoing trials will shed light on the role of PAP-based DNA vaccines in PCa (**Table 2**).

APC-based PAP vaccine: sipuleucel-T

After three phase III randomized controlled trials, the PAP-targeting vaccine sipuleucel-T, became the first cellular immunotherapy ever to be approved for any malignancy by the FDA (38, 39, 41). Sipuleucel-T is a peripheral blood mononuclear cell (PBMC)-based autologous vaccine. PBMCs are cocultured with a fusion protein, consisting of GM-CSF and PAP, for *ex vivo* activation of APCs and as tumor-associated antigen, respectively. The proposed mechanism of sipuleucel-T is inducing antigen-specific immune responses and thereby destroys PCa cells (40).

Sipuleucel-T treatment consists of three injections at 2-week intervals. In three phase III randomized controlled trials, an increase in overall survival of 4 months was noticed with no difference in progression-free survival. In general, treatment was well tolerated and only rigors and pyrexia were reported as adverse events (38, 39, 41). The trial by Kantoff et al. showed a trend of superior treatment outcome of sipuleucel-T in patients in the lowest PSA-level quartile ($\leq$22.1 ng/mL). On the contrary, in the highest PSA-level quartile treatment with sipuleucel-T showed only 2.8 months overall survival benefit (41). This suggests that treatment with sipuleucel-T should be initiated directly after the diagnosis of mCRPC, when patients have a lower tumor load, hence less immune suppression.

To date, the OS benefit of sipuleucel-T cannot be fully explained by the recorded immune responses. An elevated T-cell stimulation index was observed in the sipuleucel-T treated group. Nevertheless, T-cell proliferation responses to the fusion protein (PA2024) or PAP did not show a survival difference. Increased antibody levels against PA2024 were observed in 66.2% of the sipuleucel-T treated patients and in 2.9% of the placebo-treated patients coinciding with a slight, although not significant, survival benefit ($P = 0.08$). Increased antibody levels against PAP were noticed in 28.5% of the sipuleucel-T treated patients and in 1.4% of the placebo-treated patients, not correlating with survival (41). Research is currently ongoing to define additional biomarkers that could be related to increased overall survival.

To conclude, sipuleucel-T is the first autologous cellular immunotherapy for the treatment of PCa. Three phase III trials demonstrated crucial clinical evidence for the worthiness of sipuleucel-T. However, although an increase in overall survival of 4 months is beneficial for the patients, it is not the breakthrough for immunotherapy many researchers were hoping for. Cellular immunotherapy might not be a monotherapeutic alternative for PCa. Instead, combination with standard or novel treatment modalities might be decisive. Currently ongoing trials are focusing on combination therapies with androgen deprivation therapy, chemotherapy, and immune checkpoint inhibitor antibodies (**Table 2**).

PROSTATE-SPECIFIC MEMBRANE ANTIGEN

Prostate-specific membrane antigen, also known as glutamate carboxypeptidase II, is a zinc metalloenzyme with folate hydrolase activity that is expressed in membranes of prostate epithelial cells (87, 88). Its function in the prostate is still unknown. Low expression of PSMA is also found in the kidneys, salivary glands, duodenum, and the central and peripheral nervous system.

PSMA as tumor antigen

Prostate-specific membrane antigen is highly overexpressed in PCa and increased expression correlates with advanced disease and metastasis (89–91). It has also been shown that PSMA is involved in tumor angiogenesis of many solid tumors, and it is expressed in the endothelial lumen in tumors. Normal vascular endothelium in non-cancerous tissue is PSMA negative (92, 93). PSMA displays several features that qualify it as a suitable target for immunotherapy. In addition to its specific expression in the prostate, it is also a membrane-bound antigen that is presented on the cell surface, but not released into the circulation (94). PSMA has been exploited as a possible target for PCa treatment in different pre-clinical settings and in early-stage clinical trials (42, 43, 88, 95). Wolf et al. showed that the recombinant anti-PSMA-specific single-chain immunotoxin D7-PE40 was both specific and highly toxic for PSMA-expressing PCa cells *in vitro* and *in vivo* in prostate tumor-bearing mice (88). Usage of the ^{177}lutetium radiolabeled anti-PSMA monoclonal antibody J591 induced a 50% PSA reduction in 4 of the 35 patients with mCRPC (95). A similar PSA decrease was seen in an early clinical trial with PSMA peptide-pulsed DCs, where 9 of 33 patients displayed a partial clinical response (43). However, not all studies targeting PSMA have showed encouraging results. The PMSA-derived HLA-A2-restricted peptide (LLHETDSAV) appeared to be poorly immunogenic compared with other HLA-A2-restricted peptides, both *in vitro* as well as in patients with PCa (42). This underscores the importance of pre-clinical studies before clinical testing.

In summary, based on the highly specific expression pattern of PSMA in patients with PCa, PSMA poses as a suitable target for immunotherapy. However, early clinical trials have shown varying results. Further research concerning PSMA-based immunotherapy is warranted. **Table 2** shows several ongoing clinical studies targeting PSMA as a tumor antigen.

PROSTATE STEM-CELL ANTIGEN

Prostate stem-cell antigen (PSCA) is a glycosylphosphatidylinositol (GPI)-anchored protein expressed on the cell surface of both basal and luminal cells in the normal prostate, but overexpressed by PCa cells (44, 96). It is shown that PSCA, like other GPI-anchored proteins, is involved in the survival of stem cells, in T-cell activation and proliferation, and in cytokine and growth factor responses (97, 98). Furthermore, several studies have connected the Ly-6 family of PSCA-like GPI-anchored proteins to tumor growth and metastazation (99–102).

PSCA as tumor antigen

Its distinct expression pattern and possible function in tumor-progression makes PSCA an interesting target for immunotherapy. It has already been exploited in several studies, with promising results (45, 103–105). Anti-PSCA monoclonal antibodies have been reported to inhibit tumor growth and prolong the survival of mice bearing human PCa xenografts (46, 106). Additionally, a chaperone complex vaccine made of PSCA and the heat-shock protein GRP170 was shown to enhance T-cell-mediated immune responses, inhibit tumor growth, and prolong the life span of PCa tumor-bearing mice (107). Two DC vaccination studies have been performed in humans (45, 105). In the study by Thomas-Kaskel

et al., patients with mCRPC were treated with DCs loaded with PSCA and PSA peptides. Endpoints were safety and induction of antigen-specific immunity. The vaccine was well tolerated in all patients, and 6 of 12 patients showed stable disease after four vaccinations. One patient had a complete response. Interestingly, this patient displayed an increase in serum PSA levels. Positive delayed-type hypersensitivity skin reactions were seen in four patients after four vaccinations. A positive delayed-type hypersensitivity test was associated with increased overall survival. HLA tetramer analysis detected high frequencies of peptide-specific T cells in one patient, who had an overall survival of 27 months (105). In another study, vaccinations were performed in three patients with mCRPC using multi-epitope (PSCA, PSMA, PAP, and PSA) pulsed DCs. The treatment was well tolerated, and significant CTLs responses against all PSAs were observed. In addition, DC vaccination was associated with an increase in PSA doubling time (45).

To conclude, PSCA has been used as a target for antigen-based immunotherapy in several clinical studies due to its role in tumor growth and metastases. Unfortunately, the study results were less impressive than expected. This might be the reason that to date there is no ongoing clinical trial with PSCA registered.

MUCIN-1

The mucin family members include proteins that enclose tandem repeat structures with a high proportion of prolines, threonines, and serines. The family consists of secreted and transmembrane forms, designated Mucin-1 (MUC-1) to MUC-21 (108). MUC-1 is a large cell surface glycoprotein found on the apical surface of most glandular and ductal epithelial cells, such as the lungs, intestines, and the prostate (109). In chronic inflammation, MUC-1 expression is induced by inflammatory cytokines like TNF-α, IFNγ, and IL-6. Overexpression contributes to oncogenesis by activation of growth and survival pathways (Wnt-β-catenin and nuclear factor-κB pathways), promoting receptor tyrosine kinase signaling and downregulation of stress-induced death pathways (108). MUC-1 overexpression is associated with colon, breast, lung, prostate, and pancreatic cancer. Moreover, it is associated with tumor-progression and correlated with advanced disease (110–112). MUC-1 has also been shown to have immunosuppressive effects in mice, and secreted MUC-1 has been shown to block T-cell activation (113, 114). Moreover, human monocyte-derived DCs cultured *in vitro* with MUC-1 peptide displayed a decreased expression of both co-stimulatory molecules and antigen presenting molecules upon activation (115). Similarly, depletion of soluble MUC-1 in tumor cell line supernatants abolished the anti-proliferative effect of these supernatants on T cells, and MUC-1 has therefore been identified as a target in PCa (116). The inhibitory effect of MUC-1 has also been demonstrated *in vivo*, when synthetic MUC-1 decreased the immune response in patients vaccinated with an MUC-1 containing polyvalent peptide vaccine (117). In a recent phase I/II trial, an autologous DC vaccine loaded with an MUC-1 glycoproteine and KLH in patients with CRPC was studied. Patients received three injections biweekly followed by booster vaccinations at 6 and 12 months. The rate of PSA rise decreased in six of seven patients. The PSA doubling time increased from a median of 2.9 months prior to vaccination to 7.5 months during vaccination (118). Richman et al. also showed clinical benefit for some patients with mCRPC treated with the combination of radioimmunotherapy with an anti-MUC-1 monoclonal antibody and paclitaxel (47) (**Table 1**).

Taken together, MUC-1 is important in tumor-progression and therefore a very interesting tumor-associated antigen. Several trials focusing on MUC-1 as a target for cancer immunotherapy in PCa are ongoing (**Table 2**).

CANCER/TESTIS ANTIGENS

Cancer/testis antigens are normally only expressed in gametogenic tissue. However, this group of proteins is aberrantly expressed in several types of cancers, including PCa (48). CTAs have been shown to contribute to tumor formation and progression (119, 120). The CTAs NY-ESO-1, the MAGE family, and A-kinase Anchor Proteins (AKAP)-4 will be discussed here.

NY-ESO-1 is found to be expressed in a variety of malignancies. It is not expressed in normal adult tissue, with the exception of the testis. The expression of NY-ESO-1 is associated with level of disease, and higher NY-ESO mRNA and protein expression are observed in metastatic and advanced PCa, as compared to localized tumors (120–124). The function of NY-ESO-1 is unknown, but it is speculated to play a role in meiosis or in the assembly of the organelles that develops over the anterior half of the head in the spermatozoa (125, 126). The NY-ESO-1 is a promising candidate because of its tumor-restricted expression and the identification as one of the most immunogenic CTAs, eliciting spontaneous cytotoxic and antibody-mediated immune responses in patients with NY-ESO-1 + tumors (127–129). Humoral responses against NY-ESO-1 have been evoked by non-specific immune activation in patients with mCRPC treated with a combination of checkpoint inhibitor ipilimumab and GM-CSF, underscoring its immunogenicity (130). NY-ESO-1 has been used as target antigen in several clinical studies. Both MHC class I and II restricted T-cell epitopes specific for NY-ESO-1 are identified (131). MHC class I and/or II restricted NY-ESO-1 peptides were compared in a peptide-based vaccine trial in patients with mCRPC. The vaccine increased the PSA doubling time and yielded antigen-specific T-cell responses in all patients treated. The strongest results were seen in chemo-naive patients, most likely due to a lower tumor burden, thus less tumor-induced immune suppression (132). The immunogenic features of NY-ESO-1 are further supported by a study using a protein-based vaccine with CpG as an adjuvant. This vaccine was able to prime antigen-specific B-cell responses and induced NY-ESO-1 specific, tumor-reactive CTLs in patients with metastatic PCa, independently of autologous NY-ESO-1 expression (49). Vaccination against a tumor-specific protein without it being present, repositions this clinical vaccination protocol toward a preventive setting.

Second, the MAGE CTA subfamily is also expressed in PCa. Upregulation of these CTAs is found in CRPC and is associated with resistance to chemotherapeutic agents (50). MAGE-A2 downregulates p53 transactivation function through histone deacetylase recruitment, a possible explanation how MAGE-A2 expression leads to resistance to chemotherapy (50). Indeed, silencing of MAGE-A2 increased sensitivity to docetaxel chemotherapy in PCa tumor cells (120). Expression of MAGE-C2/CT10, another member of the MAGE-A subfamily, is

correlated with the degree of PCa malignancy. It is an indication of higher risk for biochemical recurrence after radical prostatectomy and represents a potential target for immunotherapy (133). Members of the MAGE-A subfamily and NY-ESO-1 are often co-expressed in prostate malignancies.

Third, the CTA AKAP are a family of scaffolding proteins capable of controlling intracellular signals. AKAP is involved in cytoskeletal regulation and organization by affecting cyclic AMP-dependent protein kinase-A (134). In the prostate epithelium, the anchor proteins synthesize and secrete calcitonin. It has been shown that the calcitonin secretion from malignant prostates is several-fold higher than from benign prostates (135). The calcitonin receptor is expressed in malignant PCa, and its activation stimulates growth of PCa cells via activation of cyclic AMP as well as protein kinase C (136, 137). These mechanisms suggest a marked increase in the invasiveness of PCa cells (138). Modulation of protein kinase-A activation possibly interferes with the growth, tumor genicity, and metastatic potential of advanced tumors. First, AKAP-4 has been showed to be an immunogenic CTA in patients with multiple myeloma (139). Later, Chiriva-Internati et al. showed cytoplasmic and surface expression of AKAP-4 in the LnCAP PCa cell line. AKAP-4 expression in the prostate epithelial cells was shown in 13 of 15 patients with PCa, but not in healthy subjects. Cytotoxicity assays showed that AKAP-4-loaded DC-stimulated T cells were capable of killing autologous PCa cells *in vitro*. Neither killing of AKAP-4 negative PCa cells nor normal prostate epithelial cells was observed. This underscores the antigen specificity of the response and prevention of autoimmune reactions (140). This makes AKAP-4 a very interesting target for PCa anti-tumor vaccination.

To conclude, several CTAs, especially NY-ESO-1, the MAGE-A subfamily, and AKAP-4, could serve as therapeutic targets in the fight against PCa (120, 122, 140). Especially NY-ESO-1 is of major relevance in PCa and a target in different ongoing trials (see **Table 2**). Due to the tumor-restricted expression of CTAs, these antigens can also be used in an adjuvant or a preventive setting hindering the recurrence of CTA-positive tumors (49).

MIXTURE OF TUMOR-ASSOCIATED ANTIGENS

To date, many investigators underscore the importance of a personalized approach by selecting patient-specific mutations as target antigens for immunotherapy. The group of Noguchi took a first step in a personalized direction. They performed two phase II studies with a personalized peptide vaccine (PPV). The vaccine consisted of four peptides based on each patient's immunoreactivity profile. Peptides of a variety of tumor-associated antigens were tested, including PSA, PAP, PSMA, multidrug resistance protein, and a choice of different epithelial tumor antigens. The peptides included in the vaccine were selected on their capacity to induce CTL responses. In the first phase II trial, patients with CRPC were randomized to PPV combined with chemotherapy or chemotherapy only (141). Antibody responses were seen in 64% of the patients and cytotoxic T-cells responses in 72% of the patients. An increase in progression-free survival was observed in the PPV/chemotherapy group as compared with the patients who only received chemotherapy. However, immune responses did not correlate with clinical outcome in patients treated with PPV and chemotherapy. Interestingly, the authors found that lower levels of IL-6 before PPV vaccination were favorable for overall survival. IL-6 have been associated with more aggressive cancer progression and decreased survival in PCa (142). In this perspective, IL-6 may be seen as an indicator of prognosis and a predictor of therapy effectiveness. It is also hypothesized that inhibiting IL-6 signaling may be beneficial in patients enduring other immunotherapeutic treatment.

The results of the PPV vaccinations are promising. A randomized trial with an appropriate control group before and after chemotherapeutic treatment is needed to fully identify a clinical benefit of PPV treatment. Currently, a vaccine consisting of 20 peptides is applied to patients with CRPC in an exploratory, randomized, open-label study (UMIN000008209, **Table 2**).

PROSTATE CANCER CELL LINES

GVAX

GVAX is an allogeneic, cell-based immunotherapy consisting of the PCa cell lines LNCaP and PC-3. These cell lines are genetically modified with a recombinant GM-CSF adeno-based viral vector and irradiated before administration. Clinical results in patients with PCa are indicative for a favorable clinical outcome with no toxicities (143, 144). These results have led to phase III trials, using the most promising high-dose GVAX protocol. Unfortunately, due to an even increased mortality in the GVAX-treated group, and disappointing interim results the trials were abrogated (145, 146).

van den Eertwegh et al. combined the immune checkpoint inhibitor ipilimumab (anti-CTLA-4) with GVAX (147). More than 50% decline in PSA level was seen in 25% of the patients. All the responding patients got 3.0 or 5.0 mg/kg ipilimumab. There was dose-limiting toxicity in the 5.0 mg/kg group of patients, while the lower ipilimumab regimens were well tolerated. Markedly, all patients with immune-related adverse events showed a decrease in PSA levels. A small number of patients additionally displayed an anti-PSMA antibody response. These patients had a significant increase in median overall survival (46.5 months compared to 20.6 months for patients without this humoral response). T-cell monitoring studies were performed in 28 patients receiving the combination therapy of GVAX and ipilimumab. Compared with the control group, an increase in absolute lymphocyte counts and enhanced CD4+ and CD8+ T-cell differentiation was observed. These immune responses were associated with a significantly prolonged overall survival. In addition, an OS benefit was also seen in case of high pre-treatment levels of CD4+, CTLA-4+, CD4+/PD-1+, or non-naïve CD8+ T cells. Low pre-treatment frequencies of differentiated CD4+ or regulatory T cells resulted in a prolonged OS (148). This reveals perspectives for future biomarker research.

mRNA-TRANSFECTED DCs

An alternative approach for PCa cell lines is the use of PCa cell line-derived RNA or tumor antigen encoding mRNA. Kyte et al. transfected monocyte-derived DCs with mRNA derived from the PCa cell lines LnCAP, DU-145, and PC-3. Although the generation of mRNA-transfected DC is challenging, DC vaccination appeared feasible and safe (149, 150). Furthermore, PSA-specific T-cell responses were detected in 12 of 19 patients with PCa who underwent mRNA-DC vaccination (149). To date, patients are

recruited in a phase I/II trial (NCT01197625, **Table 2**), studying the mRNA-transfected DCs in curative resected patients with PCa. More studies are needed to properly determine the strength of mRNA-transfected DCs. The usage of this immunotherapeutic modality within combination therapies might be of greater significance.

DISCUSSION

In this review, we provided overview of PCa tumor-associated antigens and how they are used to target PCa via immunotherapy (**Table 1**). PSMA and PSCA are normally expressed in the prostate gland but upregulated during cancer development and they may play a role in tumor progression (44, 89, 96, 151). Increased serum levels of secreted tumor antigens, such as PSA and PAP, can be used as biomarkers for disease and disease progression (51, 73, 74). More general tumor antigens, like MUC-1, AKAP-4, and NY-ESO-1, can also be found in PCa and might be candidates for immunotherapeutic interventions (111, 123, 140). MUC-1 is expressed in normal tissue and upregulated on several tumors, where it can exert immunosuppressive effects and attain tumor growth (110). Hence, targeting MUC-1 could have a dual role – directing the immune response toward the tumor and reducing immune suppression. This might also be valid for other immunosuppressive antigens, such as the MAGE-A subfamily or PSA (54, 120). On the other hand, the NY-ESO-1 antigen is often immunogenic *per se*, and pre-existing immune responses directed against this antigen are common in treatment-naive patients (128). Pre-existing CTL responses against PSA and PAP in healthy individuals and patients with chronic prostatitis also support the definition of PCa as an immunogenic tumor (26, 27), where tolerance against self-antigens can be broken and the immune system can be harnessed against the tumors.

Today, the only registered product for antigen-targeted immunotherapy in PCa is sipuleucel-T (38, 39, 41). Although the significance of this intervention received criticism, sipuleucel-T proves an important point: autologous cellular immunotherapy is feasible and can indeed be developed as an approved treatment modality. To date, no convincing mechanism of action has been elucidated for sipuleucel-T. Increased immune responses were observed but no correlation with clinical outcome could be established. Clinical studies aiming at identifying immunological responses and thereby hopefully providing an in-depth understanding of the mode of action of sipulecuel-T are ongoing. Unraveling the mechanism might be beneficial for further development of sipuleucel-T and other immunotherapeutic approaches.

Effective immune responses induced by immunotherapeutic treatments are still not common, and probably vary depending on tumor type, somatic differences between tumor cells, and the tumor microenvironment (66). Several recent trials have shown promising results in both clinical and immunological responses. Constructs targeting the NY-ESO-1 antigen has led to significant immunological responses, which makes NY-ESO-1 an interesting antigen to target immunotherapeutic strategies in future (49, 132). Immunological responses are also induced by several PSA-targeting vaccines, supporting the usage of PSA as an immunogenic tumor antigen (34–36).

Insight in the localization of the tumor antigen (on/in cells, normal cells vs. tumor cells, in organs) and the specificity of the antigen facilitates a precise selection of target antigens with the intention of optimizing the translation of immunotherapeutic treatments to the clinic. However, despite significant T-cell responses, tumor progression is seen most frequently in patients treated with cancer immunotherapy. This is due to the complexity of human beings and the complexity of tumors and metastases (152). The complexity of cancer is also described by Fox et al. (153). This report of the collaborating immunotherapy organizations, known as the Society for Immunotherapy of Cancer (SITC), contains the identification of nine hurdles in cancer immunotherapy that significantly delays clinical translation of promising cancer immunotherapeutics. We here discuss the hurdles relevant for this review, for a complete overview, we refer to the original article (153). The first hurdle to overcome is the complexity of cancer, tumor heterogeneity, and immune escape. The immune signature of the tumor, distinguished by genetic or histological evaluation, can predict responders to cancer immunotherapy (154, 155). The second relevant SITC hurdle for this review is the lack of definitive biomarkers for the assessment of clinical efficacy of cancer immunotherapies. Biomarkers to distinguish between patients responsive to initial treatment, patients displaying immune inhibitory features, and patients with non-immunogenic tumors, are needed. Pre-existing anti-tumor responses or the expression of inhibitory markers are examples of suggested biomarkers that could be used to predict treatment outcome and individualize the treatment regime.

A correlation of immune parameters with clinical outcome after immunotherapy is not established in patients with PCa. This can be attributed to (i) a limited number of patients per immunotherapeutic approach; (ii) a variation in clinical features of patients with PCa before treatment; and (iii) the difference in clinical signs of tumor control between conventional toxic treatments and immunotherapeutic treatments. This last argument is also one of the hurdles identified by the SITC. Effective immunotherapy does not always display initial shrinkage of the tumor, but rather a pattern of tumor growth and progression followed by shrinkage when the tumor is recognized and destroyed by the immune system (156–158). This paradox has been illustrated by the negative outcomes on progression-free survival or PSA responses in the sipuleucel-T trials and PSA-TRICOM trial. Tumor swelling, increased release of PSA due to elevated tumor cell death, and initial detrimental symptoms might be associated with a favorable clinical outcome rather than with progressive disease, as stated in the WHO and Response Evaluation Criteria In Solid Tumors (RECIST) criteria (156, 157, 159). Although clinically responding patients might have been missed, some patients do not respond, neither clinically nor immunologically. Lack of immunogenicity of the antigens used might be an explanation, but a major factor is the immunosuppressive networks within cancer patients. Infiltrating lymphocytes can be regulated by a number of inhibitory pathways within the tumor and thereby shift the direction of the ongoing immune response toward a more tolerogenic one. Other patients might have "silent" tumors that do not display an inflammatory phenotype and hence do not attract lymphocyte infiltration (66).

Novel monoclonal antibodies targeted against inhibitory receptors on T cells (anti-CTLA-4 and anti-PD-1) are able to prolong their effector functions and prevent immune inhibition. These treatment strategies are tested in combination with other immunotherapeutic approaches and showed promising results in a subset of patients (61, 147, 160). There are still ongoing combination therapy studies with ipilimumab and sipuleucel-T which will hopefully overcome the immunosuppressive signals provided by immune evading tumors (NCT01804465 and NCT 01832870). Although antigen-based immunotherapy itself seldom gives rise to severe autoimmune reactions, the combination with an immune checkpoint inhibitor likely will enhance the risk of immune-related adverse events, as recently shown in melanoma patients treated with ipilimumab (161–163).

FUTURE CHALLENGES AND OPPORTUNITIES

Until recently, patients with mCRPC had limited treatment options and a poor prognosis. With new sequential hormonal therapies, second-line chemotherapy, and new immunotherapeutic strategies, a new era has started. To date, PCa is one of the few tumor types in which immunotherapy is part of the current standard of care. Augmenting immune responses to PCa antigens is a valid therapeutic approach, and clinical responses with minimal toxic effects are observed.

In this review, we focused on commonly expressed tumor-associated antigens. Recently, patient-specific epitopes are identified as highly important to improve T-cell reactivity. Targeting these patient- and cancer-specific mutated epitopes holds promise for even better results and possibly cure of patients. By complete genome and transcriptome, sequencing and mass spectrometry-mutated HLA-binding peptides, so-called neoantigens, might be identified (164). Vaccination with individually overexpressed tumor-specific peptides could result in a unique, personalized anti-cancer vaccine (165–167). Recently, the first two demonstrations of autologous cancer exome-based T-cell responses against patient-specific neoantigens in humans were published (167, 168). This knowledge is a major step forward for both the identification of new diagnostic strategies by tumor exome analysis, as well as for the development of individualized immunotherapeutic approaches. Combination therapies harboring these patient-specific peptide vaccinations together with immune checkpoint inhibitors are likely to generate an even better immune control. There is no doubt that these are very exciting times for cancer immunotherapy.

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Gut microbiota and the paradox of cancer immunotherapy

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It is recently shown that beneficial environmental microbes stimulate integrated immune and neuroendocrine factors throughout the body, consequently modulating regulatory T-lymphocyte phenotypes, maintaining systemic immune balance, and determining the fate of preneoplastic lesions toward regression while sustaining whole body good health. Stimulated by a gut microbiota-centric systemic homeostasis hypothesis, we set out to explore the influence of the gut microbiome to explain the paradoxical roles of regulatory T-lymphocytes in cancer development and growth. This paradigm shift places cancer prevention and treatment into a new broader context of holobiont engineering to cultivate a tumor-suppressive macroenvironment.

Keywords: tumor macroenvironment, regulatory T-cells, cancer immunotherapy, inflammation and cancer, probiotic bacteria

INTRODUCTION

The neoplastic process is characterized by overwhelming complexity. Cancer is comprised of a genetically unstable population of cells that proliferate at an extraordinarily high rate. Millions of cancer deaths each year make it obvious that the battle against cancer is asymmetric, with humankind often being the weaker element (1). To date, cancer research efforts directly confront malignancy by targeting properties of individual cancer cells. In 2000, Hanahan and Weinberg described that most of the research on origins and treatment of cancer had just contributed toward "adding further layers of complexity to a scientific literature that is already complex almost beyond measure" (2).

In the same landmark paper, however, the authors were optimistic enough to predict groundbreaking upcoming advances in the conceptual rather than the technical level (2). They were proven right. One such advancement was the increased awareness for the importance of the tumor microenvironment in the etiopathogenesis of neoplasia (3, 4). We now know that initially transformed cells are much less autonomous in their growth than previously thought (5, 6). Among the microenvironment elements, immune cells and factors have emerged as fundamental players (4–6). Accumulating evidence suggests that tumor-associated inflammatory cell accumulation, whether overt or smoldering, could be viewed as a tumor-promoting event (7–9). These inflammatory responses enhance mutagenesis by oxidative DNA damage and shape the tumor stroma in favor of cancer cell survival and expansion (6, 10, 11).

Will this knowledge base in the field of inflammation, immunity, and cancer lead to new, highly effective, and biologically safe cancer immunotherapy modalities? We assert that the outcome will depend upon the philosophy and the strategic goals that will dominate the bench-to-bedside research. We propose that research in this field should focus upon stimulating systemic innate immune balance and adaptive immune resiliency, making the mammalian host more powerful to resist its cancer challenger. One possible approach utilizes gut microbiota or microbial antigens to stimulate beneficial immune cells. On the other hand, existing immunotherapy aims to selectively interrupt immune factors to better recognize and exterminate cancer cells (12–17), an approach that may ultimately lead to host instability. To further explain this point of view, we will refer to the recently discovered paradoxical roles of regulatory T-cells (T_{REG}) in cancer (10, 14).

T_{REG} ARE CENTRAL IN PRESERVING SYSTEMIC IMMUNE HOMEOSTASIS AND GOOD HEALTH

$FOXP3^+$ $CD4^+$ $CD25^{+/high}$ T_{REG} are dominant cellular elements of the professional suppressor arm of the immune system and are important for orchestrating the control of peripheral immunological tolerance (18). The transcription factor FOXP3 is a fundamental regulator of T_{REG} function in rodents and humans, and so far the most reliable phenotypic indicator of their identity. Recent studies on human T_{REG} subpopulations, however, revealed that low but discernible levels of FOXP3 expression could be detected in non-suppressive T_{REG} or even in activated effector T-cells. It is probable that this finding reflects the inherent plasticity of T_{REG}; $FOXP3^+$ cells co-expressing effector T-cell phenotypic markers or cytokines may be in stages of a progressive, epigenetically regulated, phenotypical, and functional shift process (14, 16, 19–22), ultimately favorable for healthful recovery of the host after environmental challenges. The role of T_{REG} is central in preserving immune system homeostasis for health and the balance of beneficial inflammatory responses during infections while minimizing collateral tissue damage. In cancer, however, roles of T_{REG} are traditionally considered to be negative (14–16, 23).

T_{REG} GATHER NEAR TUMORS AND FAVOR CANCER SURVIVAL

A large body of data suggest that T_{REG} gather near tumors and suppress the anti-tumor inflammatory response, thus favoring cancer

cell survival. To this end, tumor-associated T_{REG} are thought as a major impediment of anti-tumor vaccines (13–16, 23). Clinical and experimental data suggest that tumor-associated T_{REG} recognize both self and neoantigens expressed by tumor cells, counteracting antigen-specific effector T-cell responses. Consequently, immunotherapy strategies based on the vaccination with tumor-associated antigens fail to evoke an effective response against cancer cells due to the activation and expansion of tumor antigen-specific T_{REG} (14–16). This potential interplay of T_{REG} within tumors has been reviewed in detail elsewhere (12–16), and has led to the proposal of several anti-T_{REG} regimens for cancer immunotherapy. These regimens aim to deplete T_{REG}, inhibit their suppressive function, prevent their homing into tumor sites, or block their differentiation/proliferation (12–16).

Several of these T_{REG}-targeting modalities have already been tested in the clinic, with mixed results (13, 16). Blocking T_{REG} function by depleting the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) appears promising (24), due to the depletion of T_{REG} from tumor tissues (25, 26). However, a similar regimen could lead to an opposite effect with the accumulation of T_{REG} and $CD8^+$ T-cells in tumors (27, 28). A phase III study of melanoma patients using a gp100 peptide vaccine with interleukin (IL)-2 administration led to equally promising results with discovery of T_{REG} expansion in responding patients (29).

GUT MICROBIOTA INDUCE POTENT T_{REG} WITH SYSTEMIC ANTI-NEOPLASTIC PROPERTIES

As the results of these trials are anticipated, the literature reveals contradictory evidence. Indeed, the studies associating high densities of tumor-associated cells expressing T_{REG} markers including FOXP3 with a poor prognosis in several types of human cancers are now challenged by similar studies on the very same types of cancer showing the opposite outcome (30–34). The different $CD8^+$:T_{REG} ratios and the presence of $FOXP3^+$ cell subsets of undetermined identity in the tumor microenvironment have been proposed as probable explanations (16). Indeed, data from animal models show under certain conditions of microbial priming that T_{REG} not only protect but also alter the tumor microenvironment to induce remission of already established intestinal, mammary, and prostate cancers (35–41). The hypothesis that the composition of the different subsets of $FOXP3^+$, which may include effector $Foxp3^+$ cells, is intriguing (16). Indeed, it was previously shown that IFN-γ levels were increased during T_{REG}-mediated tumor regression in mice (37). Further, feeding of probiotic microbes to mice induces systemic oxytocin secretion that shifts immunity toward IFN-γ and CD25 for improved wound healing capacity and systemic good health (42). A question subsequently arising is whether gut microbiota may be engineered to harness an anti-neoplastic $FOXP3^+$ cell milieu (5, 10, 41).

GUT-CENTRIC HYPOTHESIS: PRIOR EXPOSURES TO MICROBES EXPLAIN BENEFICIAL ROLES OF T_{REG}

Stimulated by a gut-centric systemic homeostasis hypothesis, we set out to explore and explain the paradoxical roles of T_{REG} in cancer using several different mouse models of cancer and adoptive cell transfer methodologies (10). We found that T_{REG} may suppress, promote, or have no effect in carcinogenesis depending upon their timing and prior exposure to gut bacterial antigens and presence of IL-10 (35–39, 41, 43, 44). Under some conditions, adoptive transfer of T_{REG} rapidly led to apoptosis of emerging tumor cells (37, 45). Using as a model organism an opportunistic pathogen, *Helicobacter hepaticus*, commonly residing in the lower bowel of mice, we have shown in Rag2-deficient mice (otherwise lacking lymphocytes) that gut microbiota modulate inflammatory bowel disease and inflammation-associated colon cancer, a cancer process inhibited by properly functioning IL-10-dependent T_{REG} (35, 36). Subsequently, by introducing *H. hepaticus* into the large bowel flora of mice lacking the APC tumor suppressor gene ($Apc^{Min/+}$), we found that intestinal polypogenesis was greatly enhanced by bacteria and subsequently suppressed by immune-competent T_{REG}. Furthermore, adenomas of infected $Apc^{Min/+}$ mice progressed into adenocarcinoma, a transition atypical of polyps of aged-matched uninfected controls (38, 41). Interestingly, $Apc^{Min/+}$ mice having *H. hepaticus* in their gut flora were prone to develop cancer in tissues distant from intestine, such as prostate and the mammary glands (40, 41, 43, 46, 47). *H. hepaticus*-induced tumorigenic events were inhibited by supplementation with T_{REG} from immune-competent wild type donor mice.

A potent treatment to counteract these local and systemic *H. hepaticus*-induced tumorigenic events was supplementation with T_{REG} in an IL-10-dependent manner (10, 36, 38–40, 44, 46, 48). Purified T_{REG} exhibited greatest anti-cancer potency when taken from donor mice previously colonized with *H. hepaticus*. By contrast, T_{REG} taken from donor mice without prior *H. hepaticus* exposure were ineffective, and in some cases actually enhanced tumorigenesis (10). Based on these results, we theorize that the tumor microenvironment is subject to systemic inflammatory events arising from environmental exposures in the gastrointestinal tract (**Figure 1**). This microbe-inducible pro-inflammatory condition contributes to tumor trophic signaling. Interestingly, bacterial antigen triggered IL-10-dependent activities in the GI-tract impart sustained protection from the aforementioned events, resulting in immune cell recruitment, including T_{REG}, which, by being more potent in their anti-inflammatory roles, work locally and systemically to suppress sepsis, myeloid precursor mobilization, and inflammatory signaling important in extra-intestinal cancer evolution (10, 43). These systemic events comprise the tumor macroenvironment.

The roles of intestinal microflora in promoting cancer development within the bowel have been well established (35, 49–52). Linking gut microbial flora and local and systemic effects that promote (38) or suppress (45) tumors throughout the body, expands this paradigm in a challenging manner. Recent findings show that gut flora imbalances considerably undermine the response to both immune (53, 54) and non-immune chemotherapeutic regimens, such as cisplatin and oxaliplatin (53).

A WEAKENED T_{REG} FEEDBACK LOOP UNIFIES AUTOIMMUNE DISEASES AND CANCER

These gut microbe-centric findings in mice are consistent with the "hygiene hypothesis," according to which insufficient microbial exposures earlier in life predispose to allergies, autoimmune disorders, and uncontrollable inflammation-associated pathologies later in life. We have shown that the basic principles of this

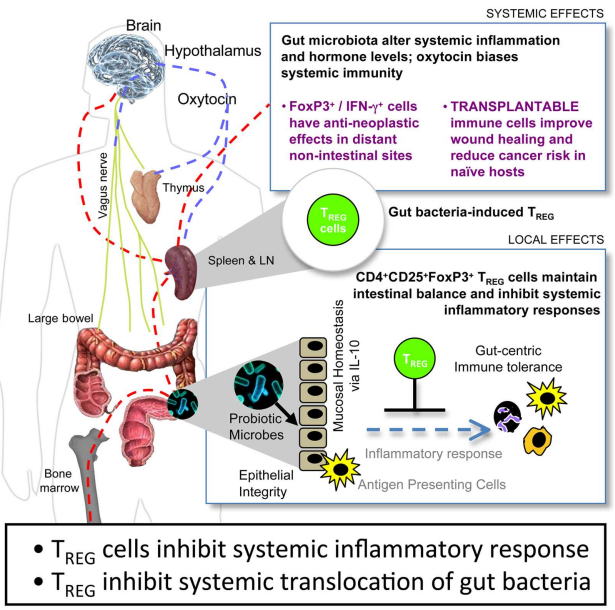

FIGURE 1 | Gut bacteria–host crosstalk is continuous and reciprocal in the cancer macroenvironment. Beneficial microbes trigger IL-10-mediated GI-tract immune and neuronal networks that lower systemic inflammatory tone and up-regulate hypothalamic–hypophyseal targets, including oxytocin, constituting a gut–systemic immunity-endocrine-axis. In this way, microbiota stimulate $CD4^+$ lymphocytes including regulatory T cells (T_{REG}) that suppress, promote, or have no effect in carcinogenesis depending upon their timing and prior exposure to gut bacterial antigens and presence of interleukin (IL)-10. This places neoplastic development and growth into a new broader context of the holobiont (comprised of the mammalian host plus resident microbes) and the cancer macroenvironment, highlighting microbes that may be engineered for sustained good health.

hypothesis may apply not only to auto-immunity, but also to neoplastic disease as well, and that T_{REG} play a central role in this phenomenon (10, 41, 55). The ability of T_{REG} to decrease risk for cancer and counteract established tumors depends upon microbe-triggered IL-10, which works to maintain immune system homeostasis and reinforce a protective anti-inflammatory, anti-neoplastic T_{REG} phenotype (41). T_{REG} display inherent phenotypic plasticity (10). Hygienic individuals with a weakened IL-10 and T_{REG} feedback loop are prone to a re-direction of unstable resting peripheral T_{REG} toward a T helper (Th)-17 pro-inflammatory process. As a result "hygienic" subjects are at higher risk to develop auto-immune diseases and cancer (10). It is tempting to postulate that this may explain why only a few people go on to develop cancer, while nearly everyone bears dysplastic and early neoplastic lesions throughout their body (56).

Depending on composition of gut microbiota, the immune system of mice may acquire different subclinical characteristics, even in the absence of overt inflammatory processes. The clinically silent immune system status may determine the risk of developing sporadic cancer in epithelia throughout the body. Further, we found that consuming beneficial probiotic bacteria led to the expansion of a $Foxp3^+$ cell population in the periphery (42, 45, 57) conferring protection to diet-related and genetic predisposition to mammary cancer (45). Targeted oral challenge with such probiotic bacteria resulted in the activation of interrelated systemic inflammatory and metabolic pathways, either through blood circulation or via the vagus nerve (**Figure 1**). Consequently, there was an upregulation of systemic hormone levels, such as oxytocin, testosterone, and thyroxin. Oxytocin serves to sustain immune and integumentary homeostasis, biasing the immune system toward IL-10 and IFN-γ, without anergy, subsequently minimizing the deleterious systemic effects of IL-17 (57). This altered immune system and metabolic profile of mice imparted healthful phenotypes including shiny fur and youthful hair follicle cycling, accelerated skin wound healing capacity, and resistance to diet-induced obesity and senility (42, 47, 57, 58). Through tightly regulated immune activities, competent T_{REG} permit brief beneficial host inflammatory responses to eliminate invading pathogens, and later inhibit chronic deleterious inflammatory tissue damage (43). The results of our wound healing assays further suggest that the probiotic microbe-induced enhancement of the T_{REG}-dominated arm of the immune system did not compromise the ability of mice to respond to invading pathogens (42).

BENEFICIAL SYSTEMIC EFFECTS OF GUT MICROBES ARE TRANSPLANTABLE VIA FOXP3$^+$ T_{REG} INTO NAÏVE HOSTS

Adoptive cell transfer models offer mechanistic insight as these beneficial effects were isolated to bacteria-primed T_{REG} (42, 47, 57–59). In fact, healthful phenotypes were entirely reproducible in naive recipient mice by the adoptive transfer of highly purified T_{REG} derived from probiotic-fed cell donors (42, 57, 59). These results suggest gut microbe-induced crosstalk with the host in a continuous and reciprocal manner. The fate of preneoplastic and neoplastic lesions arising in epithelia throughout the body depends upon this macroenvironment at the whole organism level. Consequently, the tumor macroenvironment is defined as the "holobiont," i.e., the mammalian organism plus the microbial symbionts it bears. The T_{REG} population is a central player of the tumor macroenvironment connecting gut bacteria with reproductive fitness, youthful phenotypes, and anti-neoplastic properties.

MICROBIAL ENGINEERING OFFERS NEW STRATEGIES FOR PUBLIC HEALTH

Taken together, microbial engineering strategies using food-grade bacteria highlight alternative directions in cancer immunotherapy. Modulating beneficial T_{REG} via diet is a biologically safe and efficient approach, originating from genetic programs that have been shaped during the millions of years of co-evolution of mammals with their gut bacteria symbionts. These attributes remain largely inactive in individuals with a modern lifestyle, Westernized dietary habits, and stringent hygiene practices. Awakening these latent T_{REG}-mediated capabilities may provide an alternative avenue to reduce cancer risk at a population level for public health. The perspectives presented here should be considered as an alternative paradigm – not only for fighting cancer – but also for promoting overall good health and longevity.

AUTHOR CONTRIBUTIONS

Theofilos Poutahidis, Markus Kleinewietfeld, and Susan E. Erdman wrote the paper.

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Induced pluripotent stem cells: challenges and opportunities for cancer immunotherapy

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Despite recent advances in cancer treatment over the past 30 years, therapeutic options remain limited and do not always offer a cure for malignancy. Given that tumor-associated antigens (TAA) are, by definition, self-proteins, the need to productively engage autoreactive T cells remains at the heart of strategies for cancer immunotherapy. These have traditionally focused on the administration of autologous monocyte-derived dendritic cells (moDC) pulsed with TAA, or the *ex vivo* expansion and adoptive transfer of tumor-infiltrating lymphocytes (TIL) as a source of TAA-specific cytotoxic T cells (CTL). Although such approaches have shown some efficacy, success has been limited by the poor capacity of moDC to cross present exogenous TAA to the $CD8^+$ T-cell repertoire and the potential for exhaustion of CTL expanded *ex vivo*. Recent advances in induced pluripotency offer opportunities to generate patient-specific stem cell lines with the potential to differentiate *in vitro* into cell types whose properties may help address these issues. Here, we review recent success in the differentiation of NK cells from human induced pluripotent stem (iPS) cells as well as minor subsets of dendritic cells (DCs) with therapeutic potential, including $CD141^+XCR1^+$ DC, capable of cross presenting TAA to naïve $CD8^+$ T cells. Furthermore, we review recent progress in the use of TIL as the starting material for the derivation of iPSC lines, thereby capturing their antigen specificity in a self-renewing stem cell line, from which potentially unlimited numbers of naïve TAA-specific T cells may be differentiated, free of the risks of exhaustion.

Keywords: cancer, immunotherapy, dendritic cell, cytotoxic T cell, NK cell, pluripotency, iPS cell

CANCER AND THE IMMUNE SYSTEM: A HISTORICAL PERSPECTIVE

Although it was Paul Ehrlich who, in 1909, first introduced the concept of the immune system as a means of controlling the incidence of cancer, it was 50 years later, with development of the field of cellular immunology and discovery of the role of the immune system in allograft rejection (1), that this notion first gained traction. In 1970, Burnet and Thomas introduced the concept of immunological surveillance and postulated that the immune system had a mechanism for eliminating potentially dangerous mutated cells and speculated that lymphocytes were actively involved in the process by the recognition of neo-antigens, either unique to the tumor (tumor-specific antigens; TSAs) or shared by other somatic cells (tumor-associated antigens; TAAs) (2, 3). This theory was met with skepticism, due in part, to the observation that the incidence of tumors in immune compromised *nude* mice did not differ substantially from their wild type counterparts (4, 5). These observations were, however, counter-balanced by the discovery that tumors may lack immunogenicity, not due to the absence of TAAs *per se* but rather their inability to activate the immune system (6). This subsequently gave rise to the modified concept of cancer immunoediting (7), which postulates that a developing tumor is under a constant immunological selection pressure, leading either to its elimination, the establishment of a dynamic equilibrium between the tumor and the immune system, or its escape from immune surveillance, resulting in unopposed growth. It is now accepted that one of the hallmarks of cancer is the lack of immune regulation (8) and that certain cancers therefore have the propensity to induce a state of autoimmunity in some individuals. While the underlying mechanisms remain to be clarified, mutations in specific TAAs may increase their immunogenicity, eliciting T and B cell responses that readily cross-react with the wild type protein, expressed in other cell types or anatomical locations. Indeed, a recent study has shown that certain cancer patients develop the chronic autoimmune rheumatic disease, systemic sclerosis (9), illustrating the important concept that immune surveillance harnesses elements of the autoreactive T-cell repertoire to elicit anti-tumor responses, sometimes at the cost of collateral damage to self-tissues: it is the same autoreactive repertoire that cancer immunotherapy seeks to recruit.

APPROACHES TO CANCER IMMUNOTHERAPY

Radiation, chemotherapy, and surgery are the three traditional methods for controlling the spread of cancer, which, although effective, may fail to completely eliminate neoplastic cells or the cancer stem cells that sustain a developing tumor. Additionally, the lack of specificity of these approaches and the damage to otherwise healthy tissues may lead to severe morbidity and, in extreme cases, mortality. Given its inherent specificity, adaptability, and capacity to generate a memory response, cancer immunotherapy

promises to be more effective and durable than classical treatment modalities (10).

Cytokines, such as interleukin-2 (IL-2), interferon-α (IFN-α), and tumor necrosis factor-α (TNF-α) have been used non-specifically to stimulate an anti-tumor response. These cytokines act either by directly inhibiting growth of the tumor cells or by promoting proliferation and sustained cytokine production by T cells and NK cells, thereby increasing their ability to target tumor cells. Some cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), act on antigen-presenting cells (APCs), inducing upregulation of MHC and co-stimulatory molecules, which promote their capacity to activate lymphocytes. A number of cytokines, used singly or in combination, have proven effective in increasing the anti-tumor immune response and have, in recent years, entered clinical trials for the treatment of advanced cancer (11). IL-2 has, for instance, been approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma and renal-cell carcinoma (12). However, given the non-specificity of the approach, low response rates, and toxic side effects, additional understanding of cytokine signaling pathways and their function *in vivo* are still required (11).

The identification of a number of well-defined TAAs and Tissue Specific Antigens (TSAs), along with the development of hybridoma technology (13), has facilitated the production of monoclonal antibodies (mAbs) that either directly target these antigens or block central pathways involved in tumor function. mAbs have, for instance, been used to inhibit molecules such as CTLA-4 and PD-1, since upregulation of their ligands by tumor cells may inhibit T-cell function, enhancing their ability to evade immune surveillance (14, 15). In recent clinical trials of the PD-1-specific mAb, BMS-936558, objective and durable responses were observed in approximately one in four to one in five patients with non-small cell lung cancer, melanoma, or renal-cell cancer. Immunohistochemical staining of tumor specimens prior to the onset of treatment, revealed that intra-tumoral expression of PD-1 ligand (PD-L1) correlated with the induction of anti-tumor responses, providing a means of stratifying patients in order to identify those most likely to respond to treatment and greatly increasing the likely future clinical impact of mAb therapy (16). Although it was the exquisite specificity of mAbs that first earned them the name "magic bullets," response rates remain disappointingly low when used as a single therapy (17–19). One reason for this poor performance may lie in the fact that administration of therapeutic mAbs is inherently passive, failing to generate a memory response. Furthermore, the repeated administration required as a result, may elicit neutralizing anti-idiotypic responses, which greatly limit efficacy.

In contrast, the ultimate aim of cancer immunotherapy is to activate the immune system to recognize the tumor, thereby generating a specific and durable effector T-cell response. In order to achieve this goal, adoptive transfer of TAA-specific T cells has been explored, involving their expansion *ex vivo* and re-administration to the same patients from whom they were originally derived (20). Alternatively, the ability of DCs to present TAAs to T cells has been harnessed to generate an immune response against tumor cells. Whereas adoptive T-cell transfer may generate a burst of T-cell immunity that is short-lived, DC-based vaccines have the potential to induce a sustained immune response with the capacity for subsequent recall (21). It has been shown that DC vaccination, following adoptive T-cell transfer, may further boost anti-tumor responses, suggesting a rationale for combining the two therapeutic strategies (21). In this review, we shall discuss recent experience of harnessing DCs and T cells for cancer immunotherapy and obstacles hindering their success. We shall also focus on the emerging use of patient-specific induced pluripotent stem cells (iPSCs) for the differentiation of DCs, T cells, and NK cells and discuss how this novel source holds promise for overcoming some of the shortfalls of conventional cancer immunotherapy.

CELL-BASED IMMUNOTHERAPIES AND THEIR OBSTACLES

As sentinels of the immune system, DCs play a pivotal role in eliciting the primary immune response to antigen. The ability of DCs to process and present protein antigens via the canonical endocytic pathway is crucial to this process (22). However, some subsets of DCs also possess the ability to capture exogenous antigens and cross present them via MHC class I direct to $CD8^+$ T cells, thereby eliciting a cytotoxic T-cell response (23–25). Since cancer cells are poor APCs due to constitutively low expression of MHC class I and II determinants, the generation of protective anti-tumor immunity depends upon the cross presentation of tumor antigens by DCs (22–24). Although various DC-based cancer vaccines have been exploited in the past and the properties, advantages, and disadvantages of each extensively reviewed (20, 26, 27), it is most commonly DCs cultured *ex vivo* from peripheral blood monocytes (monocyte-derived dendritic cells, moDCs) that have shown the greatest clinical benefit. Indeed, one such vaccine, Provenge, has entered the market for the routine treatment of prostate cancer and involves the co-culture of moDCs obtained by leukapheresis, with the TSA, prostatic acid phosphatase (PAP), to which T-cell responses have been detected following their reinfusion into patients (28).

The majority of the clinical trials involving cancer vaccination have used autologous moDCs cultured *ex vivo* and pulsed with soluble TAA before re-administration to patients with the hope of inducing a tumor-specific immune response (29). Although many of these trials have shown that immunotherapy based on the use of mature moDC is safe, well-tolerated, and able to elicit an immune response against the tumor (27), the overall results have been disappointing, showing significant inter-trial variability between outcomes (30–32). This may be due, in no small part, to the donor-to-donor variability in yield and quality of moDCs, which is further compounded by long-term exposure to chemotherapeutic agents. Furthermore, the poor capacity of moDCs to cross present exogenous TAAs to CTLs, limits the magnitude of the cell-mediated immunity required to clear established tumors.

The recent identification of cross presenting DCs in man, equivalent to the $CD8\alpha^+$ subset that has long been recognized in the mouse, has rejuvenated interest in the use of DCs in cancer immunotherapy (33, 34). These cells are defined by their expression of the surface marker CD141 and the chemokine receptor XCR1 and are found in the peripheral blood, tonsils, and bone marrow (35). They display an unrivaled capacity to cross present exogenous antigen to $CD8^+$ T cells, and hence to elicit effective cell-mediated immunity. While the properties of $CD141^+$ $XCR1^+$

DCs make them ideal candidates for immunotherapy against cancer, their trace numbers in peripheral blood (<0.1%) limit their therapeutic exploitation (25). Alternative sources of cross presenting DCs, including their isolation from the spleen or *in vitro* differentiation from hematopoietic progenitor cells, have so far failed to overcome these obstacles (36).

Since the holy grail of cancer immunotherapy is to stimulate tumor-specific T cells that will elicit a cytotoxic response with high specificity and minimal toxicity, adoptive transfer of TAA-specific T cells has gained popularity over the past few years (37, 38). Adoptive T-cell transfer involves the isolation of T lymphocytes from the patients and their reinfusion to treat disease. The adoptive transfer of T cells was first documented in rodents in 1955, where it was noted that T cells obtained from lymph nodes draining a tumor were able to confer immunity when transferred into the peritoneum of a secondary host, bearing a similar tumor (39). Almost three decades later, it was observed that the incubation of murine splenocytes with IL-2 generated large numbers of cells, called lymphokine-activated killer (LAK) cells, which were capable of lysing tumor cells with little effect on other somatic cells (40). These LAK cells were later shown to decrease tumor number and size in humans in a wide variety of tumors including pulmonary and hepatic metastases (41, 42). This work served as the basis for the use of tumor-infiltrating lymphocytes (TILs) in immunotherapy (43, 44). The combination of a lymphodepleting preparative regimen with adoptive transfer of TILs and administration of IL-2 has been shown to promote cancer regression in patients with metastatic melanoma, leukemias, and other types of tumor (44, 45).

The possibility of genetic modification of the T cells to overcome the immunosuppressive environment created by the tumor may lead to more effective therapies in the future, although current strategies for genetic modification are limited (46) and T cells are known to constitute particularly intractable targets. Nevertheless, the possibility of genetically engineering T cells to recognize specific TAAs makes it possible to target potentially any tumor using adoptive T-cell transfer (47), while leaving other tissues intact. The majority of clinical approaches use virus-based transduction of tumor antigen-specific T-cell receptor (TCRs) or chimeric antigen receptors (CARs) to generate T cells stably expressing tumor-specific transgenes which, although efficient, is expensive and risks insertional mutagenesis. Non-viral approaches to genetically engineer T cells have so far utilized transposon elements such as piggyback or zinc finger nucleases (46). TALEN and CRISPR/Cas-9-based-approaches, which allow for the insertion of transgenes into defined chromosomal loci, are, however, currently being actively explored (48, 49). Despite the attractiveness of using CAR technology to target cancer, only the treatment of B cell leukemia has so far proven successful using this approach. Furthermore, recent work has shown that the treatment of patients with myeloma or melanoma using T cells engineered to express affinity-enhanced TCRs for an HLA-A*01-restricted epitope of MAGE-A3 resulted in severe myocardial damage secondary to widespread T-cell infiltration leading ultimately to fatal cardiogenic shock. These findings have clearly shown how even altering the affinity of the TCR for its ligand may introduce unanticipated cross-reactivity with potentially fatal off-target toxicity (50).

Although much effort has been invested into the adoptive transfer of unmodified T cells in the treatment of cancer, outcomes have been disappointing. It is, for instance, sometimes challenging to identify tumor-specific T cells in patients with non-solid tumors. TILs can also be difficult to isolate from biopsies of most melanomas. *Ex vivo* expansion of tumor-specific CTLs can also prove difficult: in the case of EBV-specific CTLs, for instance, 3 months is required for the production of sufficient CTLs for re-administration to the patient, with obvious implications for disease progression. Often, reinfusion of T cells is required following adoptive transfer for the induction of a durable response due to the exhaustion of the expanded CTLs.

NK cells have likewise been used for adoptive transfer, due to their innate ability to recognize tumor cells deficient in MHC class I. NK cells have been isolated form peripheral blood, expanded *ex vivo*, activated using IL-2 or, more recently, the combination of IL-12, IL-15, and IL-18 (51), and re-administered to patients (52). Interestingly, although the use of autologous cells is normally preferred, several studies have demonstrated that the use of allogeneic NK cells is significantly more effective (53, 54). Accordingly, the results of several studies have shown NK cells to be well-tolerated following adoptive transfer with encouraging results of up to 20 months' survival following their administration (55). Nevertheless, low circulating numbers of NK cells in peripheral blood, coupled with the difficulty in their expansion and their inability to stimulate a robust response *in vivo*, limits their use in immunotherapy.

Given the difficulty of obtaining sufficient numbers of cells to target tumors *in vivo,* the advent of induced pluripotency offers unrivaled opportunities. The proven ability to produce iPS cells from individuals in a patient-specific manner with the capacity for indefinite self-renewal and unrestricted differentiation potential, may facilitate the scale-up in production of critical hematopoietic cell types, for many of which, protocols have already been optimized. Below, we outline the history of induced pluripotency and discuss the properties that make them attractive candidates for use in immunotherapy.

BRIEF HISTORY OF PLURIPOTENCY

Since their first description, embryonic stem (ES) cells have been regarded as the "gold standard" for pluripotency, displaying the capacity for indefinite self-renewal and differentiation into any somatic cell type, irrespective of its embryonic germ-layer of origin. Mouse ES cells were first isolated in 1981 by Martin Evans (56), work which later earned him the 2007 Nobel Prize for Physiology or Medicine. Nevertheless, it was not until 1998 that Thomson and colleagues succeeded in deriving ES cells from the inner cell mass of human blastocysts that were surplus to requirements following *in vitro* fertilization (57). Human ES cell lines, like their mouse counterparts, were found to be pluripotent, expressing embryonic markers such as SSEA-3, SSEA-4, TRA-1–60, and alkaline phosphatase and, following injection into immune compromised mice, forming teratomas containing cell types and tissues from all three embryonic germ layers.

Since their first derivation, there has been much interest in the use of human ES cells as a source of diverse cell types for drug discovery, regenerative medicine, and immunotherapy. However,

their use has been highly controversial due to the ethical sensitivities surrounding their derivation from human blastocysts, as well as the inevitable scientific constraints of using an allogeneic source of cells. In 2006, Yamanaka and colleagues demonstrated the feasibility of deriving pluripotent stem cells from adult mouse fibroblasts by retroviral transduction with genes encoding Oct3/4, Sox2, c-Myc, and Klf4 (58). These so-called iPS cells are indistinguishable at the cellular level from conventional ES cells, acquiring the capacity for indefinite self-renewal, unrestricted differentiation potential and, following injection into mouse blastocysts, the ability to generate germline-competent chimeras. These findings were subsequently translated to human dermal fibroblasts in 2007 by two independent groups (59, 60), showing, in principle, the feasibility of generating iPS cells on an individual basis. This seminal work offered a means of "personalizing" pluripotency in a manner free of the ethical concerns, while simultaneously addressing the immunological issues that limit the effectiveness of allogeneic therapies. Indeed, the production of iPS cells in an autologous fashion has paved the way for harnessing the potential of pluripotency for immunotherapeutic intervention in the pursuit of treatments for numerous indications.

Given the broad clinical applicability that iPS cells may enjoy in the future, there have been many efforts to develop and optimize the re-programming process to increase the safety profile of the resulting cell lines (**Table 1**). Protocols based on retroviral transduction may result in insertional mutagenesis while inducing the ectopic upregulation of developmental genes, which may subsequently render cells immunogenic (61). The direct delivery of re-programming proteins into somatic cells (62) and transfection with synthetic mRNA (63) have both proven successful, albeit yielding iPS cells at very low efficiency. Interestingly, small molecules, such as the histone deacetylase inhibitor, valproic acid, have been demonstrated to increase this efficiency by up to 100-fold (64). More importantly, recent work has shown that full re-programming may be achieved with a combination of seven small molecules alone, suggesting that induced pluripotency may not be dependent on the use of virus-based delivery systems (65). To achieve this, the authors screened 10,000 small molecules in order to find suitable replacements for each transcription factor. Three molecules, forskolin, 2-methyl-5-hydroxytryptamine, and D4476 were, for instance, identified as chemical substitutes for Oct3/4.

Perhaps the most dramatic advance in this rapidly evolving field has, however, been the recent description of stimulus-triggered fate conversion of cells (66), in which transient exposure of terminally differentiated cells to adverse conditions such as low pH, induces the upregulation of pluripotency genes. This approach has been shown to confer on cells such as murine lymphocytes, the capacity to form germline-competent chimeras following injection into recipient blastocysts, or the formation of entire offspring in tetraploid complementation assays. However, this method has yet to be verified independently and further characterization of the iPS cell lines produced in this way must be conducted and the translation of protocols to adult human cells has yet to be achieved, this approach may one day allow the generation of iPS cells lines with minimal intervention, compatible with downstream clinical applications.

Although traditionally much of the interest in iPS cells has focused on applications in regenerative medicine, other indications include their use as a novel source of hematopoietic cell types for cancer immunotherapy (**Figure 1**). The opportunity to derive iPS cells in a patient-specific manner, together with their tractability for genome editing using newly developed technologies such as the CRISPR–Cas-9 system (67) make them attractive

Table 1 | Methods of reprogramming and complications associated with derived iPS cell lines.

	Advantages	Disadvantages
Forced expression of genes via retrovirus	Well-characterized method, long history of use, arguably a simple approach and low cost, relatively high reprogramming rates of 0.01–0.02%	Integration into the genome may generate immunogenic cells, virus will only enter cells in mitosis, use of oncogenes such as c-Myc
Small molecules	Low cost of compounds, increases the efficiency of reprogramming	Only recent reports of full reprogramming achievable with small molecules alone: further characterization of lines generated needed
Synthetic miRNA	No integration within the genome	Very low reprogramming efficiency, miRNA degrades rapidly, modification of miRNA complicated, and time-consuming
Forced expression of genes via Sendai virus	No integration into the genome, higher efficiency of reprogramming than using retrovirus, diluted out of culture upon passage rapidly, high reprogramming rate of 0.1%	Difficult to work with, therefore most commonly used as pre-packaged "kits," which are expensive compared to other viral methods of reprogramming
Episomal plasmid vector system	No genomic footprint	Very low efficiency of reprogramming (0.0002%), loss of episomal plasmid
Stimulus-triggered acquisition of pluripotency (STAP)	No nuclear transfer or introduction of transcription factors	Limited capacity for self-renewal when compared to ES cells. Reports have yet to be independently verified

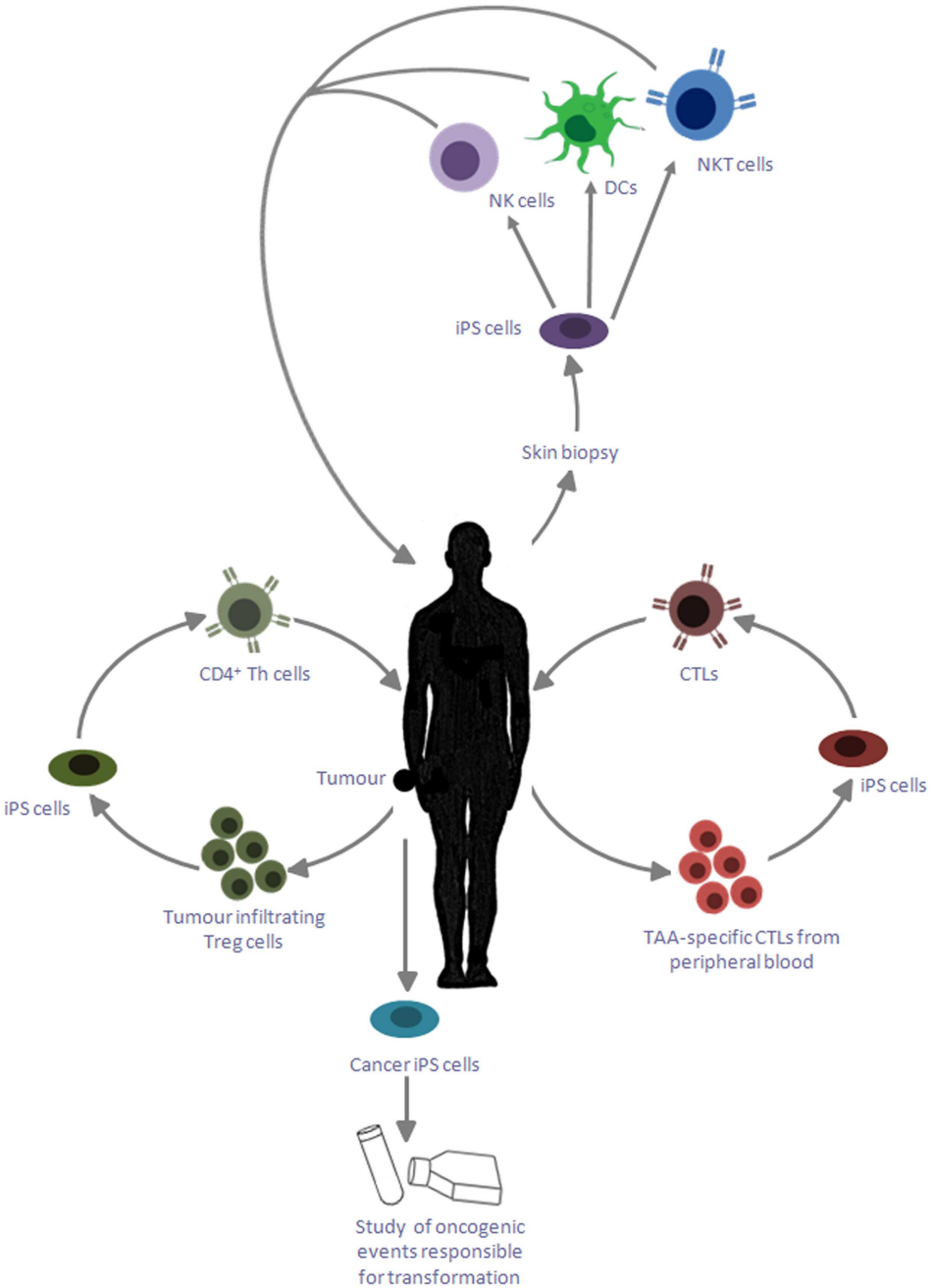

FIGURE 1 | Applications of iPS cells for cancer immunotherapy. iPS cells reprogramed from skin biopsies of cancer patients can be differentiated into DCs, which can be reintroduced into patients to cross present TAA. These iPS cells can also be differentiated into NK cells and NKT cells, which can be adoptively transferred into the patient to target cancer cells; iPS cells can be generated by reprogramming tumor-specific CTLs, which can provide an unlimited source of naïve $CD8^+$ T cells with the desired specificity; tumor-infiltrating Treg cells may likewise be reprogramed into iPS cells and redifferentiated into $CD4^+$ Th cells, which are capable of providing help to the CTLs to target cancerous cells; these iPS cells can also be exploited to study the genetic basis of transformation and its influence of primary cell types.

candidates for such applications. Furthermore, their indefinite capacity for self-renewal may greatly facilitate the scale-up of cell production, offering unrivaled opportunities for overcoming many of the obstacles encountered using conventional sources of cells.

EXPLOITING INDUCED PLURIPOTENCY FOR THE STUDY AND TREATMENT OF CANCER

CANCER iPS CELLS AS MODELS OF DISEASE PROGRESSION

The use of iPS cell lines to model *in vitro* a broad range of human disease states has already begun to yield important

advances in our understanding of their pathogenesis and progression. Nevertheless, the generation of iPS cells from primary cancer cells has remained a significant challenge, proving successful for only a limited number of cancers due, most likely, to the associated genetic or chromosomal abnormalities introducing a state of genetic instability (68). Although reprogramming of gastrointestinal cancer cells to a pluripotent state has been achieved by careful modification of culture conditions and reprogramming factors (69), it has proven necessary to use retroviral vectors to introduce the necessary transgenes, which risks the introduction of confounding mutations that may interfere with the phenotype of cells differentiated from the resulting iPS cell lines. Application of the latest non-viral reprogramming technologies to primary cancer cells is, therefore, paramount for gaining insight into the impact that oncogenic events may have on a range of primary cell types. The potential that such an approach offers for drug discovery and toxicity screening may facilitate the future identification of therapeutic targets as well as novel neo-antigens that may be exploited for vaccination purposes.

CANCER VACCINATION USING iPS CELL-DERIVED DCs

Given the significant donor-to-donor variability encountered in the use of moDCs for cancer immunotherapy, early research focused on the potential of pluripotency to provide a more homogenous source of DCs amenable to scale-up. Accordingly, several groups reported the successful differentiation of functional DCs from both mouse and human ES cells (70, 71). Since the use of animal products for their differentiation made them unsuitable for clinical applications, Tseng and colleagues succeeded in developing protocols for their differentiation in an animal product-free manner, compatible with their downstream use *in vivo* (72). Although these DCs were shown to be functional, possessing the ability to endocytose, process, and present foreign antigen to naïve $CD4^+$ T cells, their clinical utility was restricted both by their limited capacity for cross presentation of antigen to MHC class I-restricted $CD8^+$ T cells and their differentiation from allogeneic sources, requiring matching at certain HLA loci. Recent work in our laboratory has, however, demonstrated that $CD141^+XCR1^+$ DCs can be successfully derived from human iPS cells using a cocktail of GM-CSF, stem cell factor (SCF), vascular endothelial growth factor (VEGF), and bone morphogenetic protein-4 (BMP4). This protocol was found to be compatible with the exclusion of all animal products, ensuring the downstream clinical compliance of the DCs obtained (73). Unlike moDCs used for comparison, these DCs were shown to efficiently cross present the TAA, Melan A, supplied exogenously in recombinant form, to naïve $CD8^+$ T cells *in vitro*, stimulating a primary Melan A-specific immune response that could be tracked using tetramer technology (73). Since iPS cells have indefinite capacity for self-renewal *in vitro*, this approach provides a potentially unlimited source of autologous DCs that might bypass the issue of patient-to-patient variability and the confounding effects of long-term chemotherapy that impacts adversely on the circulating monocytes from which conventional moDCs are derived.

DIFFERENTIATION OF T CELLS FOR ADOPTIVE TRANSFER

While the use of DCs to stimulate TAA-specific immune responses *in vivo* offers the prospect of establishing durable immunological memory, an alternative strategy for cancer immunotherapy has been the adoptive transfer of antigen-specific T cells, expanded *ex vivo*. Since such expansion regimes risk the functional exhaustion of the resulting cells, the differentiation of potentially unlimited numbers of primary T cells from pluripotent stem cells has proven an attractive goal. The co-culture of mouse ES cells with the OP9 stromal cell line constitutively expressing delta-like ligand 1 (OP9-DL1), has been shown to successfully support their differentiation into T-cell progenitors (74, 75). Nevertheless, their final commitment to the T-cell lineage requires their introduction into fetal thymus organ cultures, so as to provide a microenvironment conducive to TCR gene rearrangement and subsequent positive selection of a diverse $CD4^+$ and $CD8^+$ T-cell repertoire. On transferring these ES cell-derived T cells into $RAG2^{-/-}$ mice, immune reconstitution was readily observed, strongly suggesting that T cells generated in this way were functionally mature. Although these findings suggest that pluripotent stem cells may serve as a potentially unlimited source of naïve T cells for adoptive transfer, the requirement for an appropriate thymic microenvironment to support V(D)J recombination and positive selection poses significant ethical and pragmatic barriers to the translation of protocols to the human.

A logical way to overcome this hurdle might be to harness induced pluripotency to generate iPS cells from T cells that have already undergone V(D)J recombination and are known to exhibit a desirable antigen specificity. Any T cells differentiated from the parent iPS cell line would maintain the original antigen specificity of the parent cells, and may, therefore, differentiate *in vitro* in a thymus-independent manner. Recent studies have reported the successful differentiation of antigen-specific T cells from an iPS cell line itself generated from CTL specific for an epitope from the melanoma antigen MART-1 (76). These cells were expanded by stimulation with anti-CD3 mAbs, thereby generating $CD8^+$ T cells, which were shown to respond to MART-1, demonstrating retention of their original antigen specificity.

Given the low frequency of tumor-specific T cells in the periphery of individuals and difficulties surrounding their identification, Themeli and colleagues exploited the tractability of iPS cells for genetic modification to introduce a bicistronic lentiviral vector encoding 19–28z, a CAR specific for CD19, expressed by the majority of leukemias and lymphomas. The authors were able to optimize differentiation conditions to allow for serum and feeder free generation of hematopoietic progenitor cells which, when co-cultured with OP9-DL1 stromal cells in the presence of SCF, Flt3L, and interleukin-7 (IL-7), differentiated into T cells expressing the CD19-specific CAR. T cells produced in this way were activated by $CD19^+$ APCs and, upon infusion into mice, potently inhibited tumor progression (77).

While the use of CARs may circumvent the requirement for the identification of antigen-specific T cells, an alternative method of capturing desirable antigen specificities might be to exploit TILs whose presence within a developing tumor provides compelling evidence for their specificity. Whereas $CD8^+$ CTL are readily

obtained from tumor biopsies and lend themselves to reprogramming, other T-cell subsets are also evident including regulatory T (Treg) cells. The presence of these Treg cells is known to negatively correlate with survival rates (78), due to their capacity to suppress anti-tumor immune responses and facilitate evasion of the developing tumor from the host immune system. If tumor-specific Treg cells could likewise be reprogramed to pluripotency, their redifferentiation along the T-cell lineage might provide opportunities for their phenotypic reassignment into effector T cells, providing a valuable source of $CD4^+$ T-cell help for endogenous CTL responses in danger of exhaustion.

One of the major hurdles to harnessing this approach is defining extracellular Treg-specific markers. Currently, the most widely used marker for Treg cells is Foxp3 among $CD4^+CD25^+$ cells (79). As this transcription factor is expressed solely in the nucleus, however, sorting of cells based on its expression is not feasible. Nevertheless, recent work has demonstrated that CD127 expression inversely correlates with Foxp3 and hence the suppressive function of human $CD4^+$ Treg cells (80). In addition to low CD127 expression, expression of CD45RA is also apparent in human $CD4^+$ Treg cells (81): sorting cells based on a $CD4^+CD25^+CD127^{low}CD45RA^+$ phenotype would, therefore, represent the most effective strategy currently available for isolating antigen-specific Treg cells infiltrating the tumor microenvironment.

NK CELL-BASED IMMUNOTHERAPY

Although much interest has focused on the use of NK cells in cancer immunotherapy, obtaining sufficient numbers for administration to patients remains a significant limitation. In 2005, Woll and colleagues used a two-step process to differentiate human ES cells into NK cells *in vitro*. These cells had the ability to lyse human tumor cells deficient in MHC class I expression and up-regulate cytokine production (82). Subsequently, NK cells were successfully differentiated from human iPS cells, using a similar two-stage culture system (83), the cells obtained representing a pure population that did not require cell sorting or co-culture with xenogeneic stromal cells. Moreover, sufficient cytotoxic NK cells could be differentiated from 250,000 iPS cells to treat a single patient, suggesting that iPS cells provide a scalable platform for the clinical implementation of such an approach.

In addition to *bona fide* NK cells, it has recently proven possible to derive NKT cells from iPS cells. NKT cells are characterized by the expression of an invariant TCR encoded by Vα24–Jα18 in humans and Vα14–Jα18 in mice (84). These cells share the properties of both NK cells and T cells and are thought to play an important role in cancer immune surveillance (85). Indeed, NKT cells differentiated from mouse iPS cells were shown to secrete large quantities of IFNγ and actively suppress tumor growth *in vivo* (86). The differentiation of NKT cells from iPS cells may, therefore, make this elusive cell type readily accessible for cancer immunotherapy in the future.

CONCLUSION

Although significant advances have been made in cancer immunotherapy over the past decade with the discovery of human cross presenting DCs and the use of CARs and TCR transfer for the generation of more effective T-cell therapy, the requirements for high specificity, minimal toxicity, and the capacity for immunological memory have yet to be achieved. It has been suggested that since no single therapy is likely to fulfill all these criteria, adoptive transfer of tumor-specific T cells might be combined with DC vaccination to generate a durable immune response (87). Given the unrestricted differentiation potential of iPS cells, prospects for the differentiation of either cell type from the same patient-specific cell line provide a comprehensive approach. Furthermore, their potential for the efficient scale-up of cell production and tractability for new generation genome engineering tools, such as the CRISPR/Cas-9 system and transcription activator-like effector nucleases (TALENs) (48, 49) may herald a new era in cancer immunotherapy, in which treatments are exquisitely tailored to the individual needs of the patient.

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