INNATE IMMUNE CELL DETERMINANTS OF T CELL IMMUNITY: FROM BASIC MECHANISMS TO CLINICAL IMPLICATIONS

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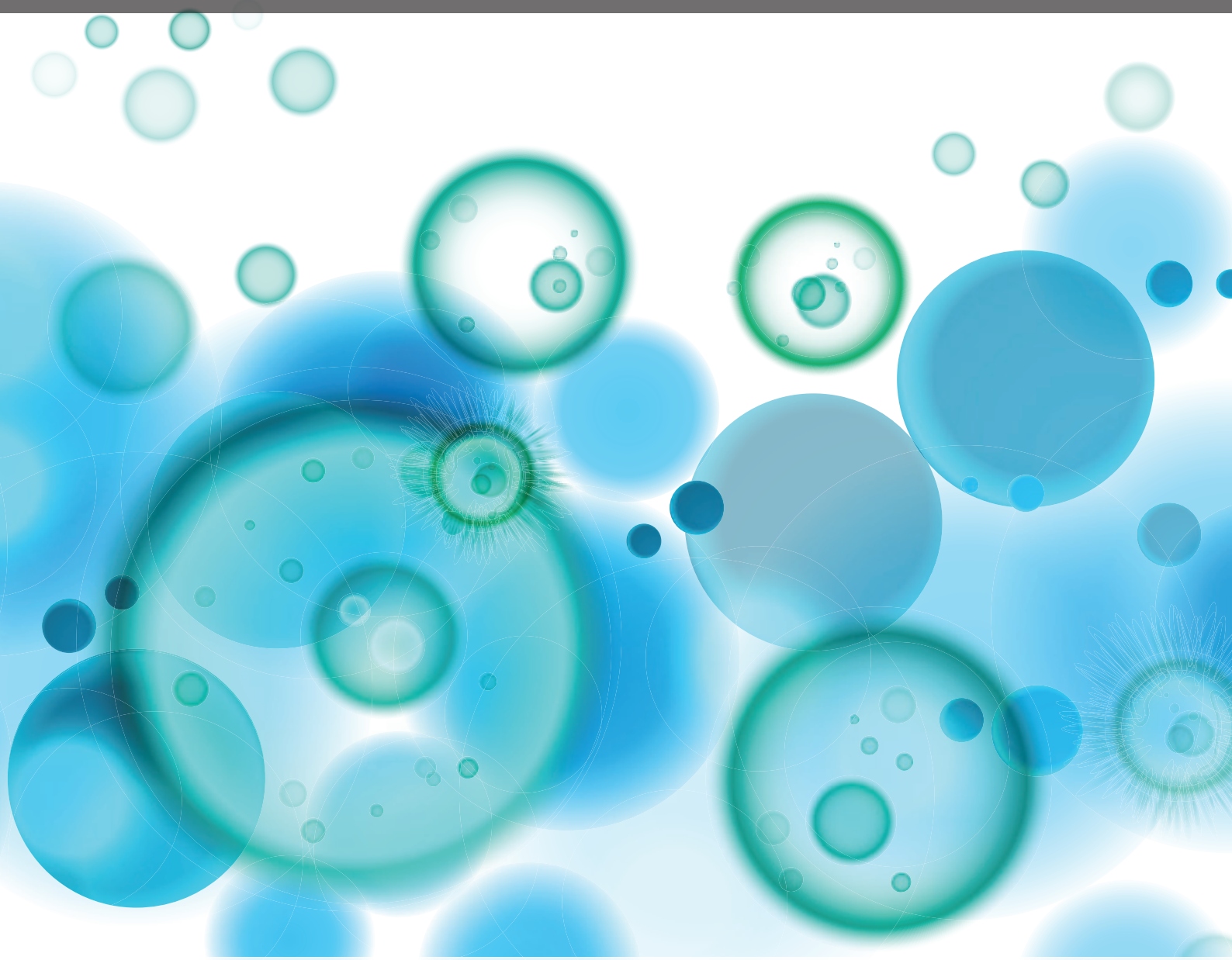

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INNATE IMMUNE CELL DETERMINANTS OF T CELL IMMUNITY: FROM BASIC MECHANISMS TO CLINICAL IMPLICATIONS

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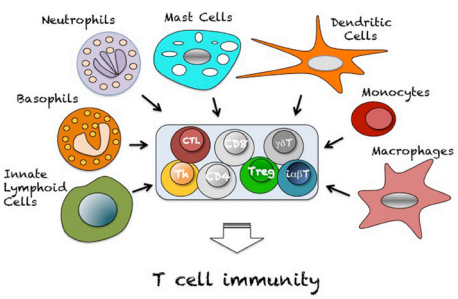

T cell immunity unfolds through the activity of different innate immune cell subsets, including Mononuclear Phagocytes (Dendritic Cells, Monocyte and Macrophages), Granulocytes (Basophils, Neutrophils and Mast Cells) and Lymphoid Cells. The molecular understanding of how these innate immune cells communicate with T lymphocytes and shape T cell immunity is leading to novel strategies of immuneinterventions against inflammatory diseases and cancers.

Figure by Elisabetta Padovan

T cell immunity assures long-lasting immune protection against an almost infinite number of noxious stimuli that might enter in contact with the host. The unfolding of this very efficient protection system, however, crucially depends on the presence and function of a variety of innate immune cell subsets, including mononuclear phagocytes, granulocytes and innate lymphoid cells. As innate immune cells are capable of homing to different tissues they can indeed impact on the priming of antigen-specific T cells in secondary lymphoid organs, as well as on their rapid mobilization and activation in the periphery, upon new exposure to the same antigen. Hence, heterogeneous Ag-specific memory T cell responses can be elicited even years after their primary induction.

This research topic provides a collection of manuscripts comprehensively describing the many roles played by innate immune cells in the orchestration of T cell immunity, from priming to memory. Contributions are organized in three different parts, as follows:

1. T cell physiology directed by innate immune cells
2. Detrimental consequences of deranged T cell immunity
3. Immune interventions

In each session, the bidirectional interaction among innate immune cell subsets and T lymphocytes is comprehensively described by focusing on molecularly defined pathways influencing T cell immunemodulation. Following the dissection of healthy and de-regulated T cell responses, a rational translation of basic research into clinical applications is presented.

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Part III: Immune interventions

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EDITORIAL
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Editorial: Innate Immune Cell Determinants of T Cell Immunity: From Basic Mechanisms to Clinical Implications

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Keywords: innate immunity, T cell memory, antigen presentation, vaccines, human diseases

The Editorial on the Research Topic

Innate Immune Cell Determinants of T Cell Immunity: From Basic Mechanisms to Clinical Implications

Long-lasting T cell immunity is delivered by individual T lymphocytes expressing clonally distributed antigen-specific receptors. Following priming in lymph nodes, naïve CD4$^+$ and CD8$^+$ T lymphocytes proliferate and generate clones of effector cells that deliver unique effector functions in peripheral tissues. Moreover, long-lasting memory T cells generated during priming are rapidly engaged upon reexposure to the same antigen, even years after their primary induction. Notably, this very efficient protection system cannot unfold without accessory cells. Our frontiers research topic features different innate immune cell subsets and the crucial roles they play in the initiation and maintenance of T cell immunity. By comprehensively describing positive and negative outcomes of these events, the contributions provide a meaningful link between basic findings and clinical applications.

T CELL PHYSIOLOGY DIRECTED BY INNATE IMMUNE CELLS

Following the seminal discovery of Steinman and Cohn in 1973 (1, 2) describing a rare cell type initiating antigen-specific responses, dendritic cells (DC) have taken up the stage for several decades as professional antigen-presenting cells (APC). In their reviews, Geginat et al. and Clausen and Stoitzner dissect the instrumental role played by specialized DC subsets in instructing protective T cell immunity, emphasizing how this specialization, conserved in mice and humans, suits at best the need of dedicated and qualitative different "classes" of T cells for immune homeostasis, defense against pathogens, and responses to vaccines and allergens.

Dendritic cells, however, do not stand alone in this process. Indeed, although DC activated through pattern-recognition receptors (PRR) are competent for CD4$^+$ T cell priming, they require feedback from other T cell subsets, including iNKT, γδT, and CD4$^+$ T helper (Th) cells, for the generation of antigen-specific CD8$^+$ T cell immunity. iNKT cells and γδT cells are innate-like T cell subsets that recognize lipid and metabolites in a non-MHC-restricted fashion. The contribution of Salio and Cerundolo highlights the specific characteristics of these cell types and their modality of activation by different tissue-resident APC, focusing on the intracellular pathways that regulate lipid and metabolite Ag presentation at steady state and upon infection. The role of these cells in "licensing" DC for CD8$^+$ T cell priming is illustrated by Gottschalk et al., presenting a comparative functional analysis of DC licensed by iNKT and Th cells.

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Immune responses to infections and other assaults are initiated in the target tissues. These do not only harbor DC but also other immune cell subtypes that are either tissue resident or become recruited. Activation of innate immune cells, such as mast cells (MC) and neutrophils, will most likely influence the activation and polarization of DC, for example, the pattern of cytokines expressed by the DC. Thereby, these cells may indirectly influence the polarization of naïve T cells by DC in the lymph node. In addition, neutrophils have been shown to migrate to lymph nodes, where they may directly contribute to T cell priming.

Secondary activation is also influenced by innate immune cell subsets. For instance, the early phase of infection is characterized by a rapid recruitment of neutrophils and monocytes into the inflamed tissue, where these phagocytes colocalize with tissue-resident memory T cells. In the most recent years, consistent evidences have accumulated in support of the capacity of these accessory cells to influence T cell immunity *in vivo*. The contributions of Leliefeld et al. and Roberts et al. address the role of, respectively, neutrophils and monocytes as "bystander activators" that favor survival and activation of T cells, independently of TCR antigen specificity. Notably, both cell types can also act as APC delivering Ag-specific and costimulatory signals to T cells, and their collaborative endeavors were found to positively and negatively modulate the activity of different effector T cell subsets, including conventional and innate-like T cells. Moreover, neutrophils and monocytes may differentiate and acquire different functional programs in response to signals provided by activated T cells and influence the quality of T cell responses even at later stages of infections and malignant transformation.

At barrier sites T cell responses become modulated also by the activity of tissue-resident MC, basophils, and innate lymphoid cells (ILC) through their bidirectional interaction with T cells.

Basophils and MC, originally regarded as "degranulating" inflammatory cells rapidly responding to the triggering of PRR, are now recognized to participate in the regulation of T cell immunity. The contributions of Sarfati et al. and Bulfone-Paus and Bahri feature the capacity of these two cell subsets to skew naïve T cell priming and modulate effector T cell responses by acting as cytokine-secreting bystanders in coordination with DC, as well as by physically interacting with T lymphocytes. The availability of suitable animal models, described by Otsuka and Kabashima, has increased our understanding of basophils as putative specialized APC for low-molecular-weight compounds, as compared to MC, although these findings await confirmation for human basophils.

Intriguingly, the effect of basophils and MC on the tissue microenvironment affects also the survival and activity of ILC. This is a heterogeneous family of rare immune cells, comprehensively described in the review of Von Burg et al., influencing T cell responses through Ag-independent modulation of T cell survival and proliferation, as well as MHC-restricted Ag presentation. Interestingly, these functions, which are required for the maintenance of immune homeostasis, are segregated among different ILC types and compartmentalized in different anatomical locations. Notably, the interaction of ILC with T cells is also bidirectional, since ILC survival and proliferation requires cytokines released by activated T cells.

NATURAL IMMUNE DEFICIENCIES DERANGING HUMAN T CELL IMMUNITY

The unfolding of Ag-specific T cell immunity and their regulation critically depends on the formation of highly organized intercellular junctions between APC and T cells, referred to as immune synapses (IS). The contribution of Kallikourdis et al. features the functions of these intercellular structures by illustrating the consequences ensuing from inborn errors of their structural components. Notably, IS instability caused by missing or dysfunctional components affects trafficking and functions of both APC and T cells and associate to autoimmunity and recurrent infections.

DETRIMENTAL CONSEQUENCES OF DERANGED T CELL IMMUNITY AT DIFFERENT BODY SITES AND IMMUNE INTERVENTION

Although the actions of innate immune cells fulfills the need of initiating and maintaining protective T cell responses, the excessive presence or activity of individual determinants may be detrimental to the host, because it could promote tissue destruction as in autoimmunity and allergy, or conversely, prevent the induction of protective immune responses against malignant tissues. The capacity of neutrophils, monocytes, and basophils to acquire different functional profiles in response to environmental triggers and also their role as "bystander activators," can indeed become detrimental, as underscored by the role of basophils and MC in allergies and chronic inflammation (Sarfati et al.; Bulfone-Paus and Bahri), of monocyte/macrophages in rheumatoid arthritis (Roberts et al.) and of neutrophils and DC in cancer (Clausen and Stoitzner; Leliefeld et al.). Based on this knowledge, it is possible to consider the possibility of exploiting different types of DC licensing for inhibiting autoimmunity or enhancing protective immunity against pathogens and tumors (Salio and Cerundolo; Gottschalk et al.). In line with this, Martin et al. expand on the promising finding that anticancer drugs act as APC modulators in antitumor immunity.

In a totally new vision, Biedermann et al. comprehensively describe the causal link between skin commensals and atopic dermatitis, highlighting how activation of skin-resident DC and myeloid-derived suppressor cells by T cell-derived cytokines and microbial components exacerbates atopic dermatitis and the clinical benefits induced by therapeutic alteration of the skin microbiome.

AUTHOR CONTRIBUTIONS

EP and SM edited and reviewed this topic.

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REVIEW
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Immunity to pathogens taught by specialized human dendritic cell subsets

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Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) that have a key role in immune responses because they bridge the innate and adaptive arms of the immune system. They mature upon recognition of pathogens and upregulate MHC molecules and costimulatory receptors to activate antigen-specific $CD4^+$ and $CD8^+$ T cells. It is now well established that DCs are not a homogeneous population but are composed of different subsets with specialized functions in immune responses to specific pathogens. Upon viral infections, plasmacytoid DCs (pDCs) rapidly produce large amounts of IFN-α, which has potent antiviral functions and activates several other immune cells. However, pDCs are not particularly potent APCs and induce the tolerogenic cytokine IL-10 in $CD4^+$ T cells. In contrast, myeloid DCs (mDCs) are very potent APCs and possess the unique capacity to prime naive T cells and consequently to initiate a primary adaptive immune response. Different subsets of mDCs with specialized functions have been identified. In mice, $CD8\alpha^+$ mDCs capture antigenic material from necrotic cells, secrete high levels of IL-12, and prime Th1 and cytotoxic T-cell responses to control intracellular pathogens. Conversely, $CD8\alpha^-$ mDCs preferentially prime $CD4^+$ T cells and promote Th2 or Th17 differentiation. BDCA-3^+ mDC2 are the human homologue of $CD8\alpha^+$ mDCs, since they share the expression of several key molecules, the capacity to cross-present antigens to $CD8^+$ T-cells and to produce IFN-λ. However, although several features of the DC network are conserved between humans and mice, the expression of several toll-like receptors as well as the production of cytokines that regulate T-cell differentiation are different. Intriguingly, recent data suggest specific roles for human DC subsets in immune responses against individual pathogens. The biology of human DC subsets holds the promise to be exploitable in translational medicine, in particular for the development of vaccines against persistent infections or cancer.

Keywords: dendritic cells, cytokines, toll-like receptors, T-cell differentiation, cytotoxic T cells

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INTRODUCTION

Human beings are constantly exposed to a myriad of pathogens, including bacteria, fungi, and viruses. These foreign invaders or cohabitants contain molecular structures that are sensed by the innate immune system, which mounts a first-line defense and also activates a pathogen-specific, adaptive immune response. The adaptive immune system is composed of B cells that produce specific antibodies, $CD8^+$ T cells that can kill pathogen-infected cells, and $CD4^+$ T cells that produce effector cytokines and coordinate the immune response. T cells express antigen receptors (T-cell antigen receptors, TCR) that recognize specific peptides presented on MHC molecules. $CD8^+$ T cells recognize peptides presented by MHC class-I molecules that are ubiquitously expressed, whereas $CD4^+$ T cells are activated by peptide-MHC class-II complexes, which are largely restricted to antigen-presenting cells (APCs). Dendritic cells (DCs) can express very high levels of MHC and costimulatory molecules, and it is generally accepted that they are the relevant cells to induce the activation ("priming") of antigen-specific "naive" T cells (1, 2) and induce their differentiation into various types of effector T cells.

The elimination or containment of different types of pathogens requires dedicated classes of adaptive immune responses (3). Thus, pathogens like viruses or intracellular bacteria require $CD4^+$ and $CD8^+$ T cells that produce IFN-γ and kill infected cells (Th1 and CTL, respectively). IL-12 is the critical cytokine that induces this type of response, but IL-12 production by DC is tightly controlled and requires several stimuli derived from pathogens and from $CD4^+$ helper T cells (4–9). Conversely, extracellular bacteria and fungi require a different type of response that can be mediated by Th17 cells (10–12). These effector cells are induced by proinflammatory cytokines produced by DC and macrophages (13) and attract neutrophils that in turn phagocytose extracellular bacteria (14). A third type of effector response is the Th2 response, which is required to expel extracellular parasites such as helminths by activating eosinophils and basophils and by inducing antibodies of the IgE class (15). IL-4 is the critical cytokine that induces this response (16), but IL-4 is normally not produced by DC (17, 18). Finally, these different effector responses have to be controlled by specialized regulatory T cells, in particular by IL-10-producing T cells ("Tr1 cells"), which are generated from effector cells and are important to avoid excessive tissue damage by adaptive immune responses (19–22). Cytokines that promote this type of regulatory T-cell response are IFN-α, IL-27, and IL-10 (23–25), and all these cytokines can be produced by DCs (26, 27).

DCs HAVE THE UNIQUE CAPACITY TO PRIME T-CELL RESPONSES

Professional APCs have to present pathogen-derived peptides on MHC molecules to activate antigen-specific T cells. DCs are phagocytic in the immature state, i.e., under steady-state conditions and upon initial pathogen encounter, and can take up antigenic material by pinocytosis or by surface receptor-mediated internalization (28). Proteins from pathogens are then shuttled to lysosomes where they are chopped to peptides and loaded on MHC class-II molecules (29, 30). These peptide–MHC complexes are then transported to the plasma membrane to activate specific $CD4^+$ T cells. The presentation of peptides derived from exogenous proteins on MHC class-I, a process called cross-presentation (31, 32), is a largely unique feature of DCs and is particularly important to activate $CD8^+$ T cells in viral infections. Virus-infected cells express viral proteins in the cytosol where they are degraded to peptides by the proteasome, translocated to the endoplasmic reticulum by TAP proteins, and loaded on MHC class-I molecules (31). However, since DCs are not necessarily infected by viruses, they must be able to process virus-derived proteins also from external sources, such as virus-infected cells, to activate $CD8^+$ T cells. The mechanism of cross-presentation is still incompletely understood, but two distinct pathways via vacuoles and peptide translocation from phagolysosomes to the cytosol have been described (32). It is believed that cross-presentation is the most important pathway leading to the induction of cytotoxic T-cell responses, and excellent reviews have been published on this relevant topic (31–33).

Naive T cells have a very high activation threshold (34), and only professional APCs that express high levels of MHC and costimulatory molecules such as DCs are able to induce proliferation of naive T cells (35). Several receptor–ligand interactions contribute to naive T-cell activation (36–38), but CD28 costimulation is particularly important to amplify the signal transduced by the TCR (39). Monocytes efficiently present peptides derived from extracellular proteins on MHC class-II to activate antigen-experienced $CD4^+$ T cells (34), and this capacity can be exploited to selectively expand antigen-specific memory T cells (40). However, monocytes have an approximately 1000-fold lower capacity to prime naive $CD4^+$ T cells as compared to DCs (Nizzoli et al., under review) and home to non-lymphoid tissues in the steady state. However, upon inflammation, they can differentiate to inflammatory DCs (41) and home to lymph nodes where they can activate T cells (42, 43). In addition, there is some evidence that $CD16^+$ subsets of human blood monocytes might contain DCs (27, 44, 45). Naive T cells constantly recirculate in the blood and migrate through secondary lymphoid organs (46), but are largely excluded from non-lymphoid tissues. In secondary lymphoid tissues, they migrate to the T-cell zone, where they encounter DCs (47). B cells are also present in secondary lymphoid organs and can potently present antigen to T cells when they internalize and process antigens that have specifically bound to their B-cell receptor (48). However, B cells are physically separated from naive T cells in lymph nodes and only following TCR activation naive T cells migrate to the B-cell zone where they interact with antigen-specific B cells to induce antibody production (49, 50). Thus, antigen presentation by B cells appears to be important for the activation of antigen-experienced T cells rather than for naive T-cell priming.

PATHOGEN-ASSOCIATED MOLECULAR PATTERNS INDUCE DC MATURATION

Dendritic cells are generated from committed precursors in the bone marrow that are released into the circulation to seed peripheral organs (51–55). Both monocytes and DCs can be derived from common myeloid progenitors (CMPs), but committed

precursors that selectively give rise to monocytes or DCs (51) or even selected DC subsets (53, 54) have been identified in humans and mice. DCs are poorly stimulatory in the immature state and can induce a partial T-cell activation, leading to deletion of autoreactive $CD8^+$ T cells (56–59). In addition, they promote self-tolerance by inducing $Foxp3^+$ regulatory $CD4^+$ T cells that suppress autoreactive T cells (60). Pathogens induce the maturation of DCs that consequently acquire the capacity to produce polarizing cytokines and to prime pathogen-specific effector T-cell responses. Pathogen-derived molecular patterns [PAMPs (61, 62)] are recognized by DCs and lead to the efficient presentation of antigens to T cells (63). There are different classes of pathogen-sensing receptors, including Toll-like receptors (62, 64), nucleotide-binding oligomerization domain (NOD)-like receptors (65), retinoic acid-inducible gene 1 (RIG-I)-like receptors (66), and C-type lectins (67). TLRs recognize different PAMPs, including nucleic acids or cell wall components such as proteins and lipoproteins (68, 69). In the case of viruses, nucleic acids are sensed not only by different TLRs in endosomes but also by cytosolic receptors like RIG-I (66, 70) and induce a potent activation of DCs. Importantly, subsets of DCs express different patterns of pathogen-sensing receptors and might thus preferentially respond to individual pathogens (71, 72). DNA viruses such as cytomegalovirus (CMV) and herpes simplex virus (HSV) and also bacteria can activate DCs via unmethylated CpG-containing DNA (69), which is sensed by TLR9. Double- and single-stranded RNAs, which are generated by both DNA and RNA viruses, are sensed by DCs via TLR3 (73) and TLR7/8 (74, 75), respectively. Of note, TLR3 is restricted to mDCs (71) and induces cross-presentation capacities (76). Viruses such as respiratory syncytial virus (RSV) and hepatitis C virus (HCV) can also activate DCs via TLR2 or TLR4, which are expressed on the plasma membrane and recognize viral proteins (77). TLR2 is also involved in immune responses to fungi (78) and Gram-positive bacteria (79, 80) while TLR4 recognizes lipopolysaccharide (LPS) (81), a cell membrane compound of Gram-negative bacteria. Many pathogens like viruses activate DCs via multiple TLRs (77). Moreover, other immune cells, including T cells themselves, feed-back on DCs to regulate the ongoing response. In particular, CD40 stimulation by $CD4^+$ helper T cells is crucial for $CD8^+$ T-cell stimulation and IL-12 production (4, 5). Moreover, IFN-γ (6) and paradoxically also IL-4 (7, 8) that can be provided by T cells further enhance IL-12 production (9).

Surface TLRs such as TLR2 and TLR4 signal via the adaptor protein Myd88 (82) to induce the activation of Map kinases and the nuclear translocation of the transcription factor NF-κB, which in turn induces the transcription of proinflammatory cytokines (62). Endosomal TLRs 7, 8, and 9 also signal via Myd88 but activate IRF7, which in turn induces type-1 interferon production (83, 84). TLR3 is an exception since it does not signal via Myd88 but utilizes TRIF (85) to activate IRF3 (86, 87) or IRF7 (88). How all these complex signaling pathways are integrated by DCs to induce the appropriate T-cell response is still incompletely understood (88–90).

SPECIALIZED DC SUBSETS INDUCE DIFFERENT CLASSES OF T-CELL RESPONSES IN MICE

Dendritic cells in mice can be subdivided into distinct subsets with specific functions. Some DCs are stably resident in lymph nodes while others are positioned in non-lymphoid tissues to sense tissue-invading pathogens, but are migratory and are recruited via the lymph following pathogen encounter in a CCR7-dependent manner (91, 92). In secondary lymphoid tissues, two major DC subsets are myeloid DCs (mDCs) and plasmacytoid DCs (pDCs; **Table 1**) (72, 93, 94). Both pDCs and mDCs upregulate MHC and costimulatory molecules like CD80 and CD86 upon maturation (72) that bind to CD28 and are required to induce full T-cell stimulation (39). However, pDCs are poorly phagocytic and have a different regulation of MHC class-II turnover upon maturation as compared to mDCs (95). Thus, mDCs stop phagocytosis and peptide loading on MHC upon pathogen recognition and stably present peptides from antigenic material they had acquired upon pathogen encounter (30, 96, 97). This maturation-induced stabilization of peptide–MHC complexes enhances the priming of pathogen-specific T cells by mDCs. In contrast, pDCs continue to present new peptides on MHC complexes even in the mature stage (95). On the one hand, this limits their capacity to stimulate pathogen-specific T cells; on the other hand, this enables them to present also late-expressed viral antigens when they are actively infected. This diverse regulation of MHC–peptide stability in mDCs and pDCs suggests that they present different antigens to T cells.

TABLE 1 | Surface markers expressed on human and mouse DC subsets.

Subsets	Mouse (spleen)			Human (blood)		
	CD8α−	CD8α+	pDC	mDC1	mDC2	pDC
CD11c	+	+	Low	++	+	−
CD11b	+	+/−	−	+	−	−
BDCA-1/CD1c	n/a	n/a	n/a	+	−	−
BDCA-2/CD303	−	−	+	−	−	+
BDCA-3/CD141	−	−	−	−	+	−
BDCA-4/CD304	−	−	−	−	−	+

This table summarizes the expression of widely used surface markers to identify DC subsets in humans and mice.

Plasmacytoid DCs are present in lymph nodes and are largely absent from non-lymphoid organs, but they can be recruited upon inflammation (98). The role of pDC in T-cell priming is still debated (99). There is consensus that they are poorly stimulatory in their resting state (100, 101), but while some groups proposed that they become potent APCs following TLR stimulation and prime $CD4^+$ and cross-prime $CD8^+$ T-cell responses (102–105), others concluded that also mature pDCs have only low priming and cross-priming capacities and might rather be tolerogenic (101). The rapid and abundant production of type-1 interferon by pDC suggests a pivotal role in viral infections, even if their capacity to prime virus-specific T cells directly appears to be limited. IFN-α can also be produced by other immune cells and by virus-infected cells, but the early and systemic IFN-α response is believed to depend on pDCs (101). Consistently, in the case of HSV infections, it was shown that pDCs were important for systemic but not local protection (106). However, in several other viral infections in mice, including vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), RSV, and mouse cytomegalovirus (MCMV), pDCs do not seem to play a major role (100). In marked contrast, in mouse hepatitis virus (MHV) infection, the antiviral response against this coronavirus was largely pDC dependent (107) (**Figure 1**). Finally, pDCs have been found by several groups to induce the production of the anti-inflammatory cytokine IL-10 by $CD4^+$ T cells, suggesting that they might be important to inhibit excessive T-cell responses. Several proteins expressed by pDCs were found to promote IL-10 induction in T cells, including the Notch ligand Delta-like 4 (108), ICOSL (109, 110), as well as IFN-α (23, Nizzoli et al., under review).

Myeloid DCs are a heterogeneous population, and different mDC subsets can be identified that preferentially initiate different types of adaptive immune responses (**Figure 1**). In the spleen of mice, mDCs can be subdivided into $CD8\alpha^+$ and $CD8\alpha^-$ subsets (**Table 1**). $CD8\alpha^+$ mDCs produce high levels of bioactive IL-12p70 and efficiently cross-prime $CD8^+$ T-cell responses (111). They express CLEC9A, a C-type lectin, that enables them to take up antigenic material from dying cells, and their generation was shown to rely on the transcription factors BATF3 and IRF8 (112, 113). Moreover, they express the chemokine receptor XCR1 that favors their colocalization with $CD8^+$ T cells (114). Altogether the present evidence indicates that $CD8\alpha^+$ DCs are specialized to induce Th1 and CTL responses in response to intracellular pathogens (115, 116). Notably, DCs in the gut that express CD103 have similar characteristics and are closely related to $CD8\alpha^+$ DC (117, 118). $CD8\alpha^-$ DCs express CD11b and can be further subdivided into $CD4^+$ and $CD4^-CD8^-$ subsets. They preferentially prime $CD4^+$ T-cell responses (119) and promote Th17 responses, but they can also induce Th2 cells (113, 120). Interestingly, $CD11b^+$ DCs produce IL-23 in the gut and are required for protection against *Citrobacter rodentium* (121). Their generation depends on the transcription factor IRF4, while KLF4 expression is required for Th2, but not for Th17 induction (122). Notably, however, $CD8\alpha^-$ DCs and also pDCs can cross-prime $CD8^+$ T-cell responses under certain conditions (102–104, 123). Moreover, it was shown that upon appropriate microbial stimulation all mDC subsets have the potential to promote either Th1 or Th2 responses (124). Thus, although the proposed functional specialization of DC subsets is an intriguing and helpful concept, it might also be an oversimplification, since DC subsets have considerable plasticity and the induction of a specific type of immune response critically depends on the stimuli they receive from pathogens as well as from other immune cells (125).

DIFFERENT PATHOGEN SENSING BY DC SUBSETS IN HUMANS AND MICE

High numbers of human DCs can be generated *in vitro* by culturing monocytes with cytokines (41), and the large majority of studies on human DCs have been done with these monocyte-derived DCs. They are primary cells and show many behaviors of *in vivo* occurring DCs, including cytokine production as well as stable and potent antigen presentation upon maturation with TLR ligands (125). However, monocyte-derived DCs are not the appropriate model to study the role of specialized DC subsets in human immune responses.

Dendritic cells circulating at low frequency in human peripheral blood share several features with murine splenic DC subsets (126) (**Table 1**). Human pDCs have been identified more than 15 years ago as the natural IFN-α-producing cells (127, 128). They express TLR7 and TLR9 and produce large amounts of IFN-α in response to CpG DNA or influenza virus. Similar to their murine counterparts, they are poorly stimulatory (94), express the C-type lectin BDCA-2 (93), and induce IL-10 production in $CD4^+$ T cells (129). In addition, subsets of mDCs can also be found in human blood and in tissues (130–133). As their murine homologues, they express CD11c and potently prime $CD4^+$ and $CD8^+$ T-cell responses. The expression of CD1c/BDCA-1 and CD141/BDCA-3 identifies two subsets among human mDCs in peripheral blood (93) and also in secondary lymphoid organs (105, 132, 134, 135). $BDCA\text{-}3^+$ "mDC2" (**Table 1**) are rare, but it could recently be demonstrated that they represent the human counterpart of murine $CD8\alpha^+$ DCs (136–140). Thus, as $CD8\alpha^+$ DCs, they selectively express CLEC9A and XCR1 and are dependent on the transcription factor BATF3 (112, 136, 138, 140, 141). Importantly, they can cross-present exogenous antigens on MHC class-I to $CD8^+$ T cells and produce IL-12 (134–136). $CD1c^+$ "mDC1" (**Table 1**) are more frequent and share some features with $CD8\alpha^-$ DC, including CD11b expression and IL-23 production (121, 142, Nizzoli et al., under review). Also TLR3 expression in DC subsets appears to be similar in humans and mice, since it is expressed at high levels on $CD8\alpha^+$ DCs and mDC2, at lower levels on $CD8\alpha^-$ DCs and mDC1, and absent on pDC. Surprisingly, TLR3 in mice is not required for immune responses against several viruses, including LCMV, VSV, MCMV, and Reovirus, suggesting that TLR3 has not a pivotal role in antiviral immune defense (143). Consistently, TLR3 deficiency in humans selectively leads to uncontrolled HSV1 infections in the central nervous system (CNS) (144).

Different subsets of DC have also been identified in human non-lymphoid tissues where they are strategically positioned to recognize invading pathogens, in particular at barrier surfaces. These migratory DC subsets play a crucial role to transport antigenic material of pathogens that invade specific tissues to draining

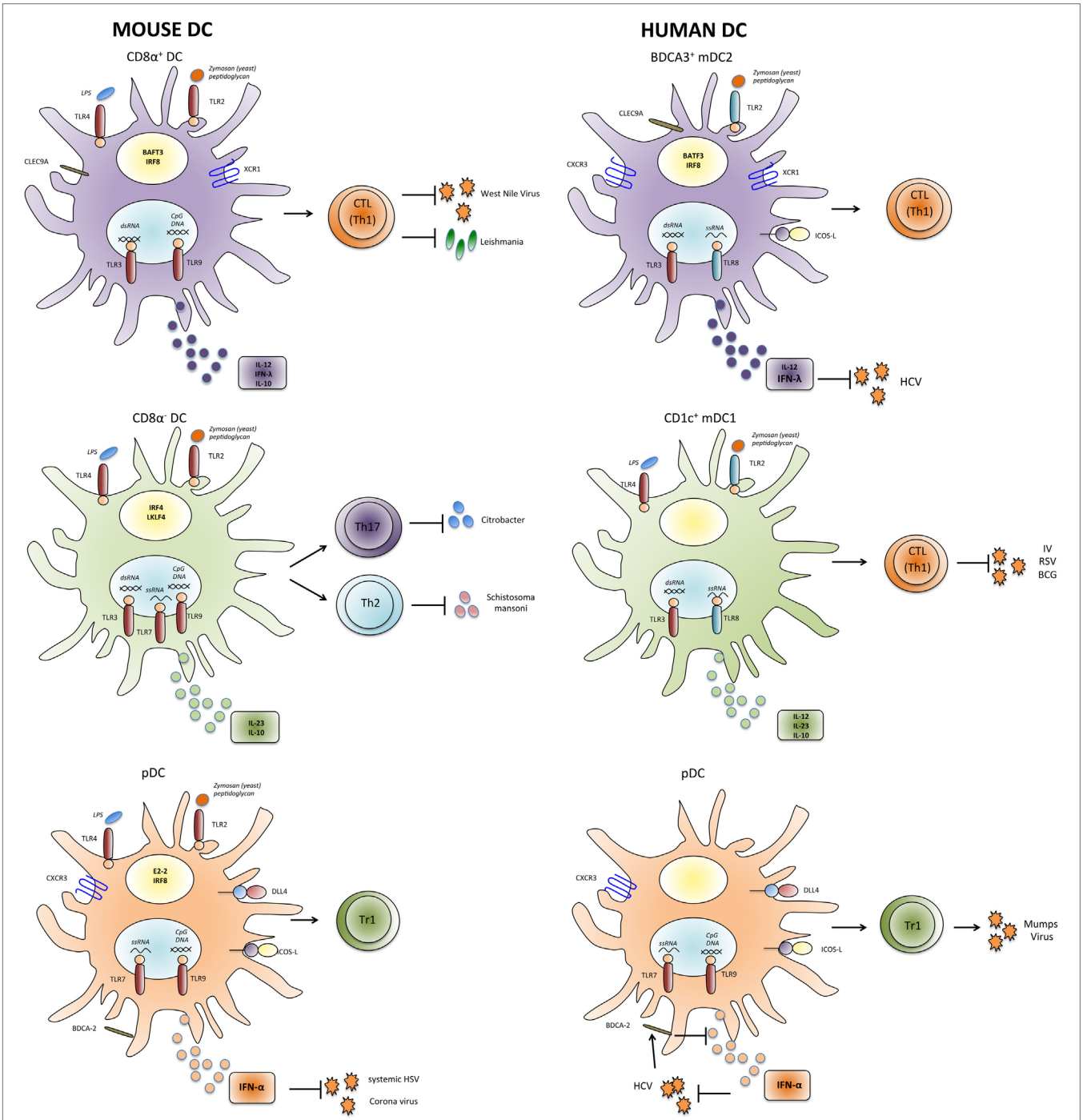

FIGURE 1 | Properties and functions of human and mouse DC subsets. Human and mouse mDC and pDC subsets express partially different patterns of pathogen-sensing receptors and cytokines and might thus have unique functions in inducing appropriate types of T-cell responses against individual pathogens. IV, influenza virus; HCV, hepatitis C virus; RSV, respiratory syncytial virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; BCG, Bacillus Calmette–Guérin.

lymph nodes and thus to initiate a tissue-specific T-cell response (130, 145, 146). Human Langerhans cells were first described more than a century ago and reside in the epidermis and are thus the first DCs that encounter skin-invading pathogens. Upon activation, they mature and migrate to draining lymph nodes to activate $CD4^+$ and $CD8^+$ T cells. In the dermis, different subsets of interstitial DCs are present and can be classified according to CD14, CD1a, and CD141 expression. Dermal $CD14^+$ cells might represent monocyte-derived macrophages rather then DCs (147), but $CD1a^+$ and $CD141^+$ DCs, respectively, resemble the

CD1c$^+$ and CD141$^+$ DC subsets in peripheral blood (148). Also in the lung and the liver, DC subsets that are related to CD1c$^+$ and CD141$^+$ DCs could be identified (133). Finally, in the human intestine, DC subsets that express CD11b and CD103 are similar to CD1c$^+$ and CD141$^+$ DCs, respectively, and these intestinal DC subsets are also largely conserved between humans and mice (149).

Although the similarities between human and mouse DC subsets are often emphasized, there are also some important differences in pathogen sensing by DCs in humans and mice (150). Importantly, the expression of several relevant TLRs is not conserved (**Figure 1**), presumably because humans and mice have evolved under the selective pressure of different pathogens. Thus, in mice, TLR7 and TLR9 are expressed by both pDC and mDC subsets (71), whereas in humans, they are restricted to pDCs (72). Also TLR4 expression is more restricted in human DCs, since it is expressed by mDC1 but not by mDC2 (136). Moreover, TLR8 is not expressed by human pDCs (72), and some agonists of human TLR8 such as the resiquimod R848 do not activate murine TLR8 (75, 151). Another relevant difference seems to be the role of the adaptor protein Myd88, which transduces signals from all TLRs with the notable exception of TLR3. Thus, mice deficient for Myd88 are highly susceptible to several infections by bacteria, viruses, parasites, and fungi. Conversely, Myd88-deficient patients are selectively affected by infections with pyogenic bacteria in childhood (152). Finally, human CD1c$^+$ DCs and also Langerhans cells seem to have superior capacities to cross-present antigens and to induce CTL responses as compared to their murine homologues (105, 134, 153, 154). Overall, these differences in pathogen sensing and T-cell activation between human and murine DCs are likely to have an important impact on their role in immune responses against specific pathogens.

SUBSET-SPECIFIC CYTOKINE PRODUCTION BY HUMAN DCs

Dendritic cell subsets in humans and mice express not only different patterns of toll-like receptors, but they have also partially distinct cytokine profiles (**Figure 1**). In particular, human mDC1 have a complex and quite unique regulation of cytokine production. Thus, while LPS triggers only low levels of cytokine production by mDC1, dual TLR stimulation with LPS or Poly-I:C (TLR3 ligand) in combination with R848 induces very high levels of a broad panel of cytokines, including TNF, IL-6, IL-10, IL-12, and IL-23 (Nizzoli et al., under review). The very potent cytokine-producing capacity of mDC1 has been missed in several studies where mDC1 were activated with single TLR ligands (45, 155, 156). Of note, single TLR stimulation is sufficient to induce antiviral cytokines by mDC2 and pDCs (see below) and proinflammatory cytokines by monocytes. Although mDC1 can secrete several proinflammatory cytokines that promote Th17 cell generation including IL-23 (142), it is unclear if they are the physiological inducers of Th17 cells or if monocyte-derived, inflammatory DCs do the job (12, 157). Also the identity of the DC subset that induces human Th2 responses is still enigmatic. It was originally proposed that mDCs induce Th1 polarization and pDCs Th2, but later it was shown that also pDCs can drive Th1 responses (158, 159). More recently, mDC2 but not mDC1 were found to induce Th2 cells in an aberrant response to influenza virus (160).

In apparent contrast to CD8α$^-$ DCs, mDC1 can produce high levels of IL-12 (134, 135), suggesting a relevant role in immune responses against intracellular pathogens. Moreover, the production of the anti-inflammatory cytokine IL-10, which can be produced by all mDCs in mice, is largely restricted to mDC1 in humans (Nizzoli et al., under review). Stimulation of mDC1 with the intestinal bacterium *Escherichia coli* or with LPS alone induces IL-10 and was proposed to induce a tolerogenic state in mDC1 (155). Although IL-10 is indeed a tolerogenic cytokine and a well-established negative regulator of DC maturation and cytokine production (161), it can paradoxically also have positive effects, in particular on CD8$^+$ T-cell responses (162, 163). Consistently, we found that IL-10 produced by mDC1 completely blocked the cross-priming of low-affinity CTL and enhanced the responsiveness of CD8$^+$ memory T cells to the homeostatic cytokine IL-15. Thus, mDC1-derived IL-10 appears to play an important positive role in CTL responses, since it selects high affinity cells upon priming and inhibits CTL memory attrition at the same time (Nizzoli et al., under review).

While mDC1 can secrete a broad panel of pro- and anti-inflammatory cytokines, mDC2 and pDC are largely dedicated to secrete high levels of antiviral cytokines. The subset-specific production of IFN-α by pDC (128) and of IFN-λ by CD8α$^+$ and mDC2 (134, 137) appears to be largely conserved between humans and mice. The very potent IFN-λ-producing capacities of BDCA-3$^+$ DC (134, 137) suggest that analogous to pDCs they might be the relevant source of early and systemic IFN-λ in viral infections. Notably, IFN-λ has antiproliferative and antiviral activities similar to type-I interferon, but the expression of the IFN-λ receptor is much more restricted and found mainly on epithelial cells at barrier surfaces and in the liver (164). MDC2 can also secrete selected isoforms of IFN-α (165) and some IL-12 (134–136, 138), consistent with the view that they play an important role in antiviral immune responses. As previously mentioned for murine pDCs, IFN-α is not only a powerful antiviral cytokine that activates several different types of immune cells, but it also induces IL-10 production in CD4$^+$ T cells, suggesting that pDCs induce Tr1-like regulatory T cells also in humans (21, 23, 108, Nizzoli et al., under review).

SPECIFIC ROLES OF HUMAN DC SUBSETS IN RESPONSES TO INDIVIDUAL PATHOGENS

The more restricted expression of TLRs and the specific cytokine-producing capacities of human DC subset suggest that they play unique roles in immune responses against individual pathogens. The roles of human DC subsets in pathogen-specific immune response are however difficult to address directly because patients that selectively lack a DC subset of interest have not

been identified so far. Nevertheless, some interesting findings were reported. In particular, mDC2 appear to be highly relevant in HCV infection. Single-nucleotide polymorphisms in the IFN-λ3 gene locus are strongly associated with spontaneous clearance and response to therapy in HCV patients (166). All DC subsets can secrete some IFN-λ1 (134, 167), but mDC2 produce much higher amounts. Moreover, IFN-λ2/3 are largely restricted to mDC2, and importantly HCV induces IFN-λ3 production by mDC2 (168, Nizzoli et al., under review). Thus, mDC2 appear to be a highly relevant source for protective IFN-λ3 in HCV infection (169). Interestingly, an important role for mDC1 rather than for mDC2 was recently proposed in tuberculosis (170, 171). Thus, mDC1 were more efficiently infected with the Bacillus Calmette–Guérin (BCG) vaccine than other DCs and induced the activation of pDCs and $CD8^+$ T cells. Notably, mDC1 could not be replaced by mDC2 in this system, suggesting that mDC1 could play a non-redundant role in the defense against selected intracellular pathogens. MDC1 and mDC2 have also been suggested to play different roles in RSV infection (172, 173). Thus, mDC subsets produced different cytokines in response to RSV, consistent with their different cytokine profiles upon stimulation with purified TLR ligands (134, Nizzoli et al., under review). Moreover, they induced different classes of T-cell responses, with mDC1 inducing preferentially Th1 cells and mDC2 inducing predominantly Th2 and T-regulatory cells. Similarly, mDC2, but not mDC1, were found to induce Th2 response to influenza virus (160). Also the capacity of pDCs to induce IL-10-producing regulatory T cells has been documented with a relevant pathogen, since pDCs were shown to induce IFN-γ and IL-10 production in antigen-experienced $CD4^+$ T cells specific for mumps virus (129). Conversely, $CD11c^+$ mDCs, which contain both mDC1 and mDC2, induced IFN-γ and, surprisingly, IL-5.

It is largely accepted that pDC-derived IFN-α is important to contain human viral infections. Thus, stabilized pegylated IFN-α is a widely used therapy for HCV patients. IFN-λ appears to be similar effective, but is less toxic presumably because of the more restricted expression of its receptor (164). Interestingly, the HCV glycoprotein E2 is a ligand for BDCA-2, which is specifically expressed on pDCs (**Table 1**) and inhibits IFN-α production (174, 175). In this way, HCV might inhibit IFN-α production to establish chronic infection. Finally, pDCs are also targeted by human immunodeficiency virus (HIV), but whether they play a protective or detrimental role is still unclear (176).

EXPLOITING DC BIOLOGY: VACCINES THAT INDUCE HUMORAL AND CELLULAR IMMUNE RESPONSES

Vaccines have been a major breakthrough for human health. Attenuated or killed pathogens are highly efficient to induce protective cellular and humoral immune responses, and the induced protective memory can last for a lifetime (177, 178). However, since these pathogen-based vaccines also have considerable side effects, proteins in combination with adjuvants that activate APCs are more often used. Protein vaccines induce $CD4^+$ T-cell responses and neutralizing antibodies, but they are poorly efficient in inducing cytotoxic T-cell responses and are also rather inefficient in inducing Th1 cells (179, 180). Frequently used adjuvants are alum, oil-in-water emulsions like MF59, and more recently also monophosphoryl lipid A (MPL), a detoxified form of LPS. In mice, different adjuvants were shown to induce different proinflammatory cytokines. Thus, alum acts via uric acid on inflammatory DCs (181), which leads to NOD-like receptor protein-3 (NALP3)-dependent IL-1β production (182). Conversely, MPL does not induce IL-1β (183) but induces specific antibodies through an IL-6-dependent mechanism (184), while MF-59 and alum act independently of IL-6 (185). However, the different TLR expression and cytokine production by human APC subsets should be considered when translating this knowledge from animal models to patients. A recent interesting report analyzed the response of APC subsets to 13 different vaccines and concluded that different vaccines activate indeed different APC populations (186). More direct information on the effect of DCs was obtained by vaccinations with peptide-pulsed monocyte-derived DCs in cancer patients, which can induce tumor-specific $CD8^+$ T cells (187), but the clinical responses were so far largely insufficient. MDCs might be more potent and are currently tested in clinical trials.

Nucleic acid-sensing TLRs are particularly potent to induce $CD8^+$ T-cell responses in mice (188) and have recently been employed as adjuvants in vaccines. Examples are CpG-DNA that stimulates TLR9 (189), and the TLR7 ligand imiquimod, which is used as a cream to stimulate DC locally in the skin, and was shown to induce $CD8^+$ T-cell responses *in situ* (190). Vaccines consisting of plasmid DNA coding for relevant protein antigens are a novel approach that efficiently induces humoral and cellular immune responses in animals. However, in humans, these DNA vaccines are often poorly immunogenic (191), presumably because they have only low adjuvant activity and stimulate mainly cytosolic DNA sensors rather than TLR9 (192), which in addition is restricted to pDCs and B cells in humans. An alternative promising approach is the vaccination with mRNA (193, 194), which delivers not only the antigenic protein directly to the cytosol, thereby bypassing the requirements for cross-presentation (195), but also induces mDC and pDC maturation and cytokine production via TLR7/8 at the same time (196). Indeed, intradermal injection of naked mRNA results in local uptake and translation of the nucleic acid (197) followed by the development of an adaptive immunity in mice (198) and in humans (199, 200). Since also lymph node-resident DCs are expected to be appropriate APCs to process antigens encoded by mRNA, direct injection of nucleic acid into lymph nodes has also been evaluated. In animal models, intra-lymph node injections of mRNA result in expression of the protein encoded by the mRNA in DCs. Furthermore, the injected mRNA activated lymph node-resident APCs and induced potent $CD4^+$ and $CD8^+$ T-cell responses as well as prophylactic and therapeutic antitumor immunity (201). The approach is currently being evaluated through two clinical studies exploring the efficacy of intra-lymph node mRNA vaccination in advanced melanoma patients. As a further development, systemic administration of a liposomal formulation of mRNA that delivers the nucleic acids

to APCs present in secondary lymphoid organs is also being evaluated. Using the functional diversity of DCs *in vivo*, and their specific capabilities in generating appropriate adaptive immune responses, those systemic synthetic vaccines might recapitulate the natural mechanisms of immunity developed during pathogen infection and guarantee the development of therapeutically efficacious immune responses.

CONCLUSION AND PERSPECTIVE

Dendritic cells continue to attract much interest of immunologists because they are the most potent APCs in the immune system and are the principal inducers of naive T-cell differentiation. Intensive research in the last years has established that different subsets of DC exist in mice that have specialized functions and preferentially induce different types of immune responses. In humans, much has been learned from *in vitro* differentiated monocyte-derived DCs, and more recently, also different subsets of DC populating human tissues have been analyzed at the molecular and functional levels. It is fundamental to further define the biology of these *in vivo* occurring human DC subsets to understand and cure pathogenic immune-mediated processes in so different settings as autoimmunity, infections, and cancer. In particular, appropriate targeting of DC subsets by vaccines holds the promise to induce cytotoxic T-cell responses to eradicate persistent intracellular pathogens or tumors.

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REVIEW
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Functional specialization of skin dendritic cell subsets in regulating T cell responses

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Dendritic cells (DC) are a heterogeneous family of professional antigen-presenting cells classically recognized as most potent inducers of adaptive immune responses. In this respect, Langerhans cells have long been considered to be prototypic immunogenic DC in the skin. More recently this view has considerably changed. The generation of *in vivo* cell ablation and lineage tracing models revealed the complexity of the skin DC network and, in particular, established the existence of a number of phenotypically distinct Langerin$^+$ and negative DC populations in the dermis. Moreover, by now we appreciate that DC also exert important regulatory functions and are required for the maintenance of tolerance toward harmless foreign and self-antigens. This review summarizes our current understanding of the skin-resident DC system in the mouse and discusses emerging concepts on the functional specialization of the different skin DC subsets in regulating T cell responses. Special consideration is given to antigen cross-presentation as well as immune reactions toward contact sensitizers, cutaneous pathogens, and tumors. These studies form the basis for the manipulation of the human counterparts of the murine DC subsets to promote immunity or tolerance for the treatment of human disease.

Keywords: contact hypersensitivity, cross-presentation, dendritic cells, immunotherapy, infectious skin disease, Langerhans cells, Langerin, skin cancer

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INTRODUCTION

The skin is the second largest barrier organ to the outside world besides the intestine. As such it is not only exposed to physical stress but also to a wide variety of environmental antigens, including chemicals, commensal bacteria, and pathogens. Hence, the skin immune system must be prepared to detect and discriminate between these diverse antigens and subsequently induce appropriate tolerogenic or protective immune responses. To this aim, the skin contains a heterogeneous population of dendritic cells (DC, from Greek *dendron* "tree") that represent key regulators of both innate and adaptive immune responses. While skin DC play a critical role in guarding the host against invading pathogens and at the same time limiting collateral tissue damage, they are also associated with the breakdown of peripheral tolerance leading to chronic immune-mediated inflammatory diseases such as allergic contact dermatitis and psoriasis. As essential mediators of cutaneous immune reactions and homeostasis, considerable work has been focused to unravel the origins, phenotypic, and functional differences of the cells of the skin DC network (1–3).

Anatomically, the skin can be divided into an outer epidermis and the underlying dermis, which are separated by a basement membrane. The cell-free basement membrane acts as a mechanical barrier, however, its primary function is to anchor the epithelium (epidermis) to the loose connective tissue (dermis) underneath. The epidermis represents a stratified epithelial layer composed of keratinocytes that generate the water-impermeable *stratum corneum*. The dermis is a cell-poor layer consisting of fibroblasts that produce the extracellular matrix containing proteoglycans and entwined collagen and elastic fibers. Together they enable the skin to resist stretching and tearing forces. In addition to forming the primary physical barrier, keratinocytes also actively contribute to the immunological barrier of the skin. They are equipped with most toll-like receptors (TLR), except TLR7 and TLR8 (4–6). Following TLR triggering and NOD-like receptor (NLR)-mediated inflammasome activation, keratinocytes secrete antimicrobial peptides and many proinflammatory cytokines as well as chemokines for the recruitment of neutrophils. Thereby keratinocytes participate in adaptive immune activation, via inducing DC mobilization and migration to skin-draining lymph nodes (LN), and innate immune modulation (7).

DC can be subdivided into conventional DC and plasmacytoid DC (pDC). Healthy skin contains no or very few pDC (8, 9); they only enter inflamed skin to promote wound healing through type-I interferons (9) or mediate the proinflammatory reaction that develops after TLR7 stimulation, for example, during psoriasis (10). An excellent overview of pDC biology and plasticity has recently been published elsewhere and these cells are not further discussed here (11).

In the steady state, the conventional DC residing in the skin are not inactive. Rather as immature cells, they constantly probe their environment for invading pathogens and continuously sample self- and environmental antigens (**Figure 1**). To this aim, epidermal Langerhans cells (LC) exhibit a unique behavior characterized by rhythmic extension and retraction of their dendrites through intercellular spaces between keratinocytes, which is amplified during inflammation (12). In fact, LC can extend dendrites through tight junctions to survey the skin surface and elicit humoral immunity to antigens that have not yet violated the epidermal barrier, providing preemptive immunity against potentially pathogenic skin microbes (13).

A small fraction of LC and dermal DC undergoes spontaneous maturation through a mechanism that is not yet understood (14). This *homeostatic* or *phenotypic maturation* involves the upregulation of chemokine receptor CCR7, which enables DC migration to the skin-draining LN (15), and in the case of LC downregulation of E-cadherin to detach themselves from the surrounding keratinocytes (16). Moreover, disruption of E-cadherin binding may actively promote a tolerogenic LC phenotype via the release and nuclear localization of β-catenin (17, 18). During their migration to the T cell areas of local LN, the cells upregulate surface expression of MHC/peptide complexes for recognition of and interaction with antigen-specific naïve T cells (**Figure 1**) (19–22). Upon encounter with potentially autoreactive T cells that have escaped central tolerance or with T cells recognizing peptides derived from innocuous foreign antigens, these DC induce T cell anergy or deletional T cell tolerance (*tolerizing function*) (23–26). In addition, the frequent T cell–DC contacts during T cell scanning of DC in lymphoid organs, i.e., in the absence of cognate antigen, induce a basal activation level in T cells required for rapid responsiveness to subsequent encounters with foreign antigen during inflammation (27).

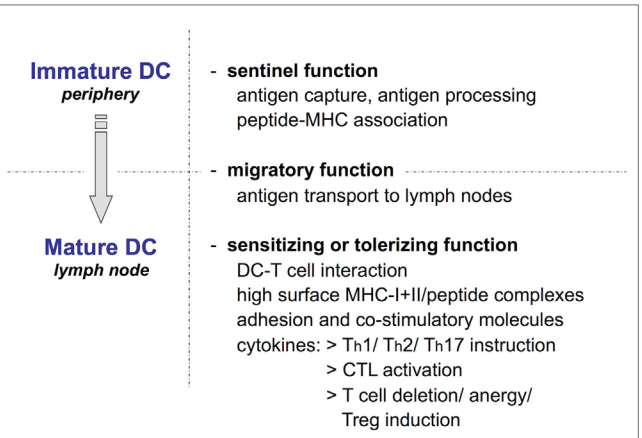

FIGURE 1 | The Langerhans cell paradigm: Ralph Steinman's scheme of key dendritic cell functions. DC, including epidermal LC, exist in two phenotypically and functionally distinct states: as immature cells highly specialized in antigen uptake and processing and as mature cells committed to antigen presentation that activate or tolerize naïve T cells. The two functional programs are connected by DC migration from peripheral tissues to draining LN, which is essential for naïve T cells to encounter their cognate antigen.

Pathogen invasion together with proinflammatory signals drive the full *functional maturation* of skin DC. Beyond the homeostatic differentiation program, the cells now also upregulate the expression of costimulatory molecules and, in particular, proinflammatory cytokines. Together these promote clonal expansion of naïve antigen-specific T cells and instruct the T cells to acquire appropriate effector functions specifically tailored to eliminate the invading pathogen (*sensitizing function*) (**Figure 1**) (19).

In this review, we describe our current understanding of the composition of the skin DC network and summarize the transcription and growth factor requirements for the development of the different skin DC populations. We then discuss the functional specialization of skin DC subsets in the context of allergic and infectious skin disease models, as well as their cross-presentation capacity and their role in skin cancer. Finally, we focus on how this knowledge may be applied to harness skin DC for therapeutic purposes and, to this aim, conclude with a comparison of mouse and human skin DC subsets.

THE SKIN-RESIDENT DENDRITIC CELL NETWORK

After the discovery of DC by Ralph Steinman and Zanvil Cohn in 1973 (28), it was only in 1985 that epidermal LC (**Figure 2**), first described by Paul Langerhans as "*Nerven der menschlichen Haut*" more than a century before (29), were unequivocally placed into the DC family (30, 31). One of the most important findings of these early studies on LC was that DC exist in two phenotypically and

functionally distinct states: as immature cells that are highly phagocytic and specialized to take up and process antigen, and as mature cells dedicated to identify and stimulate rare antigen-specific naïve T cells in secondary lymphoid organs (**Figure 1**). This observation is directly linked to another unique function of DC, namely, their migration via afferent lymphatics into the T cell areas of secondary lymphoid organs (19). In fact, in early mixed lymphocyte reaction (MLR) experiments DC turned out to be about 100-fold more efficient at inducing naive T cell proliferation than macrophages (32–34), which also do not travel from peripheral tissues to local LN (35). Owing to their easy accessibility and a large body of *in vitro* work, which almost inevitably triggers LC functional maturation, much of what we know today about the role of DC as most potent inducers of T cell immune responses stems from studying LC biology. Hence, for a long time LC were considered prototypic immunogenic DC for which Wilson and Villadangos later coined the term "*LC paradigm*" (36) and dermal DC were largely overlooked.

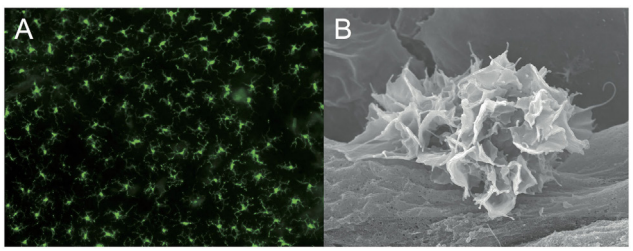

FIGURE 2 | Langerhans cells: sentinels of the skin. **(A)** LC network visualized in an epidermal sheet of mouse ear skin with MHC-II antibody staining (green fluorescence) (37). Photograph by courtesy of Julia Ober-Blöbaum and Björn Clausen. **(B)** Scanning electron microscopy of a LC sitting on a keratinocyte (38). Photograph by courtesy of Kristian Pfaller and Patrizia Stoitzner.

This picture began to change dramatically with the identification of Langerin (CD207), a novel C-type lectin specific to LC (39–41) and the generation of anti-Langerin monoclonal antibodies (42, 43). Although originally described as a LC-specific marker, in combination with constitutive and inducible Langerin$^+$ cell depletion models and a Langerin-EGFP knock-in allele (44–46), this led to the discovery of a small Langerin$^+$ dermal DC subset that is ontogenetically and phenotypically distinct of epidermal LC (47–49). Largely owing to the comprehensive analysis of the Malissen lab to disentangle the complexity of the skin DC network, we can currently distinguish five distinct DC subsets in healthy mouse skin (**Figure 3**) (50, 51). All of these DC populations express CD11c and MHC class II (MHC-II). (*i*) LC in the epidermis as well as in the dermis – en route to skin-draining LN – can be identified as Langerin$^+$CD11b$^+$EpCam$^+$Sirpα$^+$ cells, and distinguished from CD11b$^+$Sirpα$^+$ dermal DC by their absence of Langerin and EpCam staining. (*ii*) CD11b$^+$ DC are the most abundant subset and comprise about 65% of all dermal DC (51). (*iii* and *iv*) Langerin$^+$CD11b^{neg} dermal DC, on the other hand, are unambiguously recognized by expression of the chemokine receptor XCR1, lack EpCam and Sirp1α, and can be further divided into a CD103$^+$ and negative subset. Expression of XCR1 is shared by all CD11b^{neg} non-lymphoid and CD8α$^+$

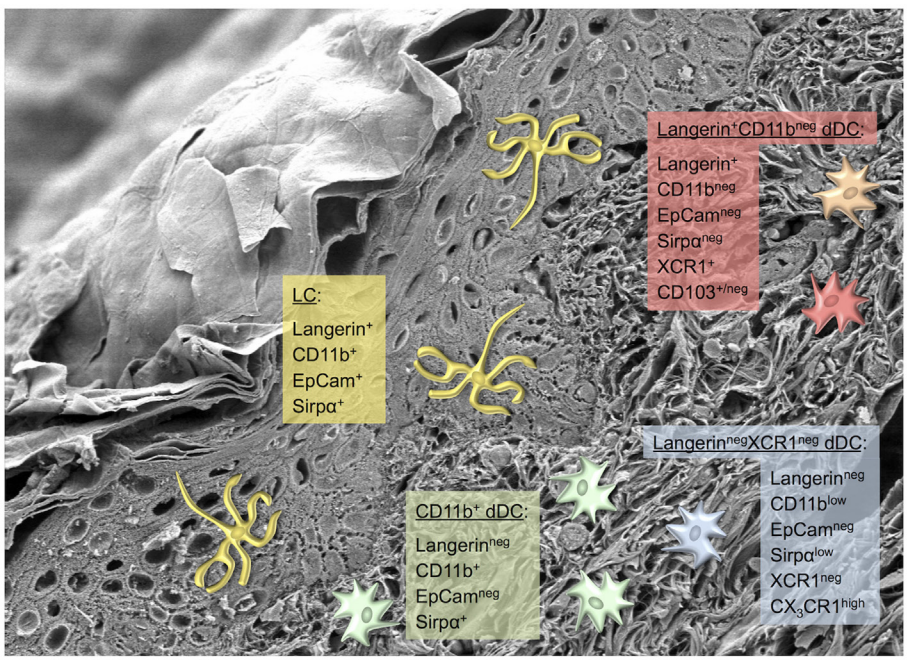

FIGURE 3 | Skin dendritic cell subsets in mice. Scanning electron microscopy picture of a skin section depicting several layers of keratinocytes and the collagen meshwork of the dermis (38). Photograph by courtesy of Kristian Pfaller and Patrizia Stoitzner. Phenotypically distinct murine skin-resident DC subsets are depicted, including the most commonly used markers for their identification. The color code matches the human counterparts shown in **Figure 4**.

lymphoid tissue DC, respectively, but only XCR1$^+$CD11b^{neg} DC in the dermis co-express Langerin (52, 53). Of note, in the skin surface expression of CD24 correlates with that of Langerin and can be used for the purification of viable LC and Langerin$^+$ dermal DC by flow cytometry. (*v*) Finally, the dermis harbors a minor population of Langerin^{neg}XCR1^{neg} double-negative DC that express low levels of CD11b and Sirpα, and are uniquely CX_3CR1^{high} (51). These five conventional skin DC populations can be separated from dermal macrophages and monocyte-derived DC by the use of CD64 (35). In particular during inflammation, large numbers of monocytes infiltrate the skin where they differentiate into CD11b$^+$Ly6C$^+$CD64$^+$ monocyte-derived DC that have very low or lack CD11c expression. These recently identified cells play a role mainly in activating skin-resident T cells and disappear after resolution of the inflammation (35). The functional specialization of LC and the different dermal DC populations that are present in mouse skin will be discussed below.

ORIGIN, TRANSCRIPTION FACTOR, AND SURVIVAL FACTOR REQUIREMENTS OF SKIN DENDRITIC CELL POPULATIONS

LC are radio-resistant cells that reside in the supra-basal layer of the epidermis, closely attached to the surrounding keratinocytes via E-cadherin containing adherens junctions. In the steady state, LC exhibit a low rate of proliferation that – unlike dermal DC – is sufficient to maintain the cells locally throughout life as has been demonstrated by parabiosis experiments in mice (54) and could also be observed in human skin of hand transplant patients (55). Only in response to inflammatory changes leading to an increased loss from the epidermis are LC replaced by blood-borne progenitors. These precursors were recruited in a CCR2-dependent way and identified to represent Ly6C^{high} monocytes that enter inflamed skin and differentiate into LC in the epidermis (56, 57). Whether these monocyte-derived LC are functionally similar and equally capable to maintain themselves *in situ* remains elusive. Recent experiments indicate that the initial wave of monocyte-derived LC reconstitution after UV radiation and contact sensitizer exposure generates only short-term LC that are transient and replaced by a second wave of steady-state precursor-derived long-term LC (58, 59). On the other hand, all dermal DC populations in healthy skin are radiosensitive, have a short lifespan, and are continuously replaced by a circulating pool of bone marrow-derived DC precursors (60).

In contrast to dermal DC that originate from DC-restricted progenitors [reviewed in Ref. (2, 60)], during ontogeny LC arise first from yolk sac-derived primitive myeloid precursors around embryonic day 18 that are largely replaced by fetal liver-derived monocytes during late embryogenesis (61). These LC precursors then acquire a DC morphology and phenotype, including CD11c and MHC-II expression immediately after birth (62), whereas Langerin expression becomes apparent only 2–3 days after birth and reaches adult levels of intensity only by 3 weeks of age (63). Moreover, between postnatal days 2 and 7 the LC undergo a massive proliferative burst (62), before reaching a typical density of about 700–1,000 LC/mm^2 in the epidermis of adult mice (37) (**Figure 2**). Intriguingly, LC share this embryonic ancestry from myeloid precursors and the capacity of self-maintenance throughout life without any input from the bone marrow with brain microglia. While macrophage colony-stimulating factor 1 (M-CSF or CSF-1) is essential for the development of most tissue macrophages and partly for CD11b$^+$ dermal DC (64), CSF-1R-deficient mice in addition lack both LC and microglia. Their development requires the presence of the alternative CSF-1R ligand IL-34 that is constitutively produced by keratinocytes and neurons (65, 66). Based on this unique life cycle and the shared pedigree with certain tissue macrophages, LC have recently been grouped into the same lineage as macrophages (67, 68). Although ontogenetically LC and macrophages are closely related cells, in stark contrast to the sessile tissue macrophages LC migrate to LN where they prime naïve T cells to induce regulatory or effector responses. Since migration and naïve T cell priming represent cardinal features characterizing conventional DC (**Figure 1**), we strongly favor to keep LC in the DC family. Reciprocally, from a semantic point of view it has to be stressed that the term "macrophage" (from Greek *makrós* "large, big" and *phagein* "eat" = "big eaters") describes a function for which DC are certainly not specialized as has been worked out so beautifully by Ralph Steinman (69–71).

Another cytokine that has been known for a long time to be essential for LC differentiation is transforming growth factor-β1 (TGF-β1) (72). Although TGF-β1 is produced by both LC and keratinocytes, LC promote their own development through an autocrine loop of TGF-β1 secretion and signaling (73). In addition, TGF-β1 is required to maintain the network of immature LC in the epidermis (74, 75). In line with the critical role of TGF-β1 for LC development, mice lacking the TGF-β1-induced transcription factor inhibitor of DNA binding 2 (Id2), or the transcription factor Runx3 that mediates DC responses to TGF-β1 both also lack LC (76–78). Moreover, Id2$^{-/-}$ mice have dramatically reduced numbers of lymphoid organ CD8$^+$ and non-lymphoid tissue CD103$^+$ DC (64).

The cytokine Flt3L is a key mediator of DC commitment during hematopoiesis (79) and injection of Flt3L into mice dramatically increased DC numbers in various tissues (80). Beyond its role in DC differentiation, Flt3L regulates the homeostatic proliferation of DC to maintain peripheral DC numbers in the steady state (81). With respect to skin DC subsets, LC are not affected by the absence of Flt3 or its ligand, whereas dermal DC were reduced in Flt3$^{-/-}$ and Flt3L$^{-/-}$ mice (64, 82). Granulocyte/macrophage colony-stimulating factor (GM-CSF or CSF-2) is essential for DC differentiation (83), and mice lacking either CSF-2 or its receptor display a reduction of LC and CD103$^+$ dermal DC (84, 85). In the absence of macrophage colony-stimulating factor (M-CSF or CSF-1), LC numbers were halved (86), and mice that lack the M-CSF receptor (CSF-1R) have no LC and reduced CD11b$^+$ dermal DC, while CD103$^+$ dermal DC develop normally (56, 64).

Moreover, a number of interferon regulatory factors (IRF) and other transcription factors have been implicated in the development of different DC subsets, albeit with incomplete available information concerning their effects on LC and dermal DC. IRF2-deficient mice exhibit reduced numbers of

splenic CD4$^+$ DC and epidermal LC, while dermal DC subsets have not been assessed (87); IRF4$^{-/-}$ mice harbor reduced numbers of splenic CD4$^+$ DC and of migratory LN DC due to a defect in dermal DC migration, which leads to an accumulation of CD103$^+$ and CD11b$^+$ dermal DC in the skin (88–90); and IRF8$^{-/-}$ mice lack splenic CD8$^+$ DC and non-lymphoid tissue CD103$^+$ DC, including CD103$^+$ dermal DC, whereas LC are unaffected (64, 76, 91–93). In addition, IRF8 also contributes to DC function: IRF8 controls CD8$^+$ DC maturation and IL-12 production (94), antigen uptake and MHC-II presentation (95), the migration of LC and dermal DC to local LN (96), and the tolerogenic function of DC by inducing the expression of indoleamine 2,3-dioxygenase (IDO) (97). Although the basic leucine zipper transcription factor ATF-like 3 (Batf3) is expressed in all conventional DC, including CD11b$^+$ DC, Batf3$^{-/-}$ mice reveal a selective deficiency of CD8$^+$ and CD103$^+$ DC, however, the penetrance of the CD8$^+$ DC defect seems to depend on the inbred background (91, 98). The transcription factor Zbtb46 represents a negative regulator of DC activation and Zbtb46-deficient mice display no alterations in DC numbers (99, 100). Nevertheless, in Zbtb46-DTR bone marrow chimeras LC and all dermal DC subsets are depleted by the injection of diphtheria toxin (82). The deletion of the late endosomal adaptor molecule p14 (LAMTOR2) caused a gradual loss of LC from newborn mice due to increased apoptosis and a defect in homeostatic LC proliferation. This effect is partly mediated by the downregulation of TGF-β receptor II on LC (59, 101). The phenotypes of different cytokine-, growth factor-, and transcription factor-deficient mice lacking distinct DC subsets are summarized in **Table 1**.

In conclusion, the various skin DC subsets vary in their dependency on different transcription and growth factors, which allows the manipulation of particular subsets to investigate their functional properties. Our current knowledge on the specific roles of cutaneous DC subsets in allergic and infectious skin diseases as well as in skin cancer will be discussed in the following sections.

FUNCTIONAL REDUNDANCY OF SKIN DENDRITIC CELLS IN CONTACT HYPERSENSITIVITY

Contact hypersensitivity (CHS) responses to topically applied haptens in mice represent a relevant model for allergic contact dermatitis. Following percutaneous penetration, the hapten covalently binds to host proteins thereby generating a neo-antigen that is eventually recognized by the immune system (102, 103). The emergence of CHS critically depends on the activation of hapten-specific naïve T cells in skin-draining LN during hapten sensitization, which then proliferate and differentiate into effector T cells that mediate a transient ear swelling reaction at the time of hapten challenge. In agreement with the LC paradigm, although haptens can passively drain to LN via afferent lymphatics, the induction of a productive T cell response hinges on the transport of haptenized antigens by migratory skin DC to the T cell areas of the nodes. Therefore, when the first *in vivo* LC ablation mouse models were introduced, it came as a surprise that CHS was similar (46) or reduced, but not absent (44), after inducible depletion of LC in the skin prior to hapten sensitization (**Table 2**). These findings suggested that LC were not essential to induce the ear swelling reaction and that dermal DC contributed to T cell activation in CHS. Moreover, LC had no role in regulating the effector T cell response as was demonstrated by comparable ear swelling following diphtheria toxin treatment after sensitization but prior to hapten challenge (46, 104).

When the Langerin$^+$ dermal DC subset was discovered in 2007 (47–49), it became clear that these initial experiments had been performed in the absence of both Langerin$^+$ skin DC

TABLE 1 | Phenotypes of transcription factor and growth factor/receptor knockout mice lacking specific skin-resident dendritic cell subsets[a].

Transcription/growth factor/ receptor knockout	Lymphoid tissue DC		Skin/non-lymphoid tissue DC			Reference
	CD8$^+$ DC	CD8^{neg} DC	LC	CD103$^+$ DC	CD11b$^+$ DC	
Batf3	–	↔	↔	–	↔	(91, 92, 98)
CSF-1 (M-CSF)	↔	↔	↓	n.d.	n.d.	(86)
Csf-1R	↔	↔	–	↔	↓	(56, 64–66)
CSF-2 (GM-CSF)	↔	↔	↓	↓	↔	(83–85)
Csf-2R	↔	↔	↔	↓	↔	(84, 85)
IL-34	↔	↔	–	↔	↔	(65, 66)
Flt3	↓	↓	↔	↓	↓	(64, 79, 81, 82)
Id2	–	↔	–	–	↔	(64, 76, 78)
IRF2	↑	↓	↓	n.d.	n.d.	(87)
IRF4	↑	↓	↔	↑	↑	(76, 88–90, 93)
IRF8	–	↔	↔	–	↔	(64, 76, 91–93, 98)
LAMTOR	↔	↔	–	↓	n.d.	(59)
Runx3	↑	↓	–	n.d.	n.d.	(77)
TGF-β1	↔	↔	–	↔	↔	(72–75)
Zbtb46[b]	↔	↔	↓	↓	↓	(82, 99, 100)

[a]*– indicates an absent cell population, ↓ indicates a reduction, ↔ no change, and ↑ an increase in cell number.*

[b]*Includes data from diphtheria toxin-treated Zbtb46-DTR bone marrow chimeras.*

n.d. = not determined.

TABLE 2 | Contact hypersensitivity reactions in mice with specific defects in skin dendritic cell subsets[a].

Mouse strain	Epidermal LC	Langerin$^+$CD103$^+$ dermal DC	Langerin^{neg}CD11b$^+$dermal DC	CHS	Reference
DT inducible cell depletion systems					
Langerin-DTR (DT days −1 to −3)	–	–	↔	↓ or ↔ (dependent on hapten conc.)	(44, 46, 47, 104, 105)
Langerin-DTR (DT days −7 to −13)	–	↓ (30%)	↔	↓ or ↔ (dependent on hapten conc.)	(47, 105)
Langerin-DTR BM → WT chimeras	↔	–	↔	↔	(106)
hLangerin-DTR	–	↔	↔	↑	(107)
Constitutive cell deficiency					
hLangerin-DTA	–	↔	↔	↑	(45)
LC/DC-specific TGF-βR1$^{-/-}$	–	↔	↔	↓	(75, 108)
LC/DC-specific p14$^{-/-}$	–	↔	↔	↓	(59)
Batf3$^{-/-}$	↔	–	↔	↔	(91)

[a]*– indicates an absent cell population, ↓ indicates a reduction and ↔ means no change in cell number, functionality or CHS intensity, and ↑ indicates an increase in the CHS reaction.*

populations and not in the selective absence of epidermal LC as one had assumed [because all Langerin$^+$ DC in the dermis were considered to be LC en route to local LN (46)]. However, in agreement with their continuous replenishment from blood-borne precursors, it turned out that following injection of diphtheria toxin the dermal Langerin$^+$ DC recovered much faster, i.e., within 7–10 days, while the long-lived self-maintaining LC stayed away for a prolonged period of time, i.e., at least 2–4 weeks (44, 47, 105). Using timed diphtheria toxin treatments, this enabled researchers to induce CHS when both Langerin$^+$ skin DC (administration of diphtheria toxin 1–3 days prior to hapten sensitization) or only LC (diphtheria toxin treatment 7–13 days before sensitization) were lacking. Alternatively, Langerin-DTR into wild-type bone marrow chimeras permitted the selective depletion of only Langerin$^+$ dermal DC before the induction of CHS (106). From this comprehensive analysis by a number of different laboratories, it became clear that the intensity of the CHS reaction is directly correlated with the efficiency of T cell priming, as was suggested by inefficient antigen transport to draining LN in the absence of Langerin$^+$ skin DC (104). Consequently, and in agreement with early dose–response studies (109), LC are required for efficient induction of CHS responses, in particular, at low hapten doses, while at higher hapten concentrations sufficient amounts of antigen can be picked up by dermal DC – both Langerin$^+$ and negative – for effective elicitation of CHS in the absence of LC (37, 47, 105, 106, 110, 111). Taken together, there is overwhelming evidence indicating functional redundancy of the different skin DC subsets in CHS (**Table 2**).

In contrast to these inducible Langerin-DTR knock-in mouse models, which harbor physiologic numbers of LC and Langerin$^+$ dermal DC until the injection of diphtheria toxin, human (h) Langerin-DTA BAC transgenic mice that constitutively lack LC throughout life mounted enhanced ear swelling responses (45). Although this observation suggested that LC may exert a down-regulatory function in CHS, the great amount of data discussed above rather support compensatory roles of the different skin DC populations during the sensitization and elicitation of CHS (37, 47, 105, 106, 110, 111). Apart from these reports, it is difficult to conceive how negative regulatory properties of LC could develop or be maintained in the highly inflammatory setting of a CHS sensitizing reaction (112). However, the reason for the discrepancy between the inducible and the constitutive LC ablation models remains elusive. On the one hand, hLangerin-DTA mice may develop increased CHS as a result of some unknown failing peripheral tolerance mechanism in the lifelong absence of LC and therefore may respond differently during hapten sensitization and/or may possess altered T cell properties (37, 111, 113). On the other hand, the Langerin$^+$ dermal DC that return after the toxin treatment in Langerin-DTR mice may differ from the cells that originally developed during ontogeny (58), and which are left untouched in hLangerin-DTA mice, presumably due to differences in the transcriptional regulation of the mouse and human *langerin* promotors. Both of these hypothetical explanations seem unlikely, however, because all transgenic mouse strains that constitutively lack LC (or Langerin$^+$ dermal DC) as a result of varying genetic defects, i.e., independently of the diphtheria toxin/DTR system, and that have been tested in CHS mount similar or attenuated ear swelling reactions than LC-competent controls (**Table 2**) (75, 91, 101, 108). Instead, hLangerin-DTA mice may develop aggravated CHS due to changes in the homeostasis of dermal DC populations, i.e., an increased number of CD103$^+$ dermal DC (92), or due to unknown DNA sequences that have been introduced with the human *langerin*-containing BAC. Although speculative as well, the latter may be implied, because to date hLangerin-DTR mice generated with the same BAC construct are the only other mouse model that mount enhanced CHS responses, i.e., after acute diphtheria toxin-mediated ablation of LC (107).

In conclusion, while LC clearly have regulatory potential that may have evolved to prevent inappropriate immune activation to keratinocyte-derived antigens or by commensal skin microbiota (see below), the vast majority of the available evidence indicates that LC promote the induction of CHS reactions, but are only essential at low hapten concentrations, and that dermal DC also contribute to CHS.

FUNCTIONAL SPECIALIZATION OF CUTANEOUS DENDRITIC CELLS IN INFECTIOUS SKIN DISEASE AND HOMEOSTASIS TO COMMENSAL MICROBIOTA

One of the first observations questioning the LC paradigm was the finding that during cutaneous herpes simplex virus-1 (HSV-1) infections not epidermal LC, but instead CD8α$^+$ LN-resident DC were responsible for T cell priming and induction of the anti-HSV-1 response (114). Notably, LC were still required to process and transport HSV-derived antigens to the LN, where they transferred their antigenic cargo to the CD8α$^+$ LN DC for cross-presentation to naïve T cells (115). Another study using an HSV-2 infection model of the vagina also revealed that epithelial LC did not present viral antigens to LN T cells (116). In this case, submucosal CD8α^{neg} migratory DC carried the viral peptides to the LN and induced the protective T helper (Th) type-1 response to HSV-2. A key question concerning these HSV infection models remains why LC played no direct role in antigen presentation and T cell activation. Was it merely because they were infected and killed by the cytopathic herpes viruses (117, 118); essentially leaving no other option for the apoptotic LC than being taken up and cross-presented to CD8$^+$ T cells by LN-resident DC (119).

This hypothesis is supported in an apoptosis-inducing vaccinia virus infection model, in which cytotoxic T cell activation was similarly taken over by CD8α$^+$ LN-resident DC, i.e., after uptake and cross-presentation of apoptotic skin-derived DC. On the other hand, in a cutaneous lentiviral infection model where LC/DC stay alive, migratory skin DC are perfectly capable to present antigen to T cells in the draining LN (120). Eventually, this concept was also confirmed for the HSV model, at least for Langerin$^+$CD103$^+$ dermal DC (121). In contrast to the primary infection via superficial skin scarification, during reactivation of the virus from its natural reservoir in the cutaneous nerves, HSV antigen presentation to CD8$^+$ T cells occurred by both Langerin$^+$CD103$^+$ skin DC and CD8α$^+$ LN DC. LC still played a minor role in direct antigen presentation, most likely due to higher sensitivity to this cytolytic virus than dermal DC. Although this concept that antigen-carrying skin DC, in particular LC, are taken up for cross-presentation by CD8α$^+$ LN-resident DC cannot be generalized (119), it was later found that Langerin$^+$CD103$^+$ dermal DC cross-present keratinocyte-derived antigens irrespective of the presence of epidermal LC (see below) (51). A comprehensive overview of the role of DC in primary HSV infections beyond these basic principles has been published recently (122).

LC were originally also considered to be critical for the induction of protective immunity in another infectious skin disease, namely cutaneous leishmaniasis, because they were shown to transport the parasites from the site of infection to skin-draining LN (123). This view was challenged when it was reported – at about the same time that the seminal HSV infection studies were published (114, 116) – that Langerin^{neg}CD8α^{neg} presumably dermal DC, but not LC, act as principal antigen-presenting cells (APC) in experimental *Leishmania major* infection (124). Resistance to *L. major* infection and healing of the skin lesions both in mice and men critically depends on the efficient induction of a Th1/T cytotoxic (Tc) type-1 response (125, 126). Langerin-DTR mice in combination with timed diphtheria toxin treatments (see above) revealed that activation of *L. major*-specific CD8$^+$ T cells is significantly reduced during the early phase of the immune response following depletion of Langerin$^+$ DC, without affecting the CD4$^+$ T cell response and clearance of the infection (127). This demonstrated that Langerin^{neg} dermal DC were indeed essential for effective priming of CD4$^+$ Th1 cells, whereas Langerin$^+$ dermal DC were involved in early priming of CD8$^+$ Tc1 cells.

Moreover, formation of CD4$^+$ follicular helper (T_{FH})/B cell conjugates is crucial for B cell differentiation and class switch recombination to generate high-affinity antibodies for host protection following infection with *L. major* parasites (128). Recently, LC were shown to promote germinal center formation and thus antibody affinity maturation in response to *Leishmania*-derived cutaneous antigens (129), although these experiments used non-physiologic high doses of parasites that might blur early events during infection. In a model of physiologic low-dose infection with *L. major* infectious-stage promastigotes (1,000 parasites), mice depleted of all Langerin$^+$ DC developed smaller ear lesions, decreased parasite loads and a reduced number of CD4$^+$Foxp3$^+$ Treg cells, which was accompanied by increased production of interferon γ (IFNγ) (130). Of note, despite repeated administration of diphtheria toxin over a prolonged period of time (20 weeks) Langerin$^+$ DC were efficiently depleted from the skin, confirming the absence of anti-diphtheria toxin neutralizing antibody formation as had previously been demonstrated (113, 131). Intriguingly, selective depletion of LC at the time of low-dose *L. major* inoculation demonstrated that the absence of LC, and not Langerin$^+$ dermal DC, was responsible for the reduced Treg cell immigration and the enhanced Th1 response, resulting in attenuated disease (130). Hence, LC act as negative regulators of the anti-*Leishmania* response in mice. This may be important to prevent complete eradication of the parasites from the host, which leads to the loss of T cell memory and susceptibility to reinfection (132, 133).

Candida albicans is a dimorphic fungus accountable for chronic cutaneous and systemic infections in immune-compromised hosts. On the *stratum corneum* of the skin, commensal *C. albicans* grows as budding yeast, while pathogenic *C. albicans* in the dermis and internal organs exists predominantly in its filamentous form, i.e., as pseudo-hyphae (134). This yeast-to-hyphae transition during epidermal invasion is required for both virulence and the generation of protective Th17 responses to cutaneous *C. albicans* (134, 135). On the other hand, systemic fungal immunity is achieved by innate immune mechanisms regulated by IL-17-mediated licensing of NK cells to promote the fungicidal activity of neutrophils (136).

Taking advantage of a superficial skin infection model that does not bypass the epidermis in combination with LC-deficient hLangerin-DTA mice, it was demonstrated that LC are essential for the induction of antigen-specific Th17, but not cytotoxic T lymphocyte (CTL) responses (137). Somewhat inconsistent, despite reduced IL-17 and similar IFNγ responses in the absence of LC, hLangerin-DTA mice mounted significantly increased DTH reactions after epicutaneous *C. albicans* infection, similar to the unique phenotype of these transgenic mice in CHS [(45) and

as discussed above]. However, using human Langerin-specific antibodies for targeted antigen delivery to LC in hLangerin-DTR mice (not treated with diphtheria toxin), LC were also found to be sufficient for inducing Th17 cell differentiation.

By contrast, Langerin$^+$ dermal DC promoted antigen-specific Th1 and efficiently cross-presented fungal antigens to activate CTL responses. At the same time, Langerin$^+$ dermal DC suppressed the ability of LC to drive the generation of Th17 cells (137). A follow-up study indicated that infection with *C. albicans* yeast but not pseudo-hyphae was capable of inducing Th17 responses through a mechanism that required Dectin-1 ligation on LC and, as a consequence, LC-derived IL-6 (138). In the dermis, absent Dectin-1 engagement by *C. albicans* pseudo-hyphae prevents Th17 induction by CD11b$^+$ dermal DC. Moreover, Th17 cells were found to provide protection against secondary cutaneous infection whereas Th1 cells were protective against systemic reinfection (138). Together these elegant studies established that distinct and opposing Th cell responses are determined by a combination of differences in *C. albicans* morphology and functional specialization of skin-resident DC subsets.

Beyond the functional specialization of skin DC subsets to deal with particular pathogens, there is accumulating evidence that the interactions between the resident skin microbiota and DC autonomously shape tissue homeostasis and local immunity (139). Skin tissue of mice housed under specific pathogen-free (SPF) conditions harbors Foxp3$^+$ Treg as well as αβ$^+$ and γδ$^+$ T cells with the potential to produce IFNγ and/or IL-17A, respectively. Microbial products from skin commensals tightly regulate this balance between Treg and effector T cells as was indicated by the increase in Treg and the reduction in IFNγ and IL-17A producing T cells in germfree mice lacking microbial products from their skin (139). Consequently, protective immunity against *L. major* is severely impaired in germfree mice, as is disease-associated pathology. Intriguingly, colonization with the single skin commensal *Staphylococcus epidermidis* was sufficient to rescue cutaneous IL-17A production in germfree mice, which was dependent on IL-1 signaling in the skin. Monoassociation of germfree mice with *S. epidermidis* at the time of infection also restored immunity to *L. major* as well as pathology with increased necrosis (139). These results suggest that defects in T cell function in the steady state or during inflammation can result from an impaired IL-1-mediated dialog with skin commensals.

Moreover, colonization of the skin of SPF mice that contained a diverse microbiota with *S. epidermidis* led to an accumulation of IL-17A$^+$ CD8$^+$ T cells in the epidermis that enhanced innate barrier immunity by upregulation of antimicrobial peptides and limited skin invasion of the pathogen *C. albicans*. In agreement with the unique role of CD103$^+$ dermal DC in antigen cross-presentation (see below), these Tc17 cells failed to develop in Batf3$^{-/-}$ and IRF8$^{-/-}$ (see **Table 1**), while the IL-17A secreting CD8$^+$ T cells developed normally in constitutively LC-deficient hLangerin-DTA mice (92). Furthermore, CD11b$^+$ dermal DC were required to promote the induction and/or maintenance of Tc17 cells through their capacity to produce IL-1 in response to *S. epidermidis* colonization of the skin. In conclusion, these findings reveal that the skin immune system is highly dynamic and can be readily reshaped by the coordinated action of the different skin DC subsets upon encounter of defined commensals (92).

Hence, in agreement with the extended *LC paradigm* (**Figure 1**), LC exhibit a great degree of functional plasticity and become tolerogenic or immunogenic depending on the nature of the invading pathogen they encounter in the skin.

CROSS-PRESENTATION BY SKIN DENDRITIC CELLS: QUESTION FINALLY ANSWERED?

For developing immunotherapeutic approaches against cancer, one prerequisite is to understand how the various skin DC subsets induce CTL responses. There has been a long-standing debate on the ability of LC to cross-present exogenous antigen to CD8$^+$ T cells (37). The start of this debate was the report that LC are dispensable for the induction of cytotoxic T cell responses against skin infection with herpes virus (see above) (114), which was later confirmed for vaccinia virus (140). Subsequent work clarified that cytopathic viruses induce apoptosis in LC rendering them sole transporters of antigen. As a consequence these cells are no longer capable of directly inducing T cell responses, however, LN-resident DC and other skin DC subsets, such as Langerin$^+$ dermal DC, can step in and cross-present antigen to CD8$^+$ T cells (115, 141, 142).

The debate was further fueled by studies on cross-presentation of self-antigen. For this approach, transgenic mice overexpressing ovalbumin protein in an inducible or constitutive way under control of the keratinocyte-specific K5- or K14-promoter in the skin were employed (143–145). Now it was possible to examine cross-presentation of self-antigen by the various skin DC subsets in the steady state and inflammation. Early studies demonstrated that Langerin$^+$ cells can cross-present ovalbumin to antigen-specific CD8$^+$ T cells (26, 146, 147). This cross-presentation ability was not necessarily restricted to Langerin$^+$ dermal DC, since LC purified from trypsinized epidermis and migratory LC from epidermal explants also efficiently cross-presented ovalbumin to CD8$^+$ T cells *in vitro* (144, 148). Chimeric mice in which antigen cross-presentation was restricted to LC proved that LC are able to cross-present self-antigen also *in vivo*. Interestingly, cross-presentation by Langerin$^+$ skin DC led to tolerance induction through deletion of antigen-specific CD8$^+$ T cells (26). After the discovery of Langerin$^+$ dermal DC, it came as a big surprise, when studies using K5-ovalbumin transgenic mice established that Langerin$^+$ dermal DC are the sole cross-presenters of keratinocyte-derived antigen (51, 121). The localization of Langerin$^+$ dermal DC adjacent to hair follicles where K5$^+$ keratinocytes are present explained how this DC subset gains access to an epidermal antigen (49, 149). Moreover, in human skin keratinocyte-derived keratin bodies were found in the dermis (150). The discrepancy to the earlier studies, proving that LC can cross-present antigen, may be due to the low migratory capacity of LC in the steady state, which ensures that the LC network stays intact until inflammation causes accelerated emigration of LC to LN (21, 43, 151). Indeed, the turnover of LC in the skin is much lower than that of dermal DC as demonstrated by BrdU incorporation assays (51).

The migration of all skin DC populations increases dramatically in an inflammatory setting, though with different kinetics, so that dermal DC arrive in LN much earlier than LC (46, 152). Hence, it would be interesting to investigate the cross-presentation of skin-derived antigen in an inflammatory setting at different time points after the onset of inflammation. Aside from this, most of the studies performed to date used transgenic mice overexpressing the model antigen ovalbumin in keratinocytes. Because of the high-affinity T cell receptor binding and very strong responsiveness of ovalbumin-specific $CD8^+$ T cells, these findings might not reflect what happens in real life (37). Thus, these studies need to be confirmed in a more physiological setting investigating the cross-presentation of genuine self-antigens in the skin.

For the development of immunotherapeutic strategies exploiting skin DC, exogenous antigen needs to be delivered through the skin (see below). Studies on skin immunization added more issues to the controversy whether LC can cross-present exogenous antigen. First of all, LC can induce CTL when they are loaded with soluble ovalbumin *in vitro* and co-cultured with $CD8^+$ T cells (148). Most importantly, topical application of ovalbumin onto the skin by either epicutaneous immunization (153) or by dissolving micro-needles (154) confirmed that $Langerin^+$ DC are involved in cross-priming of $CD8^+$ T cells and that LC are superior to $Langerin^+$ dermal DC, in particular, when the antigen is encapsulated in nanoparticles (154). In line with this, antibody-mediated targeting of the model antigen ovalbumin to $Langerin^+$ cells by intradermal injection proved that both, LC and $Langerin^+$ dermal DC, can cross-present antigen to $CD8^+$ T cells *in vivo* (155).

Finally, to answer the question asked above, yes, both LC and $Langerin^+$ dermal DC in the skin can cross-present exogenous antigen to $CD8^+$ T cells *in vitro* and *in vivo*. We would like to emphasize that cross-presentation and cross-priming must not be equated. There is the very likely possibility that the various skin DC subsets induce different functional outcomes in $CD8^+$ T cell differentiation as exemplified in a recent report. Despite initial $CD8^+$ T cell proliferation induced by LC and $Langerin^+$ dermal DC after loading them *in situ* with protein antigen (proving cross-presentation), LC did not cross-prime T cells but rather induced cross-tolerance. By contrast, $Langerin^+$ dermal DC promoted cytotoxicity, indicating that they indeed cross-primed the T cells (155). Thus, we need to better understand the differential contributions of the various skin DC subsets in $CD8^+$ T cell activation leading to either CTL differentiation or tolerance induction. This knowledge is indispensable for the future development of DC-based immunotherapy of cancer.

SKIN DENDRITIC CELLS IN CANCER

Novel immunotherapeutic strategies to vaccinate through the skin are a promising area of research for the future development of anti-cancer therapies. The rationale behind this approach comes from reports on the involvement of DC in tumor immunity and their outstanding potential in promoting T cell responses. The presence of DC has been reported in many different tumors, however, their specific role in tumor immunity is still incompletely understood (156, 157). Aside from this, tumors also strongly impair DC function and actively prevent efficient immunosurveillance by DC (158). With respect to cutaneous cancer, such as squamous cell carcinoma (SCC), basal cell carcinoma (BCC), and melanoma, several reports indicate that the numbers and function of skin DC are affected by tumor growth.

So far few studies attempted to analyze the specific role of skin DC present in cutaneous tumors. Non-melanoma skin cancer, such as SCC and BCC, are tumors of basal keratinocytes, making it very likely that LC are the first APC getting in contact with transformed cells. Two studies used patient samples from SCC to investigate LC and DC in regard to numbers, phenotype, and T cell stimulatory capacity. In the first study, the numbers of LC in the SCC tumor lesions were decreased as compared to healthy epidermis. Less myeloid cells, including dermal DC, were found around tumor nests than in normal skin. Tumor-associated myeloid DC were poor stimulators of allogeneic T cells despite displaying an activated phenotype (159, 160). The second study demonstrated that tumor-infiltrating LC are more activated and induced higher $CD4^+$ and $CD8^+$ allogeneic T cell proliferation as well as IFNγ production than LC from adjacent healthy skin (161). Thus, LC and myeloid DC found in human SCC samples display an activated phenotype, but only LC induce allogeneic T cell responses. Although suggestive, these studies do not allow any conclusion on the functional ability of these DC subsets to promote tumor immunity to non-melanoma skin cancer since tumor-specific T cell responses were not investigated. However, unhindered tumor growth despite the activation of LC/DC indicates that the immunosuppressive milieu in SCC tumors, which contains high concentrations of TGF-β1 counteracts successful tumor immunity (159).

Another study used a murine model of chemically induced SCC to investigate the role of LC during tumor development (162). Chemical carcinogenesis was induced by application of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) that causes Hras mutations, followed by the tumor-promoting agent 12-O-tetra-decanoyl-phorbol-13-acetate (TPA) leading to development of papilloma and subsequent SCC. The observation that hLangerin-DTA mice, lacking LC throughout their lifetime, are completely protected from tumor development is somehow surprising, but may be related to the intrinsically enhanced elicitation of adaptive immune responses, i.e., in CHS and DTH reactions, in this as opposed to other LC-deficient mouse models (see above). However, the authors propose the interesting concept that LC mediate the metabolic conversion of DMBA to its mutagenic metabolite that in turn leads to DNA damage and carcinogenesis. They further suggest that compared to keratinocytes LC express higher levels of CYP1B1, an enzyme of the cytochrome P-450 family responsible for the mutagenic metabolism of DMBA (162). How the metabolite is transferred from LC to keratinocytes to exert its DNA-damaging function was not investigated. Moreover, these data are difficult to reconcile with the fact that keratinocytes themselves express all required enzymes for DMBA metabolism (163, 164). In a follow-up paper, the authors demonstrated that LC exert pro-carcinogenic effects also independently of the enzyme CYP1B1 (165), possibly by aryl hydrocarbon receptor-mediated transcription of other CYP enzymes that trigger DNA damage (166).

In addition, LC played an important role in the progression of tumors by affecting the hyperproliferation of keratinocytes in the DMBA-induced SCC model (165) as well as in UVB-induced SCC (167), likely by augmenting IL-22 production by keratinocytes. Notably, this direct carcinogenic role of LC may be amplified by LC-driven immunosuppression, which is the induction of antigen-specific Treg cells upon UV radiation exposure (166, 168). It will be interesting to compare the growth of chemically and UVB-induced SCC in the hLangerin-DTA mouse model (165, 167) to one of the inducible Langerin-DTR knock-in mice (44, 46), since the constitutive absence of LC in hLangerin-DTA mice may have a distinct effect on the development of the skin immune system (see above). Intriguingly, a recent report revealed an unaltered expression profile of cytochrome P-450 enzymes in the absence of LC and Langerin$^+$ dermal DC upon DMBA application in Langerin-DTR mice (169).

Similar as in SCC and BCC, the role of the different skin DC in the immunosurveillance of melanoma is incompletely understood. Over 20 years ago first reports described decreased numbers of LC above invasive human melanoma (170, 171). In line with these findings, the presence of transplantable tumors lowered the number and impaired the migration of LC from murine skin (172). The types and relative proportions of tumor-infiltrating DC in melanoma have not been determined so far, such that information on the functional potential of distinct skin DC subsets to control melanoma is lacking. For instance, in melanoma the accumulation of mature DC of unknown origin in draining LN metastases was associated with the expansion of antigen-specific cytotoxic T cells (173). A recent effort to identify the various myeloid cell types within a transplantable murine melanoma model demonstrated that the CD103$^+$ DC subset, which most likely includes Langerin$^+$ dermal DC, was superior over CD11b$^+$CD103^{neg} DC in cross-presenting tumor antigens (174). Future studies using multi-color flow cytometry including a comprehensive panel of markers to discriminate individual DC subsets (**Figures 3** and **4**) will be required to obtain a detailed picture on the involvement of distinct skin DC in tumor immunity (175). This knowledge will form the basis for the design of novel and for the improvement of existing immunotherapies harnessing the potential of skin DC for the immunotherapy of cancer.

HARNESSING SKIN DENDRITIC CELLS AS TARGETS FOR IMMUNOTHERAPY OF CANCER

From the time of the experiments of William Coley in the early 1900s immunotherapy of cancer was in the minds of immunologists (176), though it played a rather marginal role. Next to T cells, cytokines and antibodies, DC became promising targets for immunotherapy of cancer owing to the pioneering work by the late Ralph Steinman, who received the 2011 Nobel Prize in Physiology or Medicine for "his discovery of the DC and its role in adaptive immunity" (177). His ultimate goal was to harness the outstanding immunogenic properties of DC for immunotherapy and thereby "taking dendritic cells into medicine" (178). Since the first report of treatment of a B cell lymphoma with antigen-pulsed DC from blood (179), many clinical and basic research centers have been working to improve the efficacy of DC-based strategies to treat cancer patients. The first adoptive DC therapy for cancer (Provenge™, a tumor antigen-pulsed cell suspension containing DC) was approved by the FDA in 2010, and this constituted a milestone in the development of cellular therapies. A parallel development in cancer immunotherapy occurred over the past few years when the so-called "checkpoint inhibitors" were introduced into clinical practice. These antibodies against CTLA-4, PD-1, or PD-L1 (and presumably other inhibitory mediators in the future) switch off down-regulatory signaling pathways in T cells. They are therefore able to powerfully unleash anti-cancer immunity that exists in patients but is obviously insufficient or suppressed by various mechanisms (180). Clinical responses in patients are impressive, but so are side effects (autoimmunity), particularly with the anti-CTLA-4 antibodies. Still, these clinical observations have earned this therapeutic approach the title of "Breakthrough of the Year" in 2013 by *Science* (181).

In spite of these encouraging developments targeting T cells, the potential of DC-based therapies remains high for several reasons. (*i*) Only a variable part of patients treated by checkpoint blockade responds to the treatment (180, 182, 183). (*ii*) Even though undesired autoimmunity can be clinically managed, it would be advantageous to avoid or minimize it from the beginning. (*iii*) Most importantly, checkpoint blockade can only boost those cancer-specific T cells that are already preexisting in the patient. By contrast, DC therapy would be able to generate *de novo* immune responses (182, 183). Such responses would be desired against neo-antigens ("private mutations") in patients' tumors (182, 184). DC-induced T cell responses against such mutated tumor antigens would additionally lack autoimmune danger.

The *ex vivo* generation of DC for therapy is laborious, nevertheless, therapy with tumor antigen-pulsed autologous DC proved to be safe and effective, though not curative, in patients with solid tumors (185). The continuing importance of DC therapy is highlighted by recent publications, indicating persuasive ways of improving DC vaccines. For instance, DC were loaded with peptides derived from neo-antigens identified from the patient's own tumor material, instead of the commonly used peptides from overexpressed self-antigens. This strategy augments T cell responses by broadening the antigenic diversity of the anti-tumor response in the absence of autoimmunity (186). Another approach is the conditioning of the injection site with a potent recall antigen, such as tetanus toxoid, TLR ligands, or cytokines. The pretreatment of the skin site with antigen or danger signals improved the migration of adoptively transferred DC and boosted T cell responses in tumor-bearing mice (187–189). Moreover, the co-administration of tumor-binding allogeneic antibodies enhanced the internalization of tumor antigens by DC and dramatically increased therapy outcome in murine tumor models (190). Furthermore, the choice of DC subset could enhance the efficacy of DC therapy as has been demonstrated for LC-like cells generated from CD34$^+$ precursor cells that were able to overcome tolerance to differentiation antigens commonly overexpressed in cancer patients and used for vaccination (191).

Yet another imaginative approach that has been pioneered many years ago in Ralph Steinman's laboratory (192, 193) is the use of antibody-mediated antigen targeting constructs to specifically deliver antigenic peptides or proteins to DC *in situ*. The basic idea is to target the antigen of interest to endocytic receptors specific for DC (subsets) resident in the skin or lymphatic organs to enable them to efficiently incorporate antigen for presentation to T cells. This approach would be much less laborious than the *ex vivo* generation of DC from patients' monocytes and their subsequent loading with tumor antigens. Intriguingly, antigen delivered without adjuvant can induce tolerance, whereas concomitant administration of TLR ligands and agonistic anti-CD40 antibody causes strong induction of $CD4^+$ and $CD8^+$ T cell responses (192, 194). In addition, this approach allows delivering antigen into the various skin DC subsets by aiming at different lectin receptors (195, 196). The most interesting candidate receptors for these *in vivo* antigen targeting approaches are C-type lectins, which are expressed by skin DC, e.g., DEC-205, Langerin, and Dectin-1 (197–199). Specific delivery of the model antigen ovalbumin into $Langerin^+$ cells proved that both LC and $Langerin^+$ dermal DC can cross-present antigen to $CD8^+$ T cells, however, only the dermal DC promoted the development of cytotoxicity while LC induced tolerance (155). Targeting antigens to DEC-205^+ DC led to tumor control or even eradication in murine tumor models, albeit the relative roles of skin DC and LN-resident DC were not investigated in these studies (194, 200, 201). The chemokine receptor XCR1, which is preferentially expressed on $Langerin^+$ dermal DC (52, 53) (**Figure 3**), is another promising target due to the fact that this DC subset excels in inducing cytotoxicity in $CD8^+$ T cells (155). Immunization with the chemokine XCL1 conjugated to ovalbumin protein intravenously or through skin pre-treated with a laser to form pores led to $CD4^+$ and $CD8^+$ T cell activation and inhibited the growth of transplanted tumors in mice (202, 203). First clinical trials have been initiated and so far demonstrated induction of some humoral and cellular immunity by targeting the tumor antigen NY-ESO-1 conjugated to DEC-205 antibody into DC of patients with various solid tumors (204). These data demonstrate the proof-of-concept of the targeting approach in human cancer, but they also call for intense further study to substantially improve this strategy.

Immunization strategies through the skin are very attractive for their easiness of use and, ultimately, the possibility of self-medication in case of topical treatment. Several approaches have been developed such as epicutaneous immunization, micro-needles, laserporation, or powder injection (205). The epicutaneous approach allows to topically apply antigens in protein and peptide form onto the skin (206). The disruption of the skin barrier and the addition of an adjuvant proved to be essential to elicit powerful cytotoxic T cell responses that inhibit tumor growth (153, 207, 208). The involvement of $Langerin^+$ skin DC in $CD8^+$ T cell responses was confirmed in experiments with Langerin-DTR mice in a tumor setting. In line with findings in the CHS model (see above), the antigen dose of ovalbumin protein determined which skin DC subset presented the tumor antigen. The inhibition of tumor growth by epicutaneous immunization with low-dose antigen was completely abrogated in the Langerin-DTR mice depleted for $Langerin^+$ DC, whereas application of a higher dose of antigen still partly inhibited tumor growth even in the absence of $Langerin^+$ DC (153). This supports the notion of the high plasticity of skin DC subsets. The epicutaneous immunization approach has already been clinically tested and proved to be promising for the treatment of cancer and infection (209, 210).

Another very elegant approach is the application of dissolving micro-needles that allow delivery of antigens right into the tissue where skin DC are located. This strategy has been successfully used to vaccinate against influenza (211) and to treat tumors, at least in murine models (154). The latter study demonstrated that LC can be superior to dermal DC in cross-presentation of antigen delivered with micro-needles into the skin. Interestingly, the nature of the antigen determined which skin DC subset induced $CD8^+$ T cell proliferation, in that encapsulated antigen was preferentially cross-presented by LC whereas soluble antigen required $Langerin^+$ dermal DC for $CD8^+$ T cell activation. The $CD4^+$ T cell response was promoted by all skin DC subsets, however, LC dominated the induction of Th1 and Th17 responses (154).

For the future, it will be worthwhile to investigate the potential of the various cutaneous DC subsets in skin immunization and translate the findings from murine tumor models to the patient situation. Undoubtedly, skin DC are critically involved in surveying the skin in order to prevent tumor growth and clearly fulfill an immunogenic role during vaccination against cancer. Yet their precise roles are still unclear and need to be clarified before we will arrive at urgently needed more effective DC-based treatment options for cancer.

HUMAN SKIN DENDRITIC CELL SUBSETS

The human and murine skin DC network seems to be highly conserved between the two species. While this justifies *in vivo* experiments in mice to gain mechanistic insight into the functional specialization of cutaneous DC subsets in regulating immunity and tolerance, we need to identify the human counterparts of the murine DC subtypes in order to translate this knowledge to treating patients. In the recent years, human skin DC subsets were better defined and found to be homologous to murine DC (**Figure 4**) (212–214). In humans, the epidermis contains LC expressing Langerin and high levels of CD1a, whereas in the dermis three subsets of dermal DC can be distinguished (37, 215). The largest population is represented by the $CD1c^+CD1a^+$ dermal DC, which correspond to the murine $CD11b^+$ dermal DC (216, 217). The smallest subset of DC in human dermis is characterized by high expression of CD141 and XCR1 and is homologous to the murine $Langerin^+CD103^+$ dermal DC, which also express XCR1 (214, 218). Very recently evidence for yet another small subset of (weakly) $Langerin^+CD1c^+$ dermal DC was presented, highlighting that similar to mice Langerin expression may not be strictly confined to LC in human skin (219). The $CD14^+$ dermal DC are monocyte-derived cells that are transcriptionally aligned rather to monocytes/macrophages than to DC (216). A corresponding tissue-resident DC subset with the phenotype $CD11b^+Ly6C^-CD64^{lo\text{-}hi}$ has been described in murine dermis (35).

In regard to functional aspects, both human LC and $CD1c^+$ dermal DC can polarize Th1 and Th2 responses (220), depending

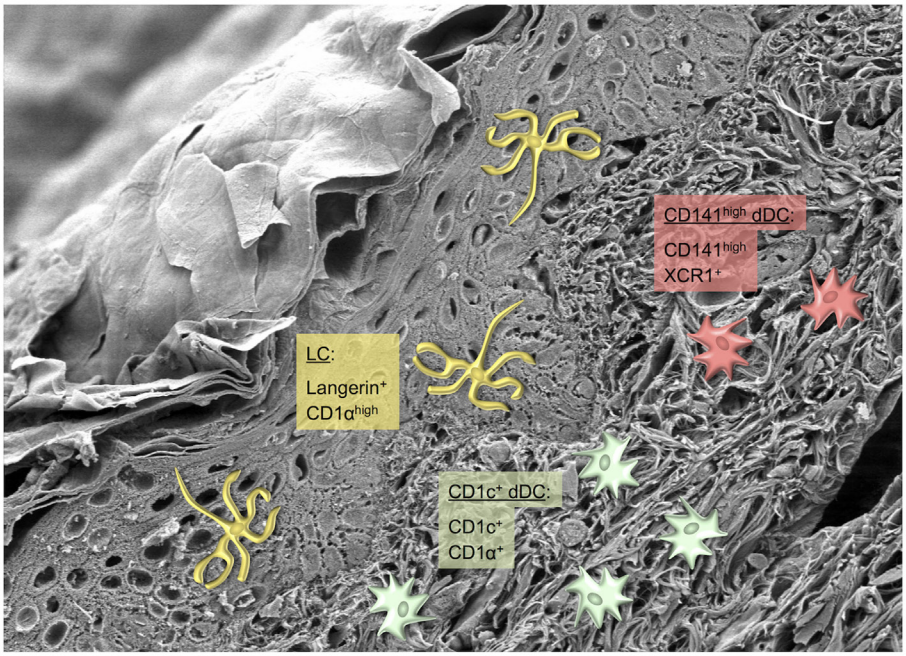

FIGURE 4 | Dendritic cell subsets in human skin. Scanning electron microscopy picture of a section through the skin depicting several layers of keratinocytes and the collagen meshwork of the dermis (38). Photograph by courtesy of Kristian Pfaller and Patrizia Stoitzner. Phenotypically distinct human skin-resident DC subsets are described by their most prominent markers. The color code represents the murine counterparts shown in **Figure 3**.

on the cytokine milieu in the skin (221), and cross-present exogenous antigen to $CD8^+$ T cells (215). However, the recently discovered $CD141^{hi}$ dermal DC excel in cross-priming of $CD8^+$ T cells comparable to murine $Langerin^+$ dermal DC (214). There still exists some controversy in the field on the suitability of XCR1 and CD141 as markers for cross-presenting DC since both molecules can also be expressed by some $CD1a^+$ and $CD14^+$ dermal DC (218, 222, 223). Importantly, the methods used for the preparation of skin DC differ strongly between the many studies published. Notably, DC isolated from skin tissue by enzymatic digestion or derived from skin explant culture after emigration from the tissue ("crawl-outs") represent immature and mature DC, respectively. This has major influence on their phenotype/function and the presence/absence of cell surface markers. One example is CD141, which is differently expressed on freshly isolated dermal DC and migratory dermal DC. While CD141 is upregulated on $CD14^+$ dermal DC upon emigration from skin explants as compared to DC enzymatically isolated from skin, its expression on $CD141^{high}$ $CD14^{neg}$ dermal DC remains unaltered upon migration (214, 223). Humoral immunity is mainly modulated by $CD14^+$ dermal DC since they can activate the differentiation of T_{FH} cells (220). Moreover, $CD14^+$ dermal DC support memory T cell activation, most likely *in situ* in the skin, but they are poor stimulators of naïve T cells (216). During inflammation, several inflammatory DC subsets, e.g., inflammatory dendritic epidermal cells (IDEC), 6-sulfoLacNAc$^+$ (slan) DC, and TNF-α/iNOS-producing (Tip) DC, are recruited to the skin and have a strong impact on the course of inflammatory skin diseases such as psoriasis and atopic dermatitis (215, 224, 225).

Despite the high degree of homology between mouse and human skin DC, functional disparities do exist. Some examples are listed here: (*i*) For instance, with the help of IL-15 released into the immunological synapse, human LC cross-prime cytotoxic $CD8^+$ T cell responses (226, 227). As a consequence human LC are able to break tolerance to self-antigens and stimulate cytotoxic T cell responses (191). So far hardly any information on the production of IL-15 by murine skin DC is available. (*ii*) CD70, a molecule involved in DC–T cell interaction and important for activation of $CD8^+$ T cells and IFNγ production (228), is highly expressed by human LC (229, 230), whereas murine LC show very low levels of CD70 even after stimulation (155). These functional disparities between murine and human LC are supported by recently published gene transcription profiles of human skin DC subsets, indicating that human LC are more closely related to murine $Langerin^+$ dermal DC than to murine LC (222). (*iii*) Another example for functional differences stems from the fact that murine LC, despite initial cross-presentation and induction of $CD8^+$ T cell proliferation (148), fail to stimulate cytotoxicity in $CD8^+$ T cells and instead induce cross-tolerance (155). Notably, the TLR ligands used in the latter study specifically activate $Langerin^+$ dermal DC (231), thus we need to evaluate the potential of LC in cross-priming with TLR ligands suitable for their activation. The knowledge on functional properties of the various murine and human skin DC subsets is of eminent importance when we envisage novel immunotherapeutic approaches that need first to be tested in preclinical murine studies before they can be translated into the clinics.

CONCLUSION

The skin, as one of the barrier tissues to the environment, harbors particular challenges to the resident DC network. While the cells continuously probe their surroundings for invading pathogens, DC have to discriminate harmless from dangerous microbes, prevent inappropriate immune reactions against self-antigens, and limit collateral tissue damage once inflammation occurs during protective immune responses. The original concept that immature DC confer tolerance and mature DC initiate immunity (232) turned out to be too simplistic and was further developed to give way to the hypothesis of functional specialization of particular DC subsets (233), including distinct pathway(s) of tolerogenic DC maturation (14, 17, 18). Through major advancements in multi-color flow cytometry, next-generation transcriptomics and proteomics, and the generation of novel cell type-specific gene targeting, cell labeling, and cell ablation mouse models, we can now dissect an increasing number of phenotypically distinct DC subsets in the skin (as well as other barrier tissues) and are beginning to unravel their functional heterogeneity (1, 3, 37). From these exciting *in vivo* experiments, it is becoming increasingly clear that specific DC subsets indeed exert specialized functions, but that this "*division of labor*" is not intrinsically defined by or fixed within one type of DC and rather determined by the signals the DC receive from their micro-environment (234). For example, LC induce Treg during *L. major* and Th17 cells upon *C. albicans* infection (130, 137), Th2 cells under the influence of pro-allergic TSLP (221) and strong Th1 responses when conditioned by a tumor environment (161). Thus, despite their context-dependent specialization, overall DC subsets display an amazing functional plasticity ("*multitasking*"). The future challenge lies in better understanding (*i*) the unique contribution of the different DC subsets to particular chronic inflammatory diseases and (*ii*) the context-dependent signals that control the function of individual DC subsets in a given disease state. This knowledge will be vital to harness (skin) DC subsets for the treatment of human diseases ranging from allergy and autoimmunity to chronic infections and cancer.

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Regulation of lipid specific and vitamin specific non-MHC restricted T cells by antigen presenting cells and their therapeutic potentials

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Since initial reports, more than 25 years ago, that T cells recognize lipids in the context on non-polymorphic CD1 molecules, our understanding of antigen presentation to non-peptide-specific T cell populations has deepened. It is now clear that αβ T cells bearing semi-invariant T cell receptor, as well as subsets of γδ T cells, recognize a variety of self and non-self lipids and contribute to shaping immune responses via cross talk with dendritic cells and B cells. Furthermore, it has been demonstrated that small molecules derived from the microbial riboflavin biosynthetic pathway (vitamin B2) bind monomorphic MR1 molecules and activate mucosal-associated invariant T cells, another population of semi-invariant T cells. Novel insights in the biological relevance of non-peptide-specific T cells have emerged with the development of tetrameric CD1 and MR1 molecules, which has allowed accurate enumeration and functional analysis of CD1- and MR1-restricted T cells in humans and discovery of novel populations of semi-invariant T cells. The phenotype and function of non-peptide-specific T cells will be discussed in the context of the known distribution of CD1 and MR1 molecules by different subsets of antigen-presenting cells at steady state and following infection. Concurrent modulation of CD1 transcription and lipid biosynthetic pathways upon TLR stimulation, coupled with efficient lipid antigen processing, result in the increased cell surface expression of antigenic CD1–lipid complexes. Similarly, MR1 expression is almost undetectable in resting APC and it is upregulated following bacterial infection, likely due to stabilization of MR1 molecules by microbial antigens. The tight regulation of CD1 and MR1 expression at steady state and during infection may represent an important mechanism to limit autoreactivity, while promoting T cell responses to foreign antigens.

Keywords: CD1, MR1, innate and adaptive immunity, lipids, vitamins

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Introduction

Earlier studies in the 1990s demonstrated that the antigen recognition potential of T lymphocytes is not limited to peptides presented by MHC class I and class II molecules (1, 2). Indeed, the newly identified MHC-related genes belonging to the CD1 family (3) were soon shown to present self and mycobacterial lipids to αβ and γδ T cell clones lacking CD4 and CD8 co-receptors (1, 2, 4).

Furthermore, human and murine T cells bearing semi-invariant T cell receptors (TCRs) (5, 6) were shown to be CD1d restricted (7).

A second MHC-related gene was identified in 1995, MR1 (8), which in 2003 was shown to select a population of cells known as mucosal-associated invariant T cells (MAIT) (9), also bearing semi-invariant TCRs (10). It was not until 2012, however, that microbial vitamin B2 metabolites were identified as the elusive antigens presented by MR1 molecules (11).

In the past 25 years, a number of investigators have elucidated the contribution of CD1- and MR1-restricted T cells to antimicrobial immunity, and for CD1-restricted T cells also to cancer immune-surveillance and autoimmunity. While comprehensive reviews on CD1 and MR1 antigen-presenting systems have been recently published (12–15), we will focus on recent findings that have advanced our understanding of the role of CD1- and MR1-restricted T cells, also known as non-conventional T cells or innate-like cells, as they straddle between innate and adaptive immunity.

CD1 Molecules

The human CD1 locus on chromosome 1 encodes five molecules, divided into group 1 (CD1a, b, and c) and group 2 (CD1d), based on sequence homology (3, 16). The fifth molecule, CD1e, is not expressed at the cell surface, yet plays an important role in assisting lipid antigen processing and loading on group 1 CD1 molecules (17). CD1 molecules are heterodimers of a heavy chain non-covalently associated with β-2 microglobulin, and have an overall fold similar to MHC class I molecules, however, unlike MHC class I and class II molecules, they are not polymorphic (3, 16). In comparison to MHC class I molecules, CD1 molecules have evolved a deep and narrow binding cavity that anchors the hydrophobic alkyl chains of lipid molecules: the binding cavity contains two pockets, A′ and F′, of which the A′ is deeper and closed by a narrow entrance at the top. Yet, each CD1 molecule differs in the antigen-presenting groove architecture, in the intracellular trafficking pattern, and in the overall tissue expression (18, 19). These differences underscore the non-redundant physiological role of the CD1 isoforms, which sample a variety of lipids in early, late endosomes or deep in the lysosomes, where exogenous lipids distribute according to their biophysical properties (20) (**Figure 1**). In mice, group 1 CD1 genes are absent and it is thought that they were lost during evolution, as they are present in other rodents (21). This has greatly hindered our understanding of the role and frequency of group 1 CD1-restricted T cells, until the recent development of CD1a, b, and c tetramers, which has opened the way toward enumeration and functional characterization of human lipid-specific T cells (22–24). Humanized SCID mice and group 1 CD1 transgenic mice are also proving to be useful models to study the role of CD1-restricted T cells in disease settings (25, 26).

CD1a

CD1a molecules are expressed on double-positive thymocytes, while in the periphery their expression is restricted to tissue-resident dendritic cells (DCs) and Langerhans cells (LC) in the skin (28). Unlike other CD1 isoforms, CD1a molecules have a short cytoplasmic tail, with no tyrosine-based motif to drive their recycling through late endocytic compartments. Hence, their trafficking is limited to the early endosomal compartment in a Rab22- and Arf6-dependent manner (29). Of all the CD1 molecules, CD1a has the smallest groove, which is suitable to present antigens encountered in the early endosomal compartment or at the cell surface (30–32).

CD1a-restricted cells can be autoreactive or pathogen reactive. The only microbial antigen known to bind CD1a is the mycobacterial lipopeptide didehydroxymycobactin (DDM) and DDM-restricted T cells could play a pivotal role in early detection of *Mycobacterium tuberculosis* infection (33). Like for many other lipid-specific T cells, recognition is exquisitely sensitive to the structure of the peptide and to the length and saturation of the fatty acid, which influences the positioning of the peptide residues available for recognition by the TCR (31). Despite a low affinity interaction (100 μM) between a DDM-specific TCR and CD1a–DDM soluble molecules (23), DDM–CD1a dextramers have been successfully used to stain DDM-specific T cells *ex vivo* in patients with active tuberculosis or positive tuberculin test, and could be a useful tool to determine the phenotype and function of these cells at a population level (23).

The first ever reported CD1-restricted clone was self-reactive (1). One of the first identified self-antigens presented by CD1a is sulfatide, a glycolipid abundant in myelin sheets. Of note, sulfatide can also be presented by CD1b, CD1c, and CD1d (34), which suggested a possible contribution of CD1-restricted T cells to the autoimmune response in multiple sclerosis (MS). To further characterize the pool of CD1a-autoreactive T cells, Moody, and co-workers have recently designed an experimental system based on CD1-expressing human myelogenous leukemia cells (K562 cells), with low or absent expression of MHC molecules in order to limit allo-reactivity. These studies have demonstrated that polyclonal CD1a reactive T cells are present at high frequency in the peripheral blood of healthy individuals [0.02–0.4% of memory T cells (35, 36)]. Similar results were independently obtained with C1R cells as antigen-presenting cells, although in this case higher frequencies of CD1a (and CD1c) reactive cells were observed [up to 10% of circulating T cells (36)]. Interestingly, CD1a-restricted T cells found in the blood express the skin-homing receptors CLA, CCR6, CCR4, and CCR10 and produce the cytokine interleukin 22 (IL-22) in response to $CD1a^+$ DCs. The identification of CD1a-restricted cells in skin biopsies suggests that they may be playing an important immunoregulatory role in skin homeostasis through IL-22 secretion (35). It will be very interesting to investigate whether they may also play a role in skin immunopathology in psoriasis or in other skin diseases where over production of IL-22 has been implicated (37).

To understand the nature of the antigens activating CD1a-restricted T cells, self-ligands were eluted from secreted CD1a molecules and skin samples and tested *in vitro* (38). Unexpectedly, stimulatory antigens were more efficiently extracted in chloroform than in the commonly used chloroform methanol mixture, suggesting high hydrophobicity. Indeed, CD1a molecules were found to stimulate T cell clones when loaded with oily antigens lacking carbohydrate or charged head groups [such as triacylglyceride (TAG), squalene, and wax esters], while lipids with hydrophilic head groups inhibited CD1a-restricted T cell autoreactivity (38). These results, which suggested a unique mode of

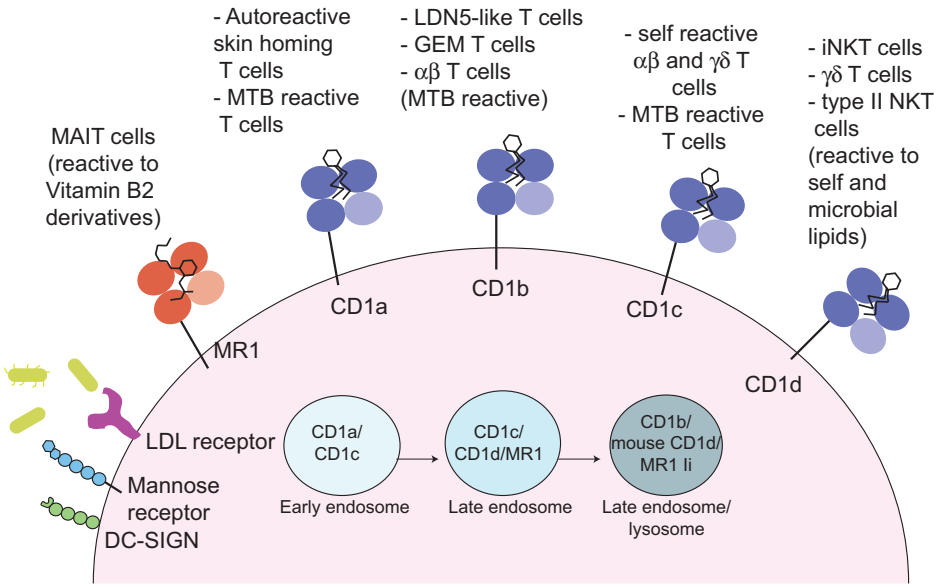

FIGURE 1 | Antigen presentation to non-peptide-specific T cells. MR1 and CD1 molecules present vitamin B2 derivatives or self and microbial lipids to a variety of αβ or γδ-bearing T cells. Through a variety of receptors (such as DC-SIGN, mannose receptor, and LDL-receptors) or via phagocytosis (not depicted) antigen-presenting cells uptake incoming pathogens. Microbial antigens are distributed through the endocytic compartment where they intersect recycling MR1 and CD1 molecules. In these compartments, antigen loading occurs, often through the help of accessory molecules such lipid transfer proteins (not depicted). The invariant chain (Ii) facilitates MR1 distribution in the late endosomal/lysosomal compartments (27).

"headless" antigen recognition by autoreactive CD1a-restricted T cells, were recently confirmed and extended with structural and mutagenesis studies (39). Although two of the studied autoreactive TCRs have binding affinities for CD1a–self complexes at the low end of the spectrum (30 and 93 μM (38, 39), CD1a tetramers loaded with a spectrum of permissive ligands [such as phosphatidylcholine and lysophosphatidylcholine (LPC)] have been shown to stain Jurkat cells transduced with one of these TCR (39). Furthermore, the ternary structure of two TCR–CD1a–self-lipid complexes showed that the TCR docks over the A′ roof of CD1a molecules without direct contact with the antigenic ligand. A comparison of these structures with those of CD1a–sulfatide (30) or CD1a–lipopeptide (31) provided a molecular explanation for the inhibitory effect of polar ligands, which are thought to disrupt the TCR–CD1a contact zone (39), revealing a mode of antigen recognition different from TCRs of peptide-specific T cells and other CD1-restricted T cells, centered on critical interactions with antigens bound to MHC or CD1 molecules (40).

TAG, fatty acids, and squalene accumulate in sebaceous glands and in the corneous stratus of the epithelium, separated from epidermal LC. So it is likely that at steady state, LC will not efficiently load these stimulatory antigens on CD1a molecules. However, upon trauma, infection or any form of barrier breach, these antigens could gain access to LC and increase the response of CD1a-autoreactive T cells. Consistent with this hypothesis, recently, CD1a-restricted responses have been documented in cohorts of patients allergic to bee and wasp venom (41). Despite the high lipidic content of wasp and bee venoms, in these patients the culprit antigens are not exogenous, but are generated *in vivo* by venom phospholipase A2 injected intradermally by wasps and bees via their sting (41). *In vitro*, it was shown that venom phospholipase A2 activates CD1a-restricted T cells cleaving non-antigenic phospholipids into lysophospholipids and antigenic headless fatty acids. Using an *in vivo* model of suction cap blisters, by mass spectrometry the authors also demonstrated the presence of lysophospholipids in the blister fluids of volunteers injected with venom. Although free fatty acids were not detected in the blister fluids, is it likely that this negative result was due to lack of sensitivity of the mass spectrometry (41).

Thus, the physical separation between antigen and antigen-presenting cells and/or the balance between stimulatory and inhibitory CD1a-ligands seem to be two of the mechanisms that the immune system deploys to keep an abundant population of autoreactive T cells under control at steady state. As endogenous or exogenous phospholipases can be activated during exposure to several allergens, it will be of interest to investigate whether in cohorts of patients with atopic dermatitis similar mechanisms may be active and account for expansion and/or activation of autoreactive T cells.

The identification of phospholipase A2 as a novel mechanism to generate autoantigens may offer new diagnostic and therapeutic opportunities. Likewise, as immunostimulatory oils and hydrocarbons are components of widely used adjuvants such as MF59, it will be important to address the role of CD1a-autoreactive T cells in shaping the adaptive T cell response during the aforementioned vaccination protocols.

CD1b

CD1b molecules are expressed on thymocytes and on peripheral DCs. Through tyrosine-based cytoplasmic motifs CD1b

molecules bind to both AP-2 and AP-3 adaptors (42, 43) and efficiently traffic to acidic LAMP1$^+$ lysosomes, where processing of complex lipid antigens may occur and loading is aided by the acidic pH and by lipid transfer proteins (17, 44, 45). Additionally, CD1b molecules have evolved the largest antigen-presenting groove, with three pockets (A′, C′, and F′) and a large tunnel, which can accommodate lipids with very long alkyl chains, such as mycobacterial antigens with up to 80 carbons (46, 47). As most cellular lipids do not exceed 40 carbons length, the architecture of the groove of nascent CD1b molecules is maintained by spacer (or scaffold) lipids, such as diacylglycerols and deoxyceramides (48, 49). The existence of spacer lipids was initially suggested from the crystal structures of CD1b bound to the ganglioside GM2 or phosphatidylinositol, where it was observed that detergent moieties occupied the channels not filled by the lipid ligands (46). Spacer lipids seat at the bottom of the antigen-presenting groove, providing support for antigens loaded in the CD1b molecule, and are displaced when loading of longer microbial lipids occurs (49, 50). Furthermore, by stabilizing the antigen-presenting molecules, they enhance presentation of microbial lipids with shorter acyl chains (49). Spacer lipids have also been found when CD1c and CD1d molecules were crystallized with short lipids (51, 52), so their use seems to be a common strategy to maintain the correct fold and antigen orientation for CD1 molecules.

While CD1b-autoreactive T cells have been described (53, 54), they are detected at lower frequency than for other CD1 members (35). Indeed, CD1b molecules are specialized in presenting bacterial antigens, perhaps because of the large volume of the groove; so far the majority of the described ligands are of mycobacterial origin. Mycolic acid from *M. Tuberculosis* cell wall was the first described lipid antigen presented by CD1 molecules (4), and it can form the scaffold for other mycolyl antigens, such as glucose monomycolate (GMM) and glycerol monomycolate (55). Other families of CD1b lipid antigens are derivatives of phosphatidyl-myo-inositol (such as phosphatidylinositol mannoside (PIM) and lipoarabinomannan (LAM)) and sulfoglycolipids. The structure–activity relationship of these classes of lipids has been recently reviewed in Ref. (56, 57) and we refer the reader to these excellent reviews for more detail.

The development of CD1b tetramers has recently allowed to track mycobacteria-specific CD1b-restricted T cells in the blood of individuals with active tuberculosis or previous *M. tuberculosis* (MTB) exposure (22). In addition to following the dynamics of lipid-specific immune responses, for those lipid antigens produced only by *M. tuberculosis* and not other mycobacterial species (such as sulfoglycolipids), tracking of antigen-specific T cells may represent an important future tool for differential diagnosis.

Until now, it was assumed that group 1 CD1-restricted T cells were expressing highly diverse TCR, like peptide-specific MHC-restricted T cells. However, the use of tetramers to study the CD1b-GMM-specific T cell response in multiple individuals has allowed the discovery of two novel T cell types in the human repertoire, germ-line encoded mycolyl reactive (GEM) T cells (58) and LDN5-like T cells (59), which stain brightly and dimly, respectively, as indication of higher (around 1 μM) and lower affinity (20–40 μM) for CD1b–GMM complexes. GEM T cells express a semi-invariant TCR using TRAV1.2 variable segments rearranged to TRAJ9 joining segments (thus differing from the TRAV1.2-TRAJ33 TCR used by MAIT cells, see later), with nearly identical CDR3 sequences and a biased TRBV6.2 usage. LDN5-like T cells have a biased TRAV17 usage, with uniform CDR3 length, and a biased TRBV4.1 usage, with variable CDR3 length. Structural data point to a role of the TCR-β chain in influencing the fine specificity of the GMM-specific TCRs (58). The evidence to date suggests that GEM and LDN5-like T cells expansion is antigen driven upon infection, and that they do not represent another population of innate-like cells, unlike the semi-invariant MAIT and iNKT cells; however, a detailed transcriptional and functional analysis of these cells is yet to be done. Furthermore, it still remains to be determined whether GEM and LDN5-like T cells show immunological memory, how long they persist, and whether they can be harnessed for vaccination purposes. Finally, it remains to be determined what drives the selection of these cells in donors with no documented mycobacterial infection.

CD1c

CD1c molecules are expressed on thymocytes and at high density on peripheral DCs, LC (together with CD1a), and B cells. Through associations with AP2 adaptor molecules, they are widely distributed through the endocytic system (but not the LAMP1$^+$ lysosomes), which allows sampling of a broad spectrum of lipids in a variety of antigen-presenting cells (60).

CD1c molecules present self and microbial lipids to T cells bearing αβ and γδ TCR. In 1989, Porcelli and colleagues demonstrated specific recognition of CD1c by a CD4$^-$CD8$^-$ γδ CTL line (1) and CD1c self-reactivity was later confirmed with other cytotoxic γδ lines bearing the Vδ1 segment (61, 62). The self-antigens recognized by these CTL lines, though, still need to be identified.

CD1c-autoreactive αβ T cells are present at high frequency in the peripheral blood of healthy donors (36). Recently, a novel self-lipid antigen (methyl-lysophosphatidic acid, mLPA) that accumulates in leukemic cells has been identified as one of the targets of CD1c-reactive T cells (63). mLPA-specific T cell clones were shown to efficiently kill *in vitro* and *in vivo* primary leukemia cells in a CD1c-restricted manner, but not normal B cells and primary DC, that despite being CD1c positive do not express the antigen at significant level (63). Selective accumulation of mLPA in human leukemia suggests that it can be considered a novel class of tumor-associated antigens and may represent a promising immunotherapeutic target.

CD1c molecules present several mycobacterial and synthetic lipids with methylated alkyl chains: mannosyl phosphodolichols (MPDs), mannosyl-β1-phosphomycoketide (MPM), and phosphomycoketide (PM) (24, 64–66). The mycobacterial enzyme polyketide synthase 12 (psk12) is crucial for the synthesis of the methyl-branched lipids, which are a molecular signature of mycobacterial infection and essential for antigenicity (24, 65). Polyclonal CD1c-restricted T cells expand *in vivo* during mycobacterial infection and can be tracked with lipid-loaded CD1c tetramers (24, 64).

The range of self and foreign antigens presented by CD1c may be larger than currently appreciated, as it has been shown that also lipopeptides can be antigenic, in analogy to CD1a. This was demonstrated as a proof of principle with a synthetic *N*-acyl

glycine dodecamer lipopeptide (lipo-12) (67). These results raise the possibility that other eukaryotic or viral *N*-terminally acylated peptides, such as post-translationally modified products of ribosomal translation, might be antigenic. Interestingly, as lipopeptide presentation is sensitive to proteolysis in late endosomes and lysosomes (67), CD1c and CD1a molecules are the two CD1 isoforms uniquely suited to lipopeptide presentation because they predominantly survey the secretory pathway and the early endosomes.

Structural studies have highlighted a partially open structure of CD1c F′ pocket (52), which may accommodate a variety of ligands, from diacylated lipids such as sulfatide (34), to lipopeptides and possibly aid antigen loading in the early endosomal compartment or at the cell surface, in the absence of specific lipid transfer proteins. Furthermore, the CD1c–MPM crystal structure has highlighted the essential role for the methyl branches of MPM in stabilizing the single alkyl chain in the A′ pocket (52). Finally, the exquisite specificity of MPM and PM reactive clones can also be explained at the structural level (68): in the absence of the mannosyl moiety, the phosphate head group of PM is shifted toward the F′ pocket. A range of TCR binding affinities (7–30 μM) have been reported for CD1c–mycoketide complexes, and while the ternary complex TCR–CD1c–antigen is not yet available, biophysical data with six CD1c-reactive TCRs showed that different TCRs used different docking strategies on the same CD1c–lipid complex, unlike what has been described for the iNKT TCR and is predicted for the GEM TCR (68).

CD1d

CD1d molecules are the most widely distributed, as they are expressed not only on hematopoietic cells (thymocytes, monocytes, DCs, and B cells), but also on epithelial cells (28). CD1d-restricted T cells are collectively known as natural killer T cells, because of co-expression of T cells and NK cell markers (most notably CD161 in humans and NK1.1 in some mouse strains). Two types of NKT cells exist: type I, (also known as invariant, iNKT), expressing a semi-invariant TCR (Vα24-Jα18 paired to Vβ11 in humans, Vα14-Jα18 paired to Vβ2, Vβ7, or Vβ8.2 chains in mice); type II, expressing a polyclonal TCR repertoire (12). The CD1d antigen presentation system is conserved across species, and both human and murine iNKT cells can be tracked with CD1d tetramers loaded with the synthetic glycolipid agonist α-galactosylceramide (α-GalCer). Furthermore, the availability of murine models lacking type I or type I and II NKT cells has greatly contributed to our knowledge of the biology of these cells. Conversely, we still lack reagents to specifically detect the majority of type II NKT cells, thus with few exceptions, their role *in vivo* has been less characterized.

Through recognition of a variety of self and microbial antigens, NKT cells have an important immune-regulatory role, spanning from autoimmunity, to protection against infection and tumor immune-surveillance. We refer the reader to recent reviews for a comprehensive discussion of NKT cell biology and CD1d antigen presentation (12, 14, 69–71), while here we highlight the role of microbiota in modulating iNKT cell reactivity and we summarize results revealing a previously unknown heterogeneity of the human NKT cell family (**Figure 1**).

Microbiota and NKT Cells

Regulation of metabolism and immunity by commensal bacteria is now well established (72). Interestingly, α-GalCer, the most potent iNKT cell agonist to date, was originally isolated from commensal bacteria of the marine sponge *Agela mauritianus* (73). The α-anomeric linkage of the sugar moiety is the quintessence of a microbial signature, and a variety of iNKT cell agonists from different microbial species have been characterized, although during microbial infection iNKT cell reactivity is often driven by cytokine-mediated signals (74). Recently, inhibitory and activatory α-GalCer species have been biochemically isolated from *Bacteroides fragilis*, a prominent species of the gut microbiota (75, 76). It has also been demonstrated that the intestinal microbiota plays an important role in the tight regulation of iNKT cell numbers and function, possibly through the balance between stimulatory and inhibitory lipids: germ-free mice have increased relative and absolute numbers of iNKT cells in the intestine, due to increased CXCL16 expression in the mucosal epithelium and CXCL16-dependent iNKT cell homing (77). The conditioning effect of the microbiota starts very early in life and has long-lasting consequences, as demonstrated by higher susceptibility of germ-free mice to intestinal immunopathology and lung inflammation (77). In turn, iNKT cells influence bacterial colonization of the intestine and lungs of mice (78) and signaling through epithelial CD1d is essential in maintaining mucosal homeostasis via IL-10 secretion (79). These findings have been recently extended in humans, where phenotypically and functionally mature iNKT cells have been detected in the sterile environment of the fetal intestine, and it is thought that they may represent an important first line of defense at birth (80). Furthermore, lysosulfatide-reactive CD1d-restricted type II NKT cells have been identified in the mucosa of ulcerative patients, and their cytotoxic activity against the intestinal epithelium suggests a pathogenic role (81). It remains to be determined whether during intestinal inflammation, T cells restricted by group 1 CD1 may also recognize self or microbial lipids.

Human NKT Cell Heterogeneity

Adipose tissue-resident iNKT cells

Like MHC-restricted CD4 cells, iNKT cells can also differentiate in Th1, Th2, Th17, T_{FH}, and T-regulatory subsets, which use the same transcription regulators as peptide-specific T cells (69). The balance between subsets could have profound regulatory effects during immune responses, through the secretion of cytokines and modulation of DC and B cell function (12). Recently, a tissue resident subset of iNKT cells with a unique transcriptional and cytokine profile has been shown to accumulate in adipose tissue and regulate the function of Tregs and macrophages, via IL-2 and IL-10, respectively (82). Adipose tissue iNKT cells do not express the master regulatory PLZF, but express the transcription factor E4BP4, which controls IL-10 production. Also, as compared to splenic or liver iNKT cells, a smaller fraction of adipose tissue iNKT cells expresses CD44 and NK1.1 markers, while expression of ICOS and PD-1 was increased. As adipocytes are CD1d positive, they could modulate iNKT cell activation through presentation of self and dietary lipids, and ultimately the cross talk between iNKT cells and adipose tissue macrophages could be very important

in preventing tissue inflammation. This hypothesis is consistent with the described protective role of iNKT cells against obesity-induced chronic inflammation (83).

CD1d-restricted γδ T cells

Two groups employed CD1d tetramers loaded with two different ligands to isolate CD1d-reactive T cells from healthy human peripheral blood. In one study, the majority of CD1-sulfatide tetramer staining cells were found to be T cells bearing the Vδ1 TCR (84), while a second study identified Vδ1 T cells amongst those binding CD1d-α-GalCer tetramers, although the majority of cells binding to the latter tetramers, as expected, were iNKT cells (85). Interestingly, human Vδ1 do not recognize mouse CD1d-α-GalCer tetramers, unlike human iNKT cells, highlighting a clear difference in the reactivity of the two populations. Also the affinity of binding of Vδ1 cells to human CD1d-α-GalCer complexes is lower than that observed for iNKT cells (Kd 16 versus 0.5 μM).

Vδ1 bearing cells are typically tissue-homing γδ cells and are abundant in the intestinal mucosa (86, 87). Preliminary results suggest that some reactivity to C1R cells expressing CD1d molecules can be detected amongst Vδ1 polyclonal lines generated from intestinal biopsies (88). Given the presence of several lipids from the microflora and the abundant expression of CD1d on the gastrointestinal epithelium (28), future studies should investigate whether intestinal Vδ1 γδ T cells can also bind CD1d-α-GalCer tetramers and if so, the role of microbiota in maintaining and expanding Vδ1 γδ T cells after birth. As Vδ1 cells are present at higher frequency than iNKT cells, they could have a marked impact on intestinal homeostasis and immunopathology, and reactivity could be modulated by the expression of stress-induced MHC-related molecules like MICA and MICB (86). Likewise, reactivity to sulfatide may underscore a possible role of these cells in MS.

The mode of γδ TCR–CD1d-α-GalCer/sulfatide recognition is markedly different from that of the iNKT TCR (85, 88). The γδ TCR docks orthogonally rather than in a parallel manner like the iNKT TCR, thus resembling type II NKT TCRs and classical peptide-specific TCRs (40); CD1d binding is dominated by the TCRδ chain, while CDR3γ residues contribute to lipid antigen binding only in CD1d-α-GalCer, but not in CD1d–sulfatide ternary complexes (85, 88).

NKT cells and chronic inflammation

Several investigators have described reactivity of human type II NKT cells toward inflammation-associated lysolipids, generated by the action of PLA A2 (89). T cells binding CD1d–LPC multimers were found at higher frequency in the blood of myeloma patients compared to healthy controls, consistent with elevated serum levels of LPC in the plasma of these patients (90). In infected hepatocytes, Hepatitis B was shown to induce the activity of secretory phospholipases and the release of lysophosphatidylethanolamine (lyso PE), capable of eliciting CD1d-restricted type II NKT cells activation in humans and mice, suggesting that they may play a role in viral recognition (91).

Glucosylsphingosine (LGL1), the deacylated product of β-glucosylceramide (GL1), accumulates in several metabolic disorders such as Gaucher disease, as a consequence of altered sphingolipid metabolism. In all metabolic disorders, lipid accumulation is associated with progressive inflammation. One of the contributing factors could be the expansion of pathogenic LGL1-reactive CD1d-restricted type II NKT cells with a T_{FH} phenotype, stimulating inflammation and B cell activation (92).

The demonstration that lysolipid species are antigenic for subsets of CD1d-restricted NKT cells is of great interest and provides the link for NKT cell activation in sterile inflammatory conditions, possibly suggesting novel therapeutic modalities through selective inhibition of the biochemical pathways generating the relevant antigens.

On the Role of DC in Regulating CD1 Reactivity

The central role of DCs in orchestrating immune responses is now well established (93). Immature DC, residing in the periphery, patrol the body for incoming pathogens and recognition of pathogen molecular patterns (PAMPs) through pattern recognition receptors (PRRs) triggers DC activation, maturation, and migration to the draining lymph nodes. Coordinated changes in expression of MHC class I and II, co-stimulatory molecules and cytokines upon DC maturation, promote efficient priming of peptide-specific CD4, CD8 T, and B cells in the lymph node (94, 95). The heterogeneity in DC subsets and their different anatomical distribution results in unique functional specialization, and ensures tailoring the adaptive immune response to the type of incoming stimulus (95).

As highlighted in the previous paragraphs, CD1d expression is constitutive and shared by all DC subsets; however, expression of group 1 CD1 molecules is much more restricted. Due to the strong autoreactivity of CD1-restricted T cells, tight regulation of steady-state cell surface expression of CD1 is required to control their activation. For example, lipids found in human serum, particularly lysophosphatidic acid and cardiolipin, inhibit group 1 CD1 expression, through a transcriptional mechanism involving activation of the peroxisome proliferator-activated receptor (PPAR) nuclear hormone receptors (96).

Monocytes express only CD1d molecules, but during *in vitro* differentiation into DC with GM–CSF and IL-4, group 1 CD1 expression is induced (2). It is likely that, *in vivo*, cytokines in the local microenvironment might influence group 1 CD1 expression in the process of monocyte to DC differentiation following transendothelial migration (97). Indeed it has been shown that monocyte infection with mycobacteria represents an efficient way to induce DC differentiation and expression of group 1 CD1 molecules (98–100). Upregulation of CD1 molecules depends on NOD and TLR signals and is enhanced by concomitant inflammasome activation and release of bioactive IL-1β (101). Interestingly, while mycobacterial infection increases group 1 CD1 expression, it downregulates CD1d and interferes with MHC-restricted antigen presentation (99, 102).

Mycobacterial cell wall lipids thus have a dual effect, by serving as antigens (i.e., mycolic acids, GMM, LAM PMK, and DDM) and adjuvants that drive CD1 expression on the infected cells, to promote antigen presentation. However, group 1 CD1 molecules are not expressed on macrophages, which instead are the infected cells during *in vivo* MTB infection, thus whether CD1b-restricted T cells might play a sizeable cytotoxic and anti-mycobacterial

function *in vivo* is debatable. The low frequencies of group 1 CD1-restricted T cells, even after infection, rather suggests a helper function, perhaps through modulation of DC function (103).

While during DC maturation a marked upregulation of MHC class I and II is observed, with increased half-life of surface MHC–peptide complexes leading to efficient peptide antigen presentation (104, 105), the surface expression of group 1 CD1 molecules is only moderately increased (CD1b, CD1c) or even decreased (CD1a) (106); furthermore, CD1 molecules continue to recycle between the plasma membrane and intracellular compartments (102). CD1-mediated lipid antigen presentation occurs very efficiently already in immature DC and this might ensure prompt licensing of DC by lipid-specific T cells via cytokines and CD40–CD40L interactions (103). The role of iNKT cells in DC licensing and memory CTL generation is discussed in depth in an accompanying review in this issue.

To ensure optimal antigen presentation through CD1 molecules, DC subsets also coordinate lipid antigen uptake and distribution through the endosomal compartment through specific receptor-mediated interactions (19). Serum lipoproteins ensure efficient delivery of self and foreign antigens for CD1-mediated presentation, through ApoE-LDL-R-mediated uptake (107).

The C-type lectin Langerin mediates *Mycobacterium leprae* antigen uptake and delivery to Birbeck granules in LC and is required for CD1a–lipid antigen presentation (108). The mannose receptor (CD206), a C-type lectin expressed on macrophages, dermal DC, and monocyte-derived DC, promotes mycobacterial LAM uptake and lysosomal delivery for CD1b presentation (109). Other C-type lectins that specifically capture pathogen-derived carbohydrate rich antigens are DEC 205 (expressed on LC, dermal DC, and monocyte-derived DCs), DC-SIGN (CD209, expressed mainly on dermal DC). Their role in enhancing peptide presentation is well described (110, 111) and it is likely that these and other related molecules involved in endocytosis of bacteria or bacterial debris might also influence CD1d-restricted lipid antigen presentation in late endosomal compartments. Selective expression of endocytic receptors in DC subsets can also be exploited therapeutically: recently it has been shown that targeted delivery of the mycobacterial antigen GMM to monocyte-derived DCs via Siglec-7 via sialic acid-coated nanoparticles induces robust CD1b-restricted T cell activation, although this was not tested on primary $CD1b^+$ $Siglec\text{-}7^+$ myeloid DCs (112).

In addition to transcriptional regulation of CD1 expression, T cell autoreactivity is controlled by the availability of self-ligands. Although determination of the repertoire of lipids bound to CD1 molecules is technically challenging, mass spectrometry analysis of lipids eluted from secreted CD1d molecules has revealed the presence of several types of phospho and sphingolipids acquired during biosynthesis (113, 114), the majority of which are non-antigenic (89). Moody and co-workers used a recently established lipidomic platform to compare self-lipids associated with all CD1 molecules and the results confirmed the ability of CD1 molecules to bind a variety of molecules (49). It is now also well established that the range of glycosphingolipids (GSL) and phospholipids expressed by cells varies amongst cell types and with cellular activation (115). TLR activation of myeloid cells has marked effect on the expression of key genes involved in GSL biosynthesis (54, 116–118), which translate in detectable biochemical changes (54, 119). This has been shown to lead to increased CD1b-restricted and iNKT cell autoreactivity (54, 116–118).

MR1 and MAIT Cells

The MHC-related molecule MR1 (8) presents antigens to a family of innate-like T cells bearing a semi-invariant TCR and known as MAIT (10). In humans, the MAIT TCR consists of the Vα7.2 TCR-α chain mostly joined to Jα33 segments (TRAJ33) and paired to a limited number of TCR-β chains (mainly TRBV6 and TRBV20).

MR1 molecules are non-polymorphic and highly conserved among mammalian species, leading to functional cross-reactivity, which is reminiscent of the species conservation in the CD1 antigen-presenting system (120). Like iNKT cells, MAIT cells are selected in the thymus by double-positive cortical thymocytes (121), but unlike iNKT cells they leave the thymus as naïve cells and complete their maturation in the periphery (122, 123). MR1 expression on peripheral B cells and the intestinal flora are crucial for MAIT cells survival, expansion, acquisition of a memory phenotype, and effector functions (9).

Due to their anatomical mucosal localization and innate-like properties with a Th1-like effector phenotype, MAIT cells are in a unique position to act as early sentinels in response to respiratory and intestinal pathogens. Indeed, they have been shown to be activated in response to a variety of bacterial and fungal infections (124, 125) and to play a role in infectious models with *BCG*, *Francisella tularensis*, *Klebsiella penumoniae* (126–128), and MTB (129). Despite the well-characterized antimicrobial activity of MAIT cells, the antigens bound to MR1 remained for a long time elusive, until a major breakthrough in 2012 demonstrated that MR1 molecules present vitamin B2 metabolites to MAIT cells (11). These vitamins are not produced by mammals, hence they can be considered as molecular signatures of microbial infection. Consistently, microbes lacking the ability to synthesize riboflavins (such as *Streptococcus pyogenes* or *Enterococcus faecalis*) are unable to induce MR1-dependent MAIT cell activation (11).

Like iNKT cells and γδ cells, however, MAIT cells can also be activated in a TCR/MR1-independent manner, through the stimulatory activity of IL-12 and IL-18 secreted by activated APCs (130). Hence, it is possible that MAIT cells may play an immunoregulatory role also during infections with viruses and with bacteria lacking the riboflavin synthetic pathway or in sterile inflammation.

MR1 Ligands

MR1 molecules are ubiquitously expressed, although barely detectable at the cell surface (131), unless cells are incubated with vitamin ligands that increase MR1 expression (11, 132, 133). Two types of vitamin ligands have been described, stimulatory (riboflavin intermediates) and not (folic acid derivatives). Both classes of ligands have been shown to stabilize MR1 molecules, covalently binding through a Schiff base complex; however, crystallographic studies revealed that TCR recognition is exquisitely sensitive to the ribityl moiety present only in the riboflavin derivatives (134).

To date, two classes of stimulatory riboflavins are known, ribityllumazine and pyrimidines [more powerful agonists, but highly unstable unless trapped by MR1 molecules (135)]. While initially the ribityllumazine rRL-6-CH_2OH was the bacterial ligand (from *Salmonella enterica serovar Typhimurium* supernatants) thought to bind to MR1 (11), subsequent elegant studies with Gram$^+$ (*Lactococcus lactis*) and Gram$^-$ (*E. coli*) bacterial strains defective for key enzymes in the riboflavin synthesis pathway unveiled the intermediate compound, 5-A-RU (5-amino-6-D-ribitylaminouracil) as the key precursor for pyrimidines and ribityllumazines (133, 135). Bacterial-derived 5-A-RU itself is not stimulatory, but it reacts with bacterial or host cell-derived small glyoxal compounds to form pyrimidines, which then can condense to form ribityllumazine.

Future studies will be needed to identify the molecular mechanisms of vitamin antigen presentation through MR1. For example, the relative contribution of host-derived versus bacterial-derived glyoxal compounds that react with 5-A-RU remains to be determined, as is the cellular compartment where this condensation and the subsequent MR1 loading occur. Furthermore, the observation that some non-activating ligands [Ac-6-FP (132)] can induce rapid and prolonged upregulation of MR1 molecules suggests different effects on MR1 trafficking. Finally, the currently identified ligands are all bound in an aromatic cradle in the A′ pocket of the MR1 binding groove [although with different orientations (134)], and there remains the possibility that other classes of ligands might extend in the more exposed F′ pocket.

MR1 Tetramers and MAIT Cell Heterogeneity

With the discovery of MR1 ligands, MR1 tetramers have been developed to characterize the MAIT cell population, previously identified solely as TRAV1.2$^+$, CD161$^+$ CD8$^+$ cells (136). In peripheral blood, rRL-6-CH2OH-loaded MR1 tetramers bind to a population of CD3$^+$ CD4$^-$ CD161$^+$ cells with comparable frequency to the TRAV1.2 antibody. The advantage of tetramer over antibody stainings, however, is that tetramers are able to detect MAIT cells that have downregulated CD161 expression, such as post activation or during HIV infection (137). Single cell sorting of CD161$^+$ TRAV1.2$^+$ cells and CD161$^+$ MR1-tetramer$^+$ cells and multiplex analysis of their TCR genes revealed the use of alternative rearrangements (particularly TRAJ20 and TRAJ12) in addition to the canonical TRAV1.2-TRAJ33 (136). These alternative rearrangements have also been identified by different investigators that performed deep sequencing of mRNA from MAIT cells sorted on the basis of TRAV1.2 and CD161 co-expression (138) and on MAIT populations that specifically secreted TNF-α in response to selected pathogens (139). Diversity in the CDR3β region due to amino acid additions and a diverse use of TCR-β chains (in addition to TRBV6.4 and TRBV20) have also been observed, suggesting an unexpected heterogeneity of the peripheral MAIT T cell repertoire.

Interestingly, the canonical MAIT TCRs as well as those bearing TRAJ12 and TRAJ33 segments have a conserved Tyr95 residue in the CDR3α-chain, which is essential in forming a hydrogen bond with the ribityl tail of activating ligands (134). These three TCRs also adopt a very similar docking mode on MR1–antigen complexes (132, 140–142). However, other recently described MR1-restricted TCRs lack the Tyr95α residue (139) and future studies will be required to confirm that these TCRs do indeed confer MR1-restricted reactivity and to determine the molecular details of their antigen recognition.

Furthermore, the non-canonical TCR α-chains paired almost exclusively with TRBV6.4 (136), raising the possibility that the TCR β-chain repertoire might impact antigen recognition, as observed with iNKT cells (143, 144). Indeed, structural and biophysical data have provided experimental evidence that the CDR3β loops can fine-tune the MAIT–TCR interaction and responsiveness to MR1, in an antigen-dependent manner (132).

The functional correlate of the phenotypic heterogeneity of the MAIT repertoire is currently unclear, and an interesting hypothesis is that it may be a surrogate signature of specific pathogen infections. Along these lines, Gold and co-workers reported selective use of MAIT cell TCRs in response to three different pathogens (*Mycobacterium smegmatis, Salmonella typhimurium, and Candida albicans*) in individual subjects (139). In this data set, however, no unique TCR sequence was found to be associated with individual pathogens across individuals. In these donors, functionally responsive MAIT cells for TCR sequencing were identified by TNF-α secretion, however, in the absence of blocking experiments with MR-1 antibodies it is unknown whether the responses were entirely TCR dependent or co-stimulated by cytokines. Nevertheless, these results are of interest, as they suggest that the MAIT cell TCR repertoire potentially reflects the host's microbial exposure history because of qualitative differences in the class of antigens presented by different pathogens, and that MAIT cells could exhibit immunological memory. However, an alternative interpretation is that different subsets of MAIT cells are differentially activated by pathogens in function of their TCR-β sequence heterogeneity (and hence of their TCR affinity), according to quantitative rather than qualitative differences in antigen availability in different microbes. Consistently, recent work by Lantz and co-workers using bacteria with mutations in the riboflavin biosynthetic pathway, suggested limited MR1 ligand heterogeneity between Gram$^+$ and Gram$^-$ bacteria (133).

Ultimately, longitudinal studies with well-defined microbial exposures (for example *MTB*, *Salmonella typhi* or *paratyphi*) will be needed to further explore these alternative hypotheses. In addition, it will be of interest to compare the MAIT T cell repertoire in the naïve thymus, in cord blood and in adults as MAIT cells undergo antigen-driven expansion at birth (122, 123). So far, the only study that analyzed by deep sequencing sorted TRAV1.2$^+$ CD161$^+$ MAIT from peripheral blood of three donors after a 5-month-interval showed that the oligoclonal TCRβ repertoire is stable in the absence of infection (138).

MAIT Cells in Sterile Inflammation

Although the predominant role of MAIT cells is protection against infections, there is some evidence that they may be implicated in autoimmune responses. Murine transgenic MAIT cells protect from the induction and progression of experimental autoimmune encephalomyelitis, and in MR1-deficient mice, which lack MAIT cells, EAE is exacerbated (145). MAIT cell TCR sequences were identified by single-strand polymorphism

analysis in autoptic material of patients with MS (146), and one study reported a decrease in the frequency of MAIT cells in the blood of MS patients, proportional with the severity and activity of the disease (147). It has been shown that IL-18 in the serum of MS patients drives MAIT cell activation and increased expression of VLA4, an integrin that mediates migration across the blood–brain barrier (148).

Reduction of MAIT cell frequencies has been reported in the small intestine of celiac disease patients (149), while an increase was observed in the inflamed mucosa of patients with inflammatory bowel disease, with a clear activated phenotype (150). In both disease settings, changes in frequency and phenotype of tissue resident and circulating MAIT cells might be driven by compromised gut barrier function and bacterial overgrowth, as also observed during HIV infection (137, 151).

Mucosal-associated invariant T cells have been identified amongst the IL-17-producing cells in psoriatic skin, although percentages were not significantly different compared to healthy skin (152). It is currently unclear whether MAIT cells are activated in the psoriatic skin via microbial ligands or as a result of the general inflammation. Finally, it has been reported that the frequency of peripheral blood CD8 and double-negative (DN) MAIT cells is reduced in lupus and rheumatoid arthritis (RA) patients, with an accumulation of MAIT cells in the synovial fluid in RA (153). This reduction was more pronounced in patients with highly active disease. Also the capacity of MAIT to secrete IFN-γ was reduced in response to both bacterial and PMA/ionomycin stimulation, although this was shown to be unrelated to their increased expression of PD-1 (153).

While enumeration of MAIT frequencies on the basis of the sole expression of Vα7.2 and CD161 might lead to some preliminary interesting observations, further studies will need to include MR1-tetramer staining or qPCR analysis of the invariant TCR, to avoid underestimation of frequencies, as CD161 is often downmodulated following activation (154). Furthermore, the lack of a suitable animal model, due to very low frequency of MAIT cells in inbred laboratory mice may hinder the understanding of the functional relevance of the above phenotypic analysis.

Recent results have brought MAIT cells to the center stage in chronic inflammatory settings associated with obesity and diabetes, possibly as a consequence of the altered composition of the gut microbiota in both diseases (155). The frequency of circulating MAIT cells, as determined by both MR1 tetramer staining and TRAV7.2 and CD161 staining, was significantly reduced as compared to healthy controls. The remaining MAIT cells showed a phenotype consistent with activation (upregulation of CD25 and CD69) and an inflammatory cytokine bias (higher secretion of IFN-γ, IL-2, IL-7, granzyme B). Conversely, MAIT cells were increased in subcutaneous and omental adipose tissue as compared to the blood, suggesting preferential tissue recruitment. Adipose tissue MAIT cells also secreted more IL-17 in obese as compared to lean patients. Interestingly, the authors observed an attenuation of MAIT cell abnormalities after weight loss following bariatric surgery. It is possible that IL-7 produced by adipose tissue stromal cells facilitates MAIT cell activation, as previously observed in the liver (156). In addition to cytokines, changes in gut microbiota and permeability might release a variety of bacterial ligands, which could react with increased endogenous levels of methylglyoxals to form MAIT cell agonists.

MAIT Interactions with APCs

As mentioned before, MR1 is ubiquitously transcribed, although cell surface expression is very low and it is only transiently upregulated following infection or incubation with some of the synthetic ligands (27, 131–133). Upon infection, MAIT cells can be activated in a MR1-dependent way by a variety of cells, including DC, macrophages, epithelial cells, and fibroblasts (124, 125). By secreting a plethora of regulatory cytokines (138), activated MAIT like iNKT cells may be able to modulate the antimicrobial function of other cells. Likewise, by secreting chemokines like CCL4 they can recruit NK, monocytes, and other inflammatory cells to infected tissues (138). Furthermore, it is likely that MAIT cell activation will provide an early source of IFN-γ during infections, facilitating the development of Th1 immunity, as described for NK cells (157), γδ (87), and iNKT cells (158). However, it is currently unknown whether MAIT cells are capable of inducing effective DC maturation *in vivo*, and if so the relative contribution of cytokines and CD40–CD40L interactions, which are key for DC licensing by CD1-restricted cells (103).

Post-natal MAIT cell expansion depends on bacterial flora and B cells (9). *In vitro*, primary B cells and EBV-transformed B cell lines have been shown to induce MAIT cell activation in an MR1-dependent manner following infection with commensal or pathogenic intestinal bacteria (159). Lack of titration of MAIT cell activation and reduced stimulation by paraformaldehyde fixed B cells, however, suggest a possible contribution of soluble factors, which was not addressed. Consistent with this, IFN-γ secretion by activated MAIT cells was only partially blocked by the anti MR1 antibody 26.5.

Finally, despite known expression of the CD161 ligand LLT1 by activated B cells and DC (160, 161), its role in modulating MAIT cell reactivity remains to be addressed, and could be of relevance considering the profound CD161 downmodulation observed with MAIT cell activation (154).

Concluding Remarks

Our current understanding of innate-like T cell populations has been widened in the past few years by few key technological advances, such as the identification of novel agonists and the capacity to refold antigen-presenting molecules to generate tetramers to enumerate qualitatively and quantitative these cells in health and disease settings. Biophysical and crystallographic studies, coupled with extensive mutagenesis, have elucidated the fine molecular details of antigen recognition, highlighting the existence of conserved (for iNKT, MAIT, and GEM T cells) and more variable (for CD1a and CD1c-restricted T cells) TCR footprints over the cognate antigen-presenting molecules (40). In the future, a better understanding of the fine details of antigen presentation through MR-1 may open new avenues aimed at therapeutically harnessing MAIT cells in promoting the cross talk between the innate and adaptive arms of the immune system. Given the higher frequencies of MAIT cells over iNKT cells in humans, and their enrichment at mucosal sites, MAIT

cell agonists might prove effective adjuvants to promote mucosal immune responses.

Finally, functional and phenotypical enumeration of MAIT cells and GEM T cells with tetramers may become a valuable immuno-monitoring tool. For example, it has been shown that MAIT cell frequencies are reduced in the blood of individuals with active MTB infection but they normalize after therapy (154), hence it should be explored whether they could be considered a marker of disease status, possibly to identify individuals at risk of progression to clinically active disease. Additional population of invariant T cells are being discovered by next-generation sequencing of the TCR-α chain repertoire (162) and may be used to probe antigenic exposure at a population level.

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The role of invariant natural killer T cells in dendritic cell licensing, cross-priming, and memory CD8$^+$ T cell generation

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New vaccination strategies focus on achieving CD8$^+$ T cell (CTL) immunity rather than on induction of protective antibody responses. While the requirement of CD4$^+$ T (Th) cell help in dendritic cell (DC) activation and licensing, and in CTL memory induction has been described in several disease models, CTL responses may occur in a Th cell help-independent manner. Invariant natural killer T cells (iNKT cells) can substitute for Th cell help and license DC as well. iNKT cells produce a broad spectrum of Th1 and Th2 cytokines, thereby inducing a similar set of costimulatory molecules and cytokines in DC. This form of licensing differs from Th cell help by inducing other chemokines, while Th cell-licensed DCs produce CCR5 ligands, iNKT cell-licensed DCs produce CCL17, which attracts CCR4$^+$ CD8$^+$ T cells for subsequent activation. It has recently been shown that iNKT cells do not only enhance immune responses against bacterial pathogens or parasites but also play a role in viral infections. The inclusion of iNKT cell ligands in influenza virus vaccines enhanced memory CTL generation and protective immunity in a mouse model. This review will focus on the role of iNKT cells in the cross-talk with cross-priming DC and memory CD8$^+$ T cell formation.

Keywords: natural killer T cells, dendritic cells, licensing, memory, CD8 T cells, cross-presentation

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Classification of Natural Killer T Cells

Natural killer T cells (NKT cells) are a subset of lymphocytes with innate and adaptive immune functions, for example, in tumor and anti-infectious defense (1). Their TCR can be either semi-invariant and encoded by a germline Valpha gene [type I invariant natural killer T cells (iNKT cells)] or may react against the self-antigen sulphatide using an oligoclonal TCR (type II NKT cells) (2–4). This review focusses on iNKT cells in dendritic cell (DC) licensing and T cell activation leading to a sustained memory response.

Invariant natural killer T cells respond to the marine sponge (*Agelas mauritianus*)-derived glycolipid alpha-galactosylceramide (αGalCer) presented by the non-polymorphic CD1d molecule and respond by rapidly producing various cytokines (5, 6). Mostly studied in mice, they represent about 0.5% of T cells in the blood, 2% in secondary lymphatic organs, and over 30% of T cells in the liver. During inflammation and infection, iNKT cell numbers can strongly increase in numerous organs, e.g., the pancreas in type I diabetes or the lung in asthma (7, 8). In human blood, only 0.1–0.2% of T cells are iNKT cells, with 5× lower numbers than in mice (9). Recently, iNKT cells came into focus as promising targets for the development of vaccine adjuvants and immunotherapies, mostly

TABLE 1 | Summary of iNKT cell activation studies in treatment of different diseases.

Therapeutic target	Species studied	Outcome	Reference, remarks
Viral and bacterial infections	Human, mouse	Effective vaccination in mice; oral and nasal route possible; no clear effect on chronic viral infections in clinical trials shown	(12–21)
Parasites and fungi	Mouse	Enhanced vaccine effects in mice	(10, 22–24) αGalCer analogs were used in Ref. (10) (7DW8–5) and Ref. (20) (α-C-GalCer) for NKT cell activation
Tumors	Human, mouse	Enhanced tumor protection and rejection in mice; clinical trials show only moderate effects in humans	(11, 14, 24–39) Antigen-pulsed DC were transferred in Ref. (32), no αGalCer or analog was added. αGalCer and α-C-GalCer were tested for tumor therapy in Ref. (34)
Autoimmune diseases	Mouse	αGalCer dose-dependent amelioration or aggravation of autoimmune diseases; NKT cell hypo-responsiveness involved in some cases	(8, 40–52) Ref. (43) used OCH, a sphingosine-truncated analog of αGalCer for NKT cell activation

iNKT cells were activated by αGalCer treatment if not indicated otherwise.

in the field of cancer treatment and in autoimmune and inflammatory diseases (**Table 1**). Preclinical studies using αGalCer demonstrated moderate therapeutic activity by activating DCs and providing Th-like functions, generating $CD8^+$ cytotoxic T cell (CTL) and antibody responses. Currently, more potent αGalCer analogs for iNKT cell activation are under investigation (10–13). Applying NKT cell immunization schemes in clinical settings is a promising therapeutic opportunity, but requires detailed knowledge on how iNKT cells activate DCs.

iNKT Cell Activation, Subsets, and Cytokine Production

Most knowledge on NKT cell activation came from the use of αGalCer, a strong and prototypical CD1-restricted agonist. In the last years, additional microbial-derived glycolipid ligands were identified, including α-glucuronosylceramides (from *Sphingomonas*), cholesteryl α-glucoside (from *Helicobacter*), or diacylglycerol-containing glycolipids (from *Borrelia*) (53, 54). These lead to sustained iNKT cell activation with inflammatory cytokine production that is independent of TLR stimulation, IL-12, or the recognition of endogenous antigens, hence relying only on engaging the invariant TCR. α-glucuronosylceramide induces IFNγ and IL-4 release similar to αGalCer (55–57). Both glycolipid antigens are structurally similar and can be recognized by the majority of mouse and human iNKT cells (58). Synthetic iNKT cell antigens have been and continue to be studied extensively for potential therapeutic application (59). However, iNKT cell activation may also promote allergic airway inflammation, and their overstimulation can induce iNKT cell anergy (1, 60).

Most microorganisms lack cognate iNKT cell antigens, hence activation of these cells relies on cytokines, such as IL-12 or IL-18, in conjunction with endogenous antigens. Even in the absence of TCR stimulation, some bacterial and viral infections induce a robust IL-12 response by DCs thereby activating iNKT cells *in vivo* (61, 62). Indirect iNKT cell activation results in the release of IFNγ but usually not IL-4 and is not restricted to TLR (62–65).

Analogous to Th cells subsets, different NKT cell subsets termed NKT1, NKT2, NKT17, NKT_{FH}, and NKT10 subsets were described with corresponding functionalities (66, 67). NKT17 cells produce the cytokines, IL-17 and IL-22, and are abundant in the lymph nodes, lungs, and skin of mice with airway neutrophilia induced by αGalCer (68). Recently, it was shown that iNKT17 cells are enriched in NOD mice, a mouse model for type I diabetes, which hint toward a possible role of those cells in disease development (69). iNKT17 cells rely on IL-7 for homeostasis and survival (70) and seem to require activation in the presence of TGF-β and IL-1β (71). The recently described NKT10 subset can dampen inflammatory responses by IL-10 production and is enriched in adipose tissue, providing protection in obesity-induced inflammation (72).

Dendritic Cell Maturation and $CD8^+$ T Cell Cross-Priming

Dendritic cells classically gather antigens in tissues and transport them into lymphatic organs, where they orchestrate the activation and differentiation of naïve $CD8^+$ T cells into CTL. Recent work showed that some DCs remain in tissues in order to regulate immigrating effector T cell responses, which is important in the defense against infections and may also promote the progression of many immune-mediated diseases. The cross-talk of myeloid cells with other immune cells, such as T cells and innate lymphocytes, is especially important in this context. Cellular encounters are orchestrated by chemokines, cytokines, and cell surface molecules. Some DCs, especially the $XCR1^+$ DC subset, are specialized in cross-presentation, which allows the presentation of extracellular antigens to activate CTL, a process important for immunity against tumors, viruses, and intracellular bacteria and for vaccination (73–76). Immunogenic cross-presentation, also referred to as cross-priming, requires the presence of pathogen-derived molecules (PAMPs) and/or of specific Th cells or NKT cells that mature the cross-presenting DC (77). This process is called "licensing," a term introduced by Lanzavecchia (78), and it aims at preventing unwanted immune answers against innocuous or self antigens. Licensing was first

described by Matzinger, Heath, and Melief (79–81), and classically is mediated by CD40 ligand provided by specific $CD4^+$ helper T cells (Th). In addition to licensing, immunogenic T cell priming requires the DCs to mature, a process that results from sensing various PAMPs, including ligands for TLR, lectins, intracellular nucleotide-binding oligomerization domain receptors, or retinoic acid-induced genes (82–85). Major consequences of DC maturation are the upregulation of costimulatory molecules like CD80 and CD86, CD40, of MHC II and the production of pro-inflammatory cytokines, especially IL-12p70 and TNF. These consequences partially can result also from CD40–CD40L interactions, but it is not clearly defined how much DC licensing and maturation functionally overlap. CD40–CD40L interactions are not only crucial for upregulation of costimulatory molecules but also for DC survival (86). Additionally, mature DCs produce chemokines to attract other immune cells and to orchestrate the ongoing immune response. In contrast to maturation-induced upregulation of MHC II, CD1 trafficking is differentially regulated during DC maturation, and CD1 molecules are already expressed on immature DCs. While human DCs express all classes of CD1 molecules, murine DCs express only CD1d (87), which is crucial for DC–iNKT cell interactions. Trafficking studies showed that antigen presentation by CD1d to iNKT cells might already occur before DC maturation and MHC II presentation (88). This notion hinted to a possible role of iNKT cells as immunological helper cells.

iNKT Cells as Immunological Helper Cells

αGalCer was found to mediate CD40-dependent activation of CTL by NKT cell-helped DC (89), directing attention to the adjuvant activity for this agent. Furthermore, αGalCer also induced resistance to tumors and intracellular pathogens (25). Compared to CD40 ligation, LPS, and CpG, αGalCer induced equally high levels of CD40, CD80, CD86, MHC II, and DEC205 in $CD11c^+$ $CD8a^+$ and $CD11c^+$ $CD8a^-$ DCs, but was unable to induce DC maturation from bone marrow progenitors. Rather than acting directly on DCs, αGalCer mediated DC maturation through iNKT cells in a MyD88-independent manner. Combining αGalCer with CD40 stimulation caused DC to produce high amounts of IL12p70, while LPS and CD40 stimulation showed no such effect. IL12p70 production might explain the results of another study (90), where the simultaneous administration of OVA and αGalCer enhanced Th and CTL responses in an iNKT cell-dependent manner. A close temporal association between αGalCer and OVA-derived peptides and additional experiments with antigen-loaded DCs led to the conclusion that αGalCer and peptides must reach the same DC. Formal *in vivo* evidence for such a tripartite cellular interaction was provided with the use of bm1/CD1d bone marrow chimeras (91). In addition, there was synergy when Th and iNKT help were combined. The means by which iNKT cells license DCs are not fully understood but in addition to providing CD40L to DCs, iNKT cells may act by promoting cross-talk of $XCR1^+$ DCs and plasmacytoid DC (92) or by abundant cytokine production upon activation. Whether iNKT cells play a role as helper cells when activated by less potent ligands remains to be elucidated.

iNKT Cells Help in CTL and $CD8^+$ Memory T Cell Formation

The knowledge on mechanisms iNKT cells use to substitute $CD4^+$ T cell help for antibody production, CTL generation, or memory formation is central for developing new vaccination strategies. An unresolved question is why some groups observed NKT cell-dependent reduction of CTL-mediated autoimmune diseases, whereas NKT cell-licensed DC induced strong CTL responses against tumors and viruses in other studies. The most obvious difference is the use of a single low dose of αGalCer for induction of protective CTL responses and the use of multiple doses or high single doses of αGalCer to inhibit unwanted T cell responses (93). In some clinical trials, αGalCer was used to treat cancer, and human $CD4^+$ iNKT cells expanded predominantly during early stages (26). $CD4^+$ iNKT cells can induce IL12p70 production by DC and thereby Th1 polarization (93). Double negative (DN) iNKT cells expanded later after αGalCer treatment and can induce apoptosis in αGalCer-loaded DC, thereby limiting the immune response (26). Functional differences between iNKT cell subsets in regards to cytokine production are evident both in mice and humans (94), but the effects on DC maturation, apoptosis, and CTL generation remain to be elucidated (**Figure 1**). A high frequency of a DN iNKT cell subset and their potential to lyse DCs may impair treatment of cancer patients and vaccination strategies. The role of iNKT cells during viral infections and the use of αGalCer as vaccine adjuvant in the context of influenza infections have been reviewed recently in Ref. (14, 95). αGalCer increased the levels of influenza-specific systemic IgG and mucosal IgA antibodies, even in the absence of Th cells and antigen-specific CTL responses (14, 95). In contrast, after combined iNKT cell activation and influenza virus vaccination, an impaired CTL response but enhanced memory CTL generation was seen (96). In line with this, enhanced CTL memory differentiation during viral infection was also shown previously (97). Another study showed that iNKT cell enrichment in the CNS during Theiler's murine encephalomyelitis virus (TEMV) infection inhibited the antiviral CTL response and delayed the accumulation of TEMV-specific CTL. Also, the magnitude of the TEMV-specific CTL response was impaired (98). CTL memory formation was not assessed in that study. Co-administration of αGalCer with suboptimal doses of irradiated sporozoites or recombinant viruses expressing a malaria antigen enhanced protective anti-malaria immunity in mice, and co-administration of αGalCer with various immunogens enhanced antigen-specific CTL responses and Th1 responses (99). In conclusion, vaccination with αGalCer as adjuvant induced iNKT cell help for DCs, which promoted CTL memory formation but impaired primary antigen-specific CTL responses.

Before we can fully understand the mechanisms of iNKT cell help in CTL formation and memory generation, it is crucial to know how "help" influences CTL responses in general. Many reports about Th help are available and most of them show a diverse picture of the requirement for $CD4^+$ T cell help in primary and/or secondary infections. Th help seems to be crucial for the clearance of some primary virus infections like HSV or influenza that do not affect DCs directly (100, 101), while in some viral

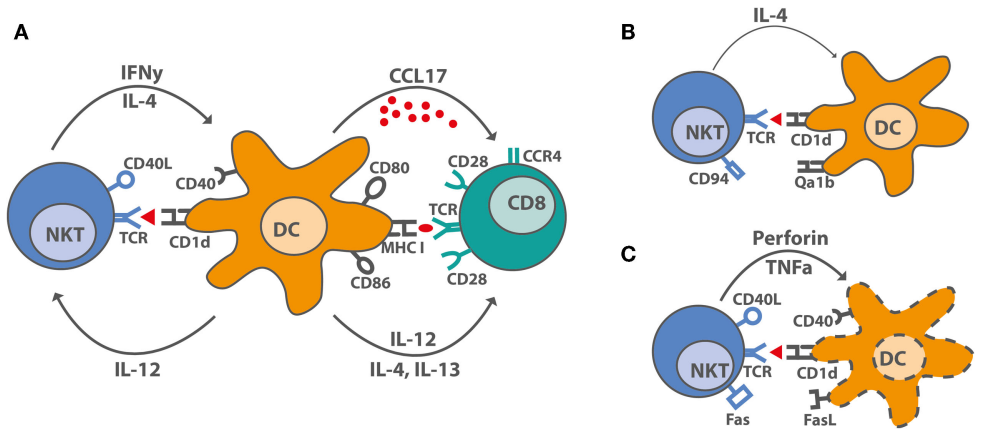

FIGURE 1 | iNKT cell–DC interactions after stimulation with αGalCer. (A) Under optimal stimulatory conditions, iNKT cells produce IL-4, large amounts of IFNγ and upregulate CD40L, thereby inducing maturation in DC. DC maturation leads to increased costimulatory capacity through upregulation of CD80 and CD86, of MHC molecules, and by producing the pro-inflammatory cytokine, IL-12, and the chemokine, CCL17. CCL17 attracts CCR4$^+$ cells, including CD8$^+$ T cells, which can be activated by the licensed DC. **(B)** Overstimulated iNKT cells upregulate inhibitory receptors like CD94 and are incapable of producing IFNγ. DC interacing with hyporesponsive iNKT cells cannot be activated and do not induce CD8$^+$ T cell activation. **(C)** Some activated iNKT cells induce DC lysis rather than maturation by yet unknown mechanisms. Proposed mechanisms suggest a role for TNFα, perforin, Fas–FasL interactions, and even CD40–CD40L.

infections, Th help can be overcome (102). Additionally, CTL responses against minor H antigens, soluble proteins, tumors, and peptide-pulsed DC require Th help for the induction of optimal primary responses (79, 103, 104). Some groups disagreed whether Th help was needed during secondary responses for proper re-expansion of CTL (105, 106) or whether Th help was merely a pre-requisite during primary infections for CTL memory formation (107). Additionally, Th help was dispensable for the expansion, but not for the cytotoxic capacity of CTL in tuberculosis (108).

These differential observations may be explained not only by the variance of pathogens and model antigens used but also by different experimental setups. Moreover, Th dependency was studied by using CD4$^{-/-}$ mice, MHC II$^{-/-}$ mice, or CD4-depleting antibodies, which are not biologically equivalent (109). For example, CD4-depleting antibodies also deplete regulatory T cells, CD4$^+$ NKT cells, and CD4$^+$ DCs. However, most older studies agreed that the requirement for Th help is not a CTL-intrinsic property but dependent on the infectious agent and DC maturation. Given the huge discrepancies in studies on Th help requirements, observations in a single model do not permit general conclusions on how CD4$^+$ help may be substituted by iNKT cells. A deeper insight into CTL generation and memory formation is required to allow predictions for the role of iNKT cell help in CTL responses.

As reviewed previously in Ref.(110), CTL in primary responses can be divided into short-lived effector cells (SLEC) that mostly die off during the contraction phase and memory precursor cells (MPEC) that received less stimulation but more survival signals (111, 112). Even a single naive CTL can differentiate into a diverse population of effector and memory cells (113, 114) by multiple mechanisms, which have been reviewed in detail by Kaech and Cui (115). Prolonged antigen exposure and pro-inflammatory cytokines like IL-12 and IL-2 promote terminal differentiation of CTL and induce superior cytotoxic capacities (116–118). NKT cells may affect CTL differentiation other than Th cells, but this hypothesis requires further experimental exploration.

iNKT Cells and Chemokines in CTL and CD8$^+$ T Cell Memory Formation

Chemokines play a major role in orchestrating primary and memory CTL responses. During infections, CTL upregulated CXCR3, which allowed them to enter peripheral tissues (119). Th help was required for enhanced recruitment of CTL to the site of infection in some situations (120) by promoting CXCL9 and CXCL10 production, with CXCL9 being especially important for rapid memory responses in the lymph node (121). Infections of the lung and intestine showed no requirement of Th help for migration as lung infections, e.g., by influenza induce on-site proliferation of CTL rather than recruitment (122, 123) but Th cells promoted development of lung-resident memory cells (124).

CXCR3 also drove CTL toward an effector fate rather than memory fate (125). In line with this, CXCR3 × CCR5 double-deficient mice showed a decreased contraction phase and harbored more memory CTL, which were unable to migrate into tissues and to clear infections (126). In humans, CCR5 expression was associated with effector memory T cells, whereas CCR7 was predominantly expressed on naïve and central memory T cells and CCR6 expression was found on early effector memory T cells (127–129).

iNKT cell-helped DCs produced high amounts of CCL17, thereby attracting CCR4$^+$ lymphocytes (91). This contrasts the situation in classical Th cell-dependent cross-priming, where DCs produced CCR5 ligands to attract CTL for cross-priming. These chemokines synergically guided CTL toward those DCs that have presented relevant antigen to helper T cell

subsets, and thereby facilitated the ensuing CTL response. Thus, CCR4- and CCR5-binding chemokines have been described as a new signal in T cell activation, distinct from signal 1, antigen, and signal 2, costimulation (130).

CCR4 is traditionally considered to be associated with skin homing Th2 and memory $CD4^+$ T cells (131–134), but also with the recruitment of Treg to the inflamed liver (135). Several studies in humans showed increased CCR4 expression also on CTL in cutaneous diseases (136–138). A $CCR4^+$ $CD8^+$ central memory subset has been described that was generated in the presence of IL-4 and produced IL4 and IL-13 upon restimulation (139). These cells were not cytotoxic and produced little IFNγ, features associated with a so-called Tc2 subset (139, 140). Kondo and Takiguchi showed that human $CCR4^+$ $CD8^+$ T cells expressed less effector molecules like perforin or granzymes compared to $CCR6^+$ early effector memory T cells, but produced more TNFα and IL-4 than $CCR7^+$ naïve or central memory $CD8^+$ T cells. They concluded that $CCR4^+CD8^+$ T cells are a "little more differentiated than $CCR7^+$ central memory ones and less differentiated than $CCR6^+$ early effector memory ones" and that they can migrate into secondary lymphoid organs where they mature after interacting with DCs expressing CCR4 ligand (141). Since iNKT cells produce IL-4 upon activation and induce CCL17 production by helped DC, they might play a role in the development or restimulation of $CCR4^+$ $CD8^+$ T cells. The physiological role of this subset in viral infections and tumors remains to be elucidated.

Concluding Remarks

CD4-helped DCs and NKT cell-helped DCs provide various costimulatory signals and cytokines deciding the fate of $CD8^+$ T cells toward effector or memory. However, the set of chemokines produced by NKT cell-helped DCs attract different subsets of naïve or memory $CD8^+$ T cells compared to chemokines produced by Th-helped DCs. Dissecting the role of those $CD8^+$ T cells subsets in effector and memory responses directed against tumors and viral infections may facilitate developing effective NKT cell-based vaccines.

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Mast cells as regulators of T cell responses

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Mast cells (MCs) are recognized to participate in the regulation of innate and adaptive immune responses. Owing to their strategic location at the host–environment interface, they control tissue homeostasis and are key cells for starting early host defense against intruders. Upon degranulation induced, e.g., by immunoglobulin E (IgE) and allergen-mediated engagement of the high-affinity IgE receptor, complement or certain neuropeptide receptors, MCs release a wide variety of preformed and newly synthesized products including proteases, lipid mediators, and many cytokines, chemokines, and growth factors. Interestingly, increasing evidence suggests a regulatory role for MCs in inflammatory diseases via the regulation of T cell activities. Furthermore, rather than only serving as effector cells, MCs are now recognized to induce T cell activation, recruitment, proliferation, and cytokine secretion in an antigen-dependent manner and to impact on regulatory T cells. This review synthesizes recent developments in MC–T cell interactions, discusses their biological and clinical relevance, and explores recent controversies in this field of MC research.

Keywords: mast cells, CD4 T cell, CD8 T cell, Treg cells, adaptive immunity

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Introduction

Mast cells (MCs) are among the most malleable and rapidly responding cells of the immune system. Within seconds of activation, they release a multitude of preformed biologically active products, followed by marked changes in cytoplasmic composition and volume that enable reconstitution of their morphology and cell content within hours (1, 2). Counterintuitively, this cell-regenerative phase coincides with a striking wave of mediator synthesis and secretion. Therefore, tissue-resident MCs have the potential to strongly shape their tissue microenvironment and direct cell–cell interactions and immune cell responses even while running through a reconstitution phase, during which they are relatively "refractory" to external stimuli.

Derived from either hematopoietic precursors or local, tissue-resident progenitors, mature MCs represent a heterogeneous collective of long lived, granulated cells located in essentially all tissues, which increase in number upon proliferation or increased recruitment, survival, and/or maturation of MC progenitors (1–3). They are particularly abundant at barrier sites, such as the skin, lung, and gut, and play an important role in defense against, and clearance of various pathogens (4, 5).

While the involvement of MCs in allergic/inflammatory reactions triggered by the crosslinking of FcεRI-bound immunoglobulin E (IgE) by antigen has been characterized in detail (6), the extent of MC function in autoimmune diseases is less well understood (7, 8). Upon activation, MCs release a plethora of mediators, including growth factors, cytokines, and chemokines (e.g., IL-1, IL-6, IL-8, IL-10, TNFα, VEGF, TGFβ, CCL2-4) as well as pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes. However, MCs are mostly known for the ability to degranulate and

very rapidly release preformed mediators from cytoplasmic granules, such as vasoactive amines (histamine and serotonin), proteoglycans (e.g., heparin), proteases (above all tryptases and chymases), and some pre-stored cytokines (e.g., TNFα) (1, 2, 9).

As players in innate immunity MCs have the capacity to initiate and amplify immune responses. Several lines of evidence have demonstrated that MCs participate in the sensitization phase of acquired immune responses via the secretion of mediators, which sustain dendritic cell (DC) maturation, function, and recruitment to the tissue or their migration to local draining lymph nodes (10). However, MCs also exert important effector function since MCs and T cells of different origin and subsets establish tight cell–cell interactions and modulate their respective effector functions in a bidirectional manner; this has been shown in a variety of models (11–13). Interestingly, MCs can even present antigen to T cells in a MHC class I- or class II-restricted mechanism (11, 13, 14).

This review focuses on MC-mediated regulation of T cell responses (**Figure 1**) since this activity not only shows MCs to be an important element of acquired immunity but also to play a cardinal role in shaping, controlling, sustaining, or arresting inflammatory responses at host–environment interfaces and, thus, of major clinical relevance.

MCs as Regulators of CD4$^+$ T Cell Effector Functions

Historically, MCs have been associated with the regulation of Th2 immune responses, and as such their modulatory activities on CD4$^+$ T cells have been amply documented in many different models (**Figure 1A**).

In 1993, the Mecheri group reported that murine bone marrow-derived mast cells (BMMCs) displayed antigen-presenting cell (APC) functions (15), with these findings later extended to MCs of rat and human origin (16–18). Efficient BMMC antigen presentation to CD4$^+$ T cells was shown to require expression of the costimulatory molecules CD80 and CD86, which are induced by IL-4 and granulocyte/macrophage-colony-stimulating factor (GM-CSF). Interestingly, in their studies, interferon (IFN) γ completely abrogated this phenomenon (19, 20); this IFNγ effect could

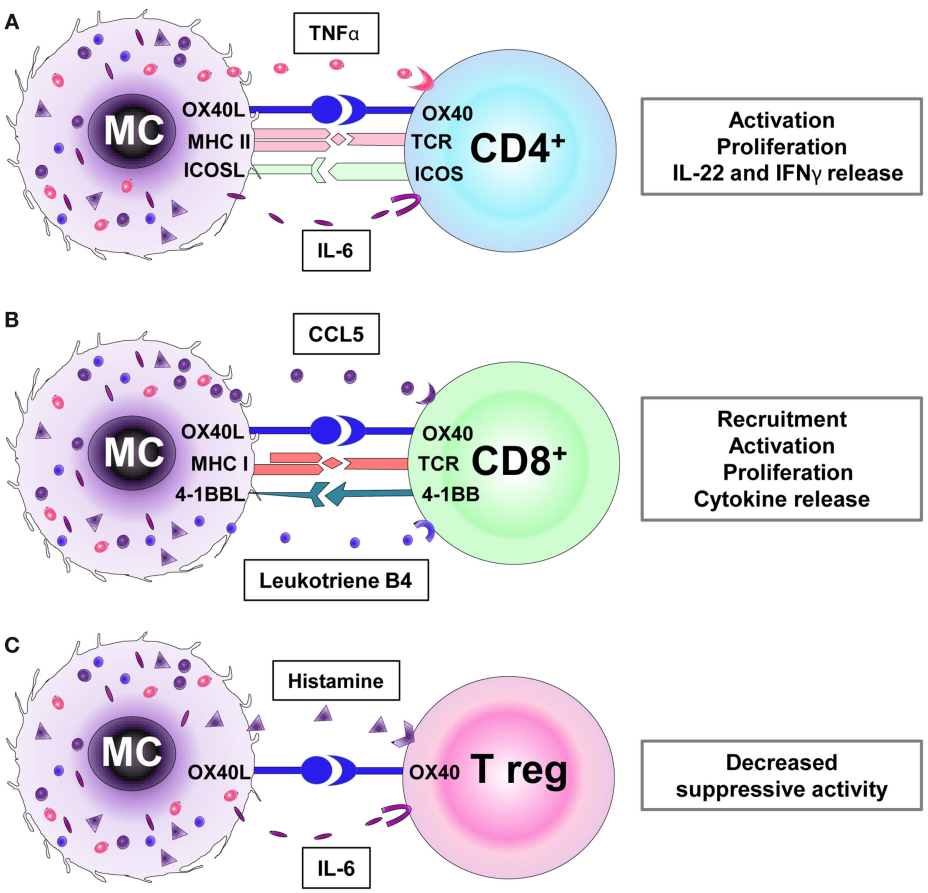

FIGURE 1 | Receptors and mediators involved in the interaction between mast cells (MCs) with CD4$^+$ (A), CD8$^+$ (B), and regulatory T cells (Tregs) (C). (A) MCs promote the activation, proliferation, and cytokine secretion (e.g., IL-22, IFNγ) of CD4$^+$ T cells via MHC II and OX40L cell–cell interactions and TNFα secretion. **(B)** MCs induce CD8$^+$ T cell recruitment via the release of chemokines (e.g., CCL5) and leukotriene B4. Furthermore, MC-mediated CD8$^+$ T cell activation requires MHC I/TCR, OX40L/OX40, and 4-1BBL/4-1BB receptors interaction. **(C)** The OX40/OX40L-directed interaction between Treg and MCs and the histamine and IL-6 production by the latter inhibit the suppressive Treg activity.

be counteracted by FcεRI-mediated antigen endocytosis (21). In contrast to the above study, IFNγ-primed mouse MCs in their antigen-mediated interaction with $CD4^+$ T cells were shown to develop a functional immunological synapse (22).

More recently, Gaudenzio and colleagues (23) have defined MCs as "tissue-localized" APCs, which (in inflamed human psoriatic skin) are primed by locally produced IFNγ to present antigen to experienced and recruited $CD4^+$ T cells. IFNγ-primed human MCs establish synaptic contacts with effector/memory $CD4^+$ T cells, thus inducing Th22 and IL-22^+IFNγ^+Th cell subsets via the release of IL-6 and TNFα. Interestingly, in inflammatory conditions in which both MCs and T cells are enriched, as seen in psoriatic skin, the majority of IL-22^+ and IFNγ^+CD4^+ T cells are in close contact with MCs and the latter act as amplifiers of inflammation (23).

Since IL-6 and TNFα are mediators commonly released by activated MCs upon a wide range of stimuli, it remains unclear whether the IFNγ-induced MC search for immune partners is broad or restricted to a specific cell type or T cell subset; and how this encounter is spatially and temporally controlled. Furthermore, whether this cognate interaction leads to bidirectional effector functions, which might shape long-term MC activities is yet to be defined.

The antigen presentation activity of murine MCs and the MC-dependent modulation of effector T cell functions correlates with the induced expression of MHC class II molecules (14, 24) together with the up-regulation of a wide variety of costimulatory molecules, including members of the B7 family (ICOS ligand, PD-L1, and PD-L2) and the TNF/TNFR families (OX40L, CD153, Fas, and 4-1BB) (25).

In conjunction with the secretion of TNFα, the up-regulation of the costimulatory molecule OX40L, in particular, has been demonstrated to be essential for the MC-$CD4^+$ T cell crosstalk and modulation of effector T cell function (25). OX40L expression was reported to be induced by exposure of mouse MCs to stimuli, such as toll-like receptor (TLR) agonists and FcεRI engagement (24). Furthermore, Notch signaling was shown to upregulate MHC class II and OX40L expression on mouse MCs thus promoting the proliferation $CD4^+$ T cells and their differentiation into T helper 2 cells producing IL-4, IL-5, IL-10, and IL-13 (26).

Interestingly, treatment of human MCs with type I IFNs had the opposite effect of down-regulating both TNFα and OX40L expression while inducing IL-10 and TGFβ production with the consequence of restraining $CD4^+$ T cell effector activities (27). This latter report underlies the key role of the inflammatory microenvironment in tightly controlling the outcome of MC–T cell interactions, and also suggests that the antigen presentation ability of MCs is possibly not intrinsic to this cell type but varies in response to time and location.

Gong and colleagues have proposed that the antigen-presenting property is restricted to an $Fc\varepsilon RI^{hi}$, MHC II^+, and c-kit^+ mouse MC subset (28). However, considering the plasticity of MCs, one could interpret the $Fc\varepsilon RI^{hi}$ and MHC II^+ expression on MCs rather as a transitory "activation" state born of environmental (allergen) or inflammatory pressure rather than as a bona fide subset of MCs.

In vitro as well as *in vivo* MCs are a heterogeneous cell population, and their MHC class II expression is variable and inducible. However, MHC class II molecules are not confined to a "professional" MHC class II compartment as it is found in professional APCs, but are stored in mature and immature forms in both lysosomal and secretory granules of MCs [Ref. (29); reviewed in Ref. (11)]. Furthermore, it has been reported that MC-mediated T cell activation is mediated via exosome release (30). It is therefore tempting to speculate that antigen presentation in MCs may be the result of both direct cognate cell–cell interactions between MCs and T cells and MC-secreted MHC class II and costimulatory molecule-loaded exosomes acting upon T cells.

MCs as Modulators of $CD8^+$ T Cell Responses

Recent evidence has suggested a protective role for MCs in antiviral immune responses (31–35). This is based on the observation that MCs are equipped with a full repertoire of pattern recognition receptors, including TLRs (36), which allow MCs to sense and respond to most microbial components, including viruses.

Upon TLR engagement, mouse MCs are activated to secrete chemokines, of which notably CCL5 can recruit effector $CD8^+$ T cells (37). Reovirus-infected human MCs have been shown, through release of chemokines including CCL3, CCL4, and CCL5, to selectively recruit cytotoxic effector cells, thus suggesting their ability to enhance viral immunity (31).

Furthermore, in a model of murine cytomegalovirus (CMV) infection, activated MCs have been described to recruit $CD8^+$ T cells to the lungs via CCL5 release and thus contribute to a reduction in the viral load and the clearance of infection (34). MC activation upon CMV infection is characterized by an immediate TLR3/TRIF signaling-dependent phase and a delayed TLR3/TRIF-independent pathway phase (38). In allergy models, the MC-mediated recruitment of effector, but not central memory, $CD8^+$ T cells to sites of inflammation was shown to be dependent on the production of MC leukotriene B4 (39).

However, the interactions between MCs and $CD8^+$ T cells go far beyond that of chemokine-induced recruitment (**Figure 1B**). MCs have been reported to be capable of antigen presentation via MHC class I molecules to T cells following phagocytosis and processing of bacterial antigens from live bacteria (40). Furthermore, physical MC/$CD8^+$ T-cell contacts have been demonstrated in healthy human skin. In lesional skin from alopecia areata (AA) patients, MCs display an activated phenotype prominently expressing MHC class I and the costimulatory molecules OX40L and 4-1BBL. Furthermore, abnormal MC numbers, effector functions, and increased interactions with $CD8^+$ T cells were observed in the grafted C3H/HeJ mouse model of AA and in a recently developed humanized mouse model for AA (41). Here, in a pathological inflammatory environment, activated MCs may contribute to the collapse of hair follicle immune privilege by initiating/sustaining $CD8^+$ T cell effector functions, thus promoting the disease (41).

Importantly, MC initiated antigen-dependent and MHC class I-mediated cross-presentation to $CD8^+$ T cells has been shown to regulate $CD8^+$ T cell effector functions including proliferation, cytokine secretion, and cytotoxic activity *in vitro*;

this was supported by complementary *in vivo* studies in which antigen-specific $CD8^+$ T cell numbers were reduced in MC-deficient mice, using the experimental autoimmune encephalomyelitis (EAE) model (42). These studies support previously published evidence that MC-deficient mice not only display defective $CD4^+$ but also $CD8^+$ T cell numbers in EAE (43) as well as in *Leishmania major* infection (44).

A specific priming of $CD8^+$ effector T cells in the tissue at the site of inflammation, delivered by resident immune cells, such as MCs, may also be a relevant strategy not only to, initially, promote protective inflammation but also to control and limit excessive and/or chronic cytotoxic activity. However, very little evidence has been published to date on $CD8^+$ T cell/MC interactions. Therefore, closing this important gap in our understanding of MCs functions in health and disease should be a prime future research focus.

MCs as Suppressors of T Cell Effector Functions

Mast cells are also able to suppress T cell effector functions, namely, by their interaction with regulatory T cells (Treg) (**Figure 1C**). Adoptive transfer of Tregs in a mouse model of sepsis correlated with increased MC numbers (45). Furthermore, MCs contribute to the induction of tolerance to alloantigens being recruited to skin allografts in response to IL-9 secreted by Tregs (46, 47).

In line with the previously reported finding that high-FcεRI expression correlates with efficient antigen-presenting abilities in MCs (24, 28), Treg cells down-regulate FcεRI expression in MCs (48). Mouse MCs have been shown to secrete histamine and IL-6 and to use the OX40/OX40L signaling pathway to inhibit Treg functions and to thus promote optimal activation of effector $CD4^+$ and $CD8^+$ T cells (49–52).

However, it remains unclear which conditions promote the suppression of MC functions by Tregs versus the inhibition of Tregs by MCs. Moreover, it is conceivable that, under some conditions, MC activation may overcome Treg-mediated immunosuppression, promote the development of effective antitumor immunity, and boost the immune response in the tissue, while a different signaling environment may contribute to allograft tolerance in transplantation. Only a better definition of the relevant molecular check points will clarify the mechanisms that underlie these opposite functional outcomes and will identify promising targets for therapeutic interventions.

Controversies in the Field

However, it should be acknowledged that the regulatory impact of MCs on T cell functions is still a controversially debated field. Namely, studies utilizing various MC-deficient mouse models have claimed that MCs are non-essential for the regulation of either $CD4^+$ or $CD8^+$ T cell immune responses (53, 54). Yet, this does not necessarily exclude T cell-regulatory MC activities under physiological conditions.

Mast cell function has been classically studied using the MC-deficient C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ or $Kit^{W/W\text{-}V}$ mice, whose MC deficiencies arise through loss of function mutations affecting Kit. However, these mice are limited in their usefulness by their perturbed immune cell composition, as such a number of new "Kit-independent" MC-deficient strains have been generated (53, 55, 56). These mice have the great advantage of deleting the MC population without apparently affecting other immune populations, with the exception of basophils (strain dependent), and have called into question findings originally obtained using Kit-dependent MC-deficient mice.

Owing to the use of multiple mouse strains and diseases models, the role of MCs in autoimmune diseases has been very controversial, with some authors tending to conclude that MCs are generally dispensable in autoimmunity (53). However, very recently, Schubert and colleagues investigated in more detail the function of MCs in arthritis using different strains of MC-deficient mice and in models, either based on autoreactive antibody transfer or effector T cells (57). Interestingly, these authors found MCs to be critically relevant in the T cell-dependent mouse model of rheumatoid arthritis [collagen-induced arthritis (CIA)], while being dispensable in the T cell-independent antibody-induced arthritis model. In the CIA model, absence of MCs resulted in dramatic loss of T cell expansion upon immunization and concomitant reduction in T cell cytokine responses (57). These recent findings underscore the critical role of MCs in T cell-dependent autoimmunity.

However, in a T cell-dependent spontaneous diabetes model, using non-obese diabetic (NOD) mice, MCs failed to impact on $CD4^+$ and $CD8^+$ T cell numbers measured at the onset of the disease (54). However, this study left it unclear whether at later time points during disease progression, i.e., when the phenotype divergence between MC deficient and wild-type mice may be greatest, or during spontaneous disease resolution, the absence or presence of MCs would have impacted upon T cell responses and clinical outcome.

Possibly, the most contentious issue has been the role of MCs in multiple sclerosis (MS), particularly in the T cell-dependent surrogate mouse model of EAE, with some studies arguing an important role for MCs (58, 59), while another one claims that they are dispensable (53). This controversial discussion has been very important and productive in the sense that it has brought to light the limitations in the use of each of the presently available MC-deficient mouse strains, and has underscored the urgent need for standardized disease-induction protocols to improve data reproducibility. Furthermore, these discrepancies have served to acutely remind us of the constitutive difficulties one faces in translating murine data to the human condition.

Conclusion and Perspectives

In this review, we have highlighted interactions between MCs and T cells, which regulate adaptive immune responses and have delineated that the antigen-presenting activity of tissue-resident immune cells, such as MCs, is fundamental to the maintenance of productive and protective inflammation. MCs may also actively participate in the fundamental processes, which minimize immune-mediated bystander damage to healthy tissues.

We have also reviewed the evidence that MC can modulate Treg activities. However, the mechanisms and dynamics that interrupt MC-mediated antigen presentation, down-regulate the MC-induced amplification of T cell-dependent immune response, and restore local Treg cell homeostasis, all remain to be dissected by future research.

This review has closed with discussing contradictory results and the ensuing controversial debate on the role of MC in experimental autoimmune disease. It is important to keep in mind that the conflicting findings were generated using different MC-deficient mouse strains. This raises the pertinent question whether models, which rely on the deletion of an entire cell population, such as MCs, which are notoriously heterogeneous, highly plastic and adaptable in nature, and excel in their capacity to rapidly shift the spectrum of mediators released and surface markers expressed in distinct signaling environments (e.g., homeostatic versus inflammatory settings), are not overly simplistic. Can such models possibly reflect the (very transitory) dynamics of MC biology *in vivo*? Therefore, the ultimate research tool for definitively clarifying the contribution of MCs to the regulation of T cell functions under physiological and pathological conditions, which fully takes into account the dynamism and heterogeneity of MCs, may still have to be developed.

Author Contributions

SP and RB drafted and revised the manuscript.

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REVIEW
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Contribution of basophils to cutaneous immune reactions and Th2-mediated allergic responses

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Basophils are potent effector cells of innate immunity and also play a role in T helper 2 (Th2)-mediated allergic responses. But, although their *in vitro* functions are well studied, their *in vivo* functions remain largely unknown. However, several mouse models of basophil depletion have recently been developed and used to investigate basophil functions. For example, in a croton oil-induced model of irritant contact dermatitis in conditionally basophil-depleted transgenic mice, we found that basophils rapidly infiltrate inflamed skin and subsequently induce infiltration of eosinophils. We also showed that basophils induce Th2 skewing upon epicutaneous sensitization with various haptens and peptide antigens. Intriguingly, basophils also promoted Th2 polarization upon protein antigen exposure in the presence of dendritic cells (DCs). The dermal DC subset associated with Th2 skewing was recently identified as CD301b$^+$ DC. Such studies with basophil-deficient mouse models have significantly improved our understanding of the mechanisms involved in human immune-related diseases. In this review, we will focus on the relative contribution of basophils and DCs to Th2-mediated allergic responses.

Keywords: basophil, dendritic cell, Th2, contact dermatitis, IgE-CAI

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Introduction

T helper 2 (Th2) immune responses, which develop in response to allergens and parasites, are characterized by high levels of immunoglobulin E (IgE) and the presence of Th2 cells (1). Basophils are intimately involved in Th2 immune responses, and upon activation of the high-affinity receptor for IgE (FcεRI) or other surface receptors, they release multiple effector molecules, including proteases, vasodilating substances, such as histamine, cytokines, pro-inflammatory chemokines, and lipid mediators (2, 3). However, the mechanisms that initiate Th2 responses are not fully understood. Previous reports have shown that dendritic cells (DCs), the most efficient antigen-presenting cells (APCs) in the immune system, play a crucial role (4). However, recent experiments in newly developed basophil-deficient mouse models have highlighted the importance of basophils as well. For example, Th2 skewing is considered to be mainly induced by DCs, but recent studies in basophil-depletion mouse models indicate that basophils also play a pivotal role in this process (5–7). Here, we review the roles of basophils in cutaneous immune reactions and Th2-mediated allergic responses associated with cutaneous allergic diseases, focusing on the possibility that basophils and DCs function cooperatively in inducing Th2-mediated allergic responses.

Cutaneous Allergic Diseases Associated with Basophil Infiltration

Basophils have been detected in the vicinity of eosinophils in several human cutaneous allergic diseases, and infiltration of basophils has been reported in several skin diseases, including atopic dermatitis (AD), prurigo, and urticaria (8). It is noteworthy that skin lesions of bullous pemphigoid, classical eosinophilic pustular folliculitis (Ofuji's disease), and Henoch–Schönlein purpura also frequently exhibit tissue basophilia (8–11) (**Table 1**). We recently demonstrated the presence of both basophils and eosinophils in inflamed skin of patients with irritant contact dermatitis (ICD) (12). Further, we showed that basophils rapidly infiltrate into the inflamed skin, and subsequently induce infiltration of eosinophils with a croton oil-induced model of ICD. But it is still unclear exactly how basophils infiltrate into the lesional skin. There are several candidate basophil attractants, such as α(1,3)-fucosyltransferases IV and VII, for the initial recruitment of basophils in chronic allergic inflammation (CAI) (13). Subsequently, basophils attract eosinophils directly or indirectly via eotaxin-mediated interaction with mesenchymal fibroblasts (12).

TABLE 1 | Dermatological diseases accompanied with basophil infiltration.

Atopic dermatitis (8)
Irritant contact dermatitis (12)
Prurigo (8)
Urticaria (8)
Pemphigus vulgaris (8)
Bullous vulgaris (8)
Drug eruption (8)
Henoch–Schönlein purpura (8)
Insect bite (tick bite) (11)
Scabies (8)
Dermatomyositis (8)
Eosinophilic pustular folliculitis (10)
Leprosy (LL type) (9)

IgE-Mediated Chronic Allergic Inflammation and Chronic Idiopathic Urticaria

Immunoglobulin E-mediated chronic allergic inflammation (IgE-CAI) is a novel type of chronic inflammation of the skin that follows the immediate-type and late-phase responses in mice (14). It has been reported that IgE-CAI is independent of mast cells and T cells, but is dependent on basophils expressing FceRIa and CD49b phenotypic markers (14). Interestingly, although the number of basophils infiltrating the lesional skin is very low, their depletion led to a marked reduction in inflammation, concomitantly with decreased numbers of eosinophils and neutrophils and attenuation of the increased ear thickness (14). Recent studies have shown that inflammatory monocytes recruited to IgE-CAI lesions acquire an anti-inflammatory phenotype via basophil-derived IL-4 (15). Collectively, these results suggest a specific and non-redundant role for basophils in the initiation and maintenance of chronic IgE-mediated inflammatory responses in mice (16).

It is important to note that there are some functional differences between human basophils and mouse basophils. In mice, activated basophils produce platelet-activating factors and contribute to the development of anaphylaxis in response to penicillin-IgG antibody complexes. On the other hand, human basophils do not respond to IgG immune complexes (17, 18). Nevertheless, some findings in mouse basophils appear to shed light on the pathogenesis of human cutaneous diseases. Antibodies to FcεRIα were found in 40% of patients with chronic idiopathic urticaria (CIU). Some CIU patients exhibited urticaria in response to anti-FcεRIα IgG and/or IgE antibodies, which may stimulate mast cells or basophils (19). In addition, an activation marker on basophils, CD203c, was upregulated upon incubation of donor basophils with sera from patients with CIU (20). Furthermore, infiltration of basophils is increased in urticarial lesions of CIU (8, 21). Consistently with this finding, basopenia in CIU appears to be due to the migration of basophils from the peripheral blood to urticarial lesions (1, 8, 21). Therefore, the phenomena seen in the mouse IgE-CAI model might explain the pathogenesis of human CIU.

Dendritic Cell-Specific and Basophil-Specific Depletion Models

Although the CD11c-based system is the most common depletion model of DCs, it has the disadvantage of imperfectly separating conventional DCs (cDCs) and macrophages (22, 23). On the other hand, since there are no natural mouse mutants with basophil deficiencies, antibodies that recognize either FcεRI (clone MAR-1) or the orphan-activating receptor CD200 receptor 3 (CD200R3) (clone Ba103) have been used to investigate the role of basophils. However, these antibody clones not only deplete basophils but also stimulate mast cells (24, 25). In addition, Ba103 activates myeloid cells and NK cells (26), and MAR-1 depletes a subset of FcεRI-positive DCs (27). **Table 2** summarizes currently available mouse strains with constitutive or inducible depletion of basophils. Three groups have developed basophil-depletion models through regulation of *Mcpt8*, a basophil-specific gene in the conserved chymase locus (24, 28–30). In these mice, basophils were depleted in peripheral blood without side effects. A different basophil-depletion model utilizing the P1-Runx gene was reported by Mukai et al. (31) These mice show depletion of basophils, but not eosinophils, neutrophils, or mast cells. Sawaguchi et al. developed Bas-TRECK Tg mice, using a diphtheria-toxin receptor (DTR) transgene under the control of the DNase I-hypersensitive site 4 (HS4) region of IL-4 (32).

Basophils are Associated with Th2 Skewing in Response to Haptens

Mature DCs are generally considered to be required for naïve T cells to proliferate and acquire Th2 effector functions in response to antigen encounters (33). Recently, however, the function of DCs in Th2 induction has been questioned because basophils also appear to play a pivotal role in this process (5–7). Basophils migrate into draining lymph nodes (LNs) from the site of papain injection or helminth infection and act as APCs by taking up and processing antigens (5–7). In addition, basophils are capable of expressing MHC class II and costimulatory molecules, such

TABLE 2 | Mouse models of basophil depletion.

Model system	Experimental strategy	Method of depletion	Depletion efficiency	Reference
Basoph8	Knock-in of IRES–YFP–Cre cassette before the Mcpt8 start codon	Cross to R-DTA mice	>90%	(28)
Mcpt8-Cre	BAC transgene (Cre inserted after the Mcpt8 start codon)	Constitutive depletion	>90%	(24)
Mcpt8^{DTR}	Knock-in of IRES–DTR–EGFP cassette in 3′-UTR of Mcpt8	DT injection	>90%	(29)
P1-Runx1	Knockout	P1-Runx1 seems to be essential for the basophil lineage	>90%	(31)
Bas-TRECK	DTR transgene (under control of HS4 region of IL-4)	DT injection	>90%	(32)

BAC, bacterial artificial chromosome; DTR, diphtheria toxin receptor; IL-4, interleukin-4; IRES, internal ribosome entry site; Mcpt, mast cell protease; NA, not applicable; NR, not reported; R-DTA, ROSA-diphtheria toxin-α; UTR, untranslated region; YFP, yellow fluorescent protein.

as CD40, CD80, and CD86. They also secrete several cytokines critical for Th2 development, including IL-4 and thymic stromal lymphopoietin (TSLP). Thus, under certain conditions, basophils alone, without DCs, can cause Th2 induction from naïve T cells. However, the role of basophils in Th2 skewing has again been questioned since several of the above experiments used bone marrow-derived basophils (BMBaso) containing FcεRI-expressing inflammatory DC (27).

Recently, we demonstrated that basophils play a role in Th2 skewing in response to haptens and peptide antigens, but not protein antigens, in a basophil-deficient mouse model, Bas TRECK Tg (34). In addition, we showed that basophils were capable of Th2 skewing by using CD11c-depleted BMBaso in order to avoid contamination with inflammatory DCs. Basophils express MHC class II, CD40, CD80, CD86, and IL-4 in the hapten-induced cutaneous Th2 model. However, using the DQ-OVA system, we confirmed that basophils did not efficiently take up or process protein antigens (34). A different experimental system using OVA coupled to fluorescein isothiocyanate showed that basophils could take up protein antigens (6), but our results showed that hapten antigens and peptides might bind directly to MHC class II on basophils, and they could be acquired and presented by basophils. On the other hand, basophils hardly digest protein and so cannot efficiently present protein antigens (**Figures 1** and **2**). Although the OVA-peptide system is totally artificial, complex inflammatory environments, such as post-*Schistosoma mansoni* or *Trichuris muris* infection, probably contain small soluble antigens as well as larger proteins. In addition, cutaneous immunization with papain protease allergen promotes MHC class II expression on basophils in LNs, probably after the generation of peptide antigens from the protein *in vivo* (6). A recent report showed that basophils are capable of inducing Th2 upon exposure to OVA proteins complexed with specific IgE (7). They pulsed basophils with various doses of DNP-OVA in the presence of monoclonal antibody to DNP (IgE anti-DNP) and showed that Th2 skewing by basophils was enhanced with the effect of IgE anti-DNP, especially when basophils were pulsed with low concentrations of DNP-OVA (7). House dust mites, which possess cysteine protease activity, are incapable of inducing Th2 when presented by basophils, even though cysteine protease may play a role in processing protein antigen into peptides *in vivo* (27), because the expressions of HLA-DM and of the invariant chain on basophils, those were sorted from the LNs 3 days after house dust mites administration, were very low (27).

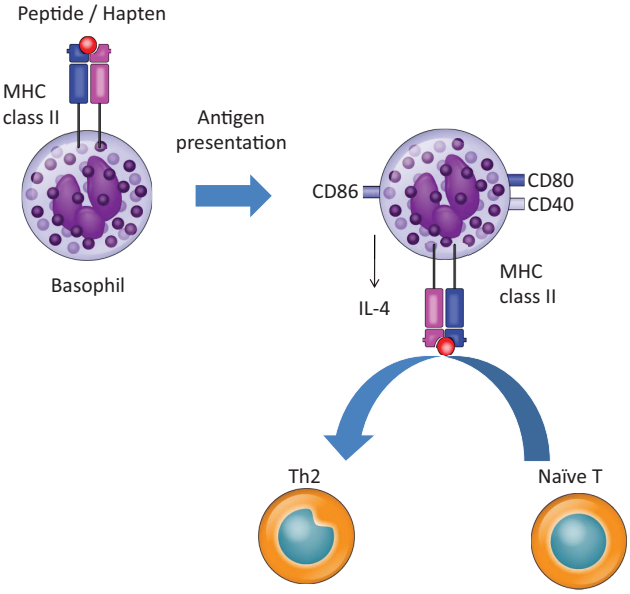

FIGURE 1 | Basophils promote Th2 skewing in response to haptens and peptide antigens. Basophils promote Th2 skewing upon peptide and hapten exposure by expressing MHC class II, CD40, CD80, CD86, and IL-4.

Although several studies show that murine basophils can serve as APCs, the situation is less clear for human basophils. Human basophils express MHC class II (35, 36), but it was not able to induce antigen-specific T cell activation or proliferation in response to house dust mite allergen exposure (36). Another group reported that HLA-DR in human basophils is upregulated by IL-3 and IFN-γ, but the basophils cannot work as APCs for pollen allergen (37). It has been confirmed that human basophils lack some features of APCs (38, 39). Additional studies are needed to determine whether human basophils can act as APCs under various pathophysiological conditions.

Interaction between DCs and Basophils for Th2 Skewing

It has been reported that basophils contribute to the strength of the Th2 response in the lungs, but they cannot present antigens or express chaperones involved in antigen presentation (27). Therefore, it was suggested that DCs are necessary and sufficient

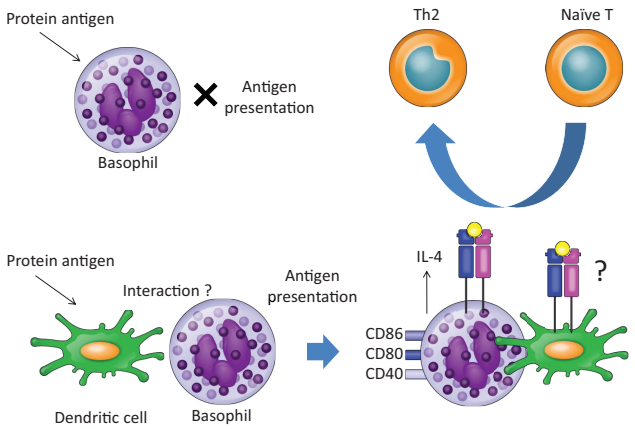

FIGURE 2 | Basophils promote Th2 skewing in response to protein antigens in the presence of dendritic cells. Upon exposure to OVA protein, basophils do not work as APCs, since basophils cannot take up or process OVA protein. In the presence of dendritic cells, however, basophils promote Th2 skewing. The precise mechanisms of interaction between basophils and dendritic cells remain unclear.

for inducing Th2 immunity to house dust mites in the lungs, and basophils are not required. In accordance with this idea, Th2 responses were severely impaired after *Schistosoma mansoni* egg injection and during active *Schistosoma mansoni* infection by depletion of $CD11c^+$ cells, but not by depletion of basophils with anti-FcεRIα antibody (4). These findings suggest that some DC subsets induce Th2 skewing upon exposure to protein antigens.

Recently, two different groups have shown that Th2 skewing in response to *Nippostrongylus brasiliensis* infection depends on dermal $CD301b^+$ DCs (40, 41). Depletion of $CD301b^+$ DCs prior to infection reduces the number of IL-4-producing $CD4^+$ T cells (40, 41). $CD301b^+$ DCs also express programed death ligand-2 (PDL2), and a subset of $PDL2^+CD301b^+$ DCs that express the transcription factor interferon regulatory factor 4 (IRF4) was shown to be required for Th2 induction *in vivo* (41). In accordance with these findings, $CD11c^+$MHC class II^+ dermal DCs expressing PDL2, and CD301b were identified as a Th2-inducing DC subset in *Nippostrongylus brasiliensis* infection (42). However, $CD301b^+$ DCs alone are incapable of inducing a Th2 response *in vitro* (41) or *in vivo* (40).

We have shown that basophils are capable of inducing Th2 skewing upon exposure to protein antigens in the presence of DCs (34). Because basophils are not able to take up or process protein antigens efficiently, DCs may prepare peptides from protein antigens for antigen presentation by basophils or may promote IL-4 production from basophils to skew Th2. In line with this, we had previously demonstrated that Langerhans cells, an epidermal DC subset, mediate epicutaneous sensitization with OVA protein antigen to induce Th2-type immune responses (43). Further studies are needed to show direct evidence whether DCs prepare peptides from protein antigens for the Th2 induction by basophils. In addition, Th2 reaction in response to schistosome infection or protein antigens was reduced in a CD11c-depletion model (4, 27). Therefore, DCs seem to be necessary for inducing Th2 reaction upon exposure to protein antigen both *in vivo* and *in vitro*.

Furthermore, basophils were found in the vicinity of T-cells in the T-cell zone of draining LNs by epicutaneous sensitization with haptens (34). Optimal localization of DCs within LNs may play a crucial role in Th2 skewing in each condition. CXCR5-expressing $CD11c^+$ DCs migrate to the LNs and localize adjacent to B cell follicles in *Heligmosomoides polygyrus* infection, whereas depletion of CXCR5 or B cell-derived lymphotoxin alters the localization of DCs and impairs the development of Th2 cells (44). Therefore, although the location of DCs on draining LNs for Th2 induction is still controversial, it is possible that basophils, T cells, and DCs promote Th2 induction in a coordinated way. Similarly, reactive oxygen species (ROS) were generated in dermal DCs and in LN DCs upon subcutaneous exposure to papain plus antigen. ROS promoted Th2 response via formation of oxidized lipids that triggered TSLP production by epithelial cells. In addition, ROS enhanced Th2 induction by inducing release of CCL7 from DCs, leading to the recruitment of basophils to the draining LNs (45). Another group showed that IL-3 plays a role in basophil recruitment to draining LNs using helminth infection model with mice deficient in IL-3 or IL-3Rβ (46). However, they found that helminth-induced Th2 response was not diminished in an MAR-1 antibody-induced basophil-depletion model. Further studies are needed to determine whether DCs present peptides to basophils directly or whether plasma membrane fragments are transferred from APCs to lymphocytes by trogocytosis.

Conclusion

Studies in basophil-deficient mouse models over the last decade have greatly improved our understanding of the mechanisms of development of Th2 immune reactions. Nevertheless, some key questions remain unanswered, including how DCs cooperate with basophils during Th2 skewing, especially in response to protein antigen exposure. In addition, the precise role of basophils in Th2 skewing, especially their function as APCs, remains controversial (27, 46). One possibility is that basophils may work as early IL-4-producing cells for the induction of Th2. An issue in some previous studies has been the imperfect separation of cDC in CD11c-based systems, and one possible approach to overcome this would be to use *CD11c*-DTR and *Zbtb46*-DTR, a marker specifically expressed by cDCs in lymphoid and non-lymphoid tissues but not by other myeloid or lymphoid cell types (47). Newly developed DC-deficient and basophil-deficient models are expected to provide further information on the mechanisms involved in Th2 skewing. Such studies may provide a basis for novel therapeutic approaches to controlling allergic diseases.

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REVIEW
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Maintenance of immune homeostasis through ILC/T cell interactions

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Innate lymphoid cells (ILCs) have emerged as a new family of immune cells with crucial functions in innate and adaptive immunity. ILC subsets mirror the cytokine and transcriptional profile of $CD4^+$ T helper (T_H) cell subsets. Hence, group 1 (ILC1), group 2 (ILC2), and group 3 (ILC3) ILCs can be distinguished by the production of T_H1, T_H2, and T_H17-type cytokines, respectively. Cytokine release by ILCs not only shapes early innate immunity but can also orchestrate T_H immune responses to microbial or allergen exposure. Recent studies have identified an unexpected effector function of ILCs as antigen presenting cells. Both ILC2s and ILC3s are able to process and present foreign antigens (Ags) via major histocompatibility complex class II, and to induce cognate $CD4^+$ T cell responses. In addition, Ag-stimulated T cells promote ILC activation and effector functions indicating a reciprocal interaction between the adaptive and innate immune system. A fundamental puzzle in ILC function is how ILC/T cell interactions promote host protection and prevent autoimmune diseases. Furthermore, the way in which microenvironmental and inflammatory signals determine the outcome of ILC/T cell immune responses in various tissues is not yet understood. This review focuses on recent advances in understanding the mechanisms that coordinate the collaboration between ILCs and T cells under homeostatic and inflammatory conditions. We also discuss the potential roles of T cells and other immune cells to regulate ILC functions and to maintain homeostasis in mucosal tissues.

Keywords: innate lymphoid cell, cytokine, T helper cell, immune response, antigen presentation

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Introduction

Adaptive immune responses are tightly controlled by the selection of the T and B cell receptor repertoire and by transcriptional networks regulating commitment, expansion, and contraction of the responses. Upon cognate antigen (Ag)–peptide–major histocompatibility complex (MHC) recognition Ag-specific T helper (T_H) cells proliferate and differentiate into effector T_H cell subsets with distinguishable cytokine profiles. Almost 30 years ago, interferon (IFN)-γ-secreting T_H1 cells were discriminated from T_H2 cells, whose cytokine profile includes interleukin (IL)-4, IL-5, and IL-13 (1). Additional subsets of T_H cells, such as T_H17 (2), regulatory T (T_{reg}) cells (3), T_H9 (4), T follicular helper cells (5), and more recently granulocyte-macrophage colony-stimulating factor (GM-CSF) producing T_H cells (6–8), were described.

In the past 5 years, new subsets of innate immune cells have emerged as a first line of defense at mucosal barriers. Like conventional natural killer (cNK) cells, they belong to the lymphoid lineage and develop from common lymphoid progenitor (CLP) cells but unlike T and B cells, they lack rearranged Ag-receptors. Hence, they were termed innate lymphoid cells (ILCs). ILCs are found in

various tissues including mucosa, lymphoid tissue, liver, skin, and fat. They depend on the expression of the common cytokine receptor γ chain (γ_c chain) and the transcriptional repressor inhibitor of DNA binding 2 (ID2) for their development (9–11). The factors involved in regulating different stages of ILC commitment from CLPs have been recently reviewed in Ref. (12). ILCs resemble T_H cells in their developmental requirements, transcriptional regulation, and in their cytokine secretion pattern. Thus, they were classified into three groups, which are able to immediately react to microbial and inflammatory challenge with cytokine production thereby limiting pathogen spread and tissue injury (9). Group 1 ILCs consist of cNK cells and so-called helper ILC1s; both secrete the T_H1-type cytokine IFN-γ. Group 2 ILCs are characterized by the production of T_H2-type cytokines IL-4, IL-5, and/or IL-13. Group 3 ILCs include fetal lymphoid tissue-inducer (LTi) cells, as well as adult ILC3s either expressing the natural cytotoxicity receptor (NCR) NKp46 (NCR^+ILC3s) or lacking this molecule (NCR^-ILC3s). Cells within this group produce the T_H17-type cytokines, IL-17 and/or IL-22 (9). The classification into ILC1, 2, and 3 is sometimes unhelpfully restrictive because ILCs have the potential to modulate their phenotypic and transcriptional signature upon activation and inflammation. When exposed to inflammatory conditions, NCR^-ILC3s can produce IFNγ (13, 14), and NCR^+ILC3s are able to convert into IFNγ-producing ILC1-like cells (15, 16). Moreover, in multiple sclerosis patients, blockade of CD25 (IL-2Rα) induces phenotypic changes of ILC3s toward cNK cells (17). Additional evidence for heterogeneity among ILC subsets comes from clonal analysis in humans demonstrating that the spectrum of cytokines produced by ILC3s is diverse (18) and in some cases, both ILC2 and ILC3 cytokines are produced (19). Finally, environmental factors, such as retinoic acid, short chain fatty acids, vitamins, aryl hydrocarbon receptor (AHR) ligands, stearyl sulfate, and probably bacterial metabolites, can shape ILC phenotypes and functions (20–24). Together, these data now provide convincing evidence that, similar to T_H cells, ILCs have a degree of plasticity in their cytokine profile. As for T_H cell commitment, cytokine-mediated conditioning, as well as epigenetic (25, 26) and transcriptional regulation (27) may account for changes of ILC subset-determining transcription factors and cytokines.

The biological relevance of ILCs is based on their capacity to sense environmental and inflammatory signals, and to respond with the secretion of cytokines important for immune defense, allergic reactions, and tissue repair. Recent data provide additional evidence that ILCs can condition T cell responses, either through cytokines, direct cell–cell contact, or through effects on accessory cells. This review will focus on the effects of ILC–T cell interactions for maintaining immune homeostasis. We will highlight major questions on how ILCs may cooperate with T cells thereby regulating T cell responses.

Induction and Skewing of T Cell Responses

Dendritic cells (DCs) are professional Ag-presenting cells (APCs) known for their robust capacity to activate naïve T cells and to modulate innate and adaptive immune responses (28). Distinct DC subsets have decisive roles in engaging pathways responsible for skewing the type of effector T_H cell response (29, 30). Moreover, DCs can suppress immune responses in order to maintain peripheral immune homeostasis and tolerance to self-Ags (31). As a key step in shaping the type of T_H cell response, cytokines secreted by innate immune cells including APCs can account for the expression of T_H subset-specific transcription factors (32). For example, IL-12 activates signal transducer and activator of transcription (STAT)-4 and induces the expression of the T-box transcription factor T-bet, which is critical for T_H1 cell commitment (33, 34). T-bet expression and T_H1 cell differentiation are further promoted by IL-2 (35). IL-4 induces STAT6 activation, which enhances Gata3 expression thereby initiating differentiation into T_H2 cell lineage (36). Additionally, IL-2 signaling followed by STAT5 activation plays a crucial role in T_H2 cell commitment by the induction of IL-4 transcription (37, 38). IL-6 signaling through STAT3, together with transforming growth factor (TGF)-β, induces retinoic acid-related orphan receptor (ROR)-γt expression and consequently the differentiation of pathogenic T_H17 cells from naïve T_H cells (39). A key issue in establishing immune homeostasis is the induction of T_{reg} cells that prevent immunopathology by maintaining tolerance. In addition, active suppression of inappropriate T cell responses is mediated by the induction of immune-regulatory cytokines, such as IL-10 (40), the expression of inhibitory receptors including cytotoxic T-lymphocyte-associated protein (CTLA)-4 or programed cell death (PD)-1 or the lack of co-stimulation and bystander signals. Altogether, cytokines and activating or inhibiting receptors of innate immune cells are pivotal for generating and conditioning T_H cell responses.

Group 1 ILCs

The group 1 ILCs comprised cNK cells and helper ILC1s. Both subsets secrete IFNγ and express the transcription factor T-bet (15, 16, 41–43). The expression of Eomesodermin (Eomes) is considered as a key factor for distinguishing cNK cells ($Eomes^+$) from ILC1s ($Eomes^-$) (43). However, splenic $NK1.1^+$ $CD127\ (IL\text{-}7R\alpha)^+$ cells, which are in some studies referred to as ILC1s, express considerable levels of Eomes (44). Nfil3, another transcription factor, has been attributed a role in specifying cNK cells versus ILC1s. Although important for the development of all ILC lineages, studies of Nfil3-deficient mice (42, 45, 46) revealed that cNK cells have greater dependency on Nfil3 than ILC1s (47, 48). This is probably due to direct transcriptional control of Eomes expression by Nfil3 (49). Thus, NK cells resident in the salivary gland appear to be a prototype of ILC1s, as they also do not require Nfil3 for their development (48). Cells defined as ILC1s in the intestinal epithelium in humans and mice express the epithelial homing marker CD103 and readily produce IFNγ upon stimulation (41). $CD103^+$ intraepithelial ILC1s, similar to cNK cells, express Eomes and T-bet, and are Nfil3-dependent, but in contrast to cNK cells do not require IL-15 for their development. Phenotypically, cNK cells express DX5 and, unlike most ILC1s, lack Trail or CD127 expression (43, 47, 48). Some ILC1-like cells derive from $ROR\gamma t^+$ ILC3s by a process that is accompanied by the loss of RORγt expression and the upregulation of T-bet in both mice and humans (15, 16, 50). Future research on $T\text{-}bet^+$

IFNγ-secreting subsets will help to clarify the developmental and functional relationship of group 1 ILCs.

Group 1 ILC–T Cell Interactions

Unlike group 2 and group 3 ILCs, murine cNK cells and ILC1s do not express MHC class II (MHC II) molecules, thus being incapable of direct Ag-dependent interaction with $CD4^+$ T_H cells (**Table 1**). Nevertheless, in recent years, a number of reports described new aspects of a direct crosstalk between T and cNK/ILC1 cells. Several studies defined a regulatory role for cNK cells in controlling T cell-dependent immune responses by direct cytotoxic activity toward $CD4^+$ and $CD8^+$ T cells (51–53), as well as toward APCs required for T cell priming. Two recent publications demonstrated that type 1 IFN confer the resistance to cNK cell-mediated lysis of activated $CD8^+$ T cells (54, 55). $CD8^+$ T cells isolated from IFN-α-receptor-1-deficient (*Ifnar1*$^{-/-}$) mice were preferentially targeted by cNK cells resulting in the elimination of cytotoxic $CD8^+$ T cells in response to viral infection through a perforin-dependent pathway. Another study proposed a role for NKp46 in limiting graft versus host disease (GVHD) (56), although it has remained obscure whether NKp46 is required for the direct killing of host-reactive T cells, or if it operates via targeting of accessory APCs. More recently, Schuster et al. reported that cNK cells specifically limit the number of virus-reactive $CD4^+$ T cells in a model of chronic murine cytomegalovirus (MCMV) infection in the salivary gland (57). Intriguingly, this process is dependent on the TNF-superfamily ligand Trail, which is, in addition to NKp46 also expressed by ILC1s. This suggests a possible contribution of ILC1s to the processes described above. Additionally, in humans, activated cNK cells could be shown to positively regulate $CD4^+$ T_H cell activity (58). cNK cells stimulated by cytokines or through activating receptors were shown to upregulate the co-stimulatory molecules, OX40L and members of B7 family (CD80/CD86). Interaction with such cNK cells led to augmented IFNγ production and enhanced T cell receptor-dependent proliferation of autologous $CD4^+$ T_H cells.

Conventional natural killer/ILC1 and T cell crosstalk operates in a reverse direction as well. Two studies showed that T_{reg} cells play an important role in keeping cNK cell activity in check (59, 60). Gasteiger et al. demonstrated that upon depletion of T_{reg}

TABLE 1 | Phenotype of mouse and human ILCs.

	Mouse				Human			
	cNK	**ILC1**	**ILC2**	**ILC3**	**cNK**	**ILC1**	**ILC2**	**ILC3**
SURFACE MOLECULES								
CD90	+	+	+	+	ND	ND	ND	ND
CD127	−[a]	+	+	+	lo	−[a]	+	+
CD117	lo	+	+[c]	+	lo	sub[l]	±	+
NK1.1	+	+	−	lo	+	+	+	+[o]
NKp46/NKp44	+	+	+	sub	sub	+[a]	−	sub[o]
CD25	−	−[b]	+	+	+	−	+	+[o]
ST-2	−	−	+[d]	−	−	−	+	−
Sca-1	−	−	+[e]	lo	ND	ND	ND	ND
TRANSCRIPTION FACTORS								
ID2	+	+	+	+	ND	ND	ND	+[o]
Gata3	−	lo	+	lo	lo	lo	+	lo
RORγt	−	−	lo	+	−	lo	lo	+
T-bet	+	+	−	sub	+	+	−	−
Eomes	+	−	−	−	+	−	−	−
NFIL3	+	+	+	+	ND	ND	ND	ND
MOLECULES INVOLVED IN ILC–T CELL INTERACTION/ILC ACTIVATION								
CD69	lo	lo	−[f]	ind, +[h]	+	sub[m]	sub[m]	sub[m, p]
MHC class II	−	−	+	+[i]	ind, +[k]	ND	+[n]	+
CD80	−	−	ind[g]	ind[l]	ind, +[k]	ND	+[n]	ND
CD86	−	−	ind[g]	ind[l]	ind, +[k]	ND	+[n]	ND
CD40	−	−	−	ind[l]	−	ND	ND	ND
CD30L	−	lo	−	+	ind, +[k]	ND	ND	ND
OX40L	−	−	−	+	ind, +[k]	ND	ND	+
ICOS	−	−	+	ND	ind, +[k]	ND	+	+
ICOSL	−	lo	+	ND	ND	ND	+	lo
RANKL	−	−	ND	+	−	ND	ND	+
TRAIL	−	+	ND	lo	ind, +[k]	ND	ND	ND

+ indicates expression; − indicates no expression; lo indicates low expression; sub indicates expression on a subset; ind indicates activation-induced expression; ND indicates expression is not determined.

[a]Expressed in certain tissues; [b]Intestinal ILC1s are $CD25^+$ (44); [c]Skin ILC2s are $CD117^-$ (62); [d]Small intestinal ILC2s are ST-2^- (73); [e]Liver ILC2s are Sca-1^- (71); [f]Fat-associated lymphoid cluster-derived and intestinal ILC2s are $CD69^+$ (69); [g]Expressed on mediastinal LN-derived ILC2s from IL-33 treated mice (93); [h]Expressed on splenic ILC3s under inflammatory conditions (130); constitutively expressed on intestinal ILC3s (44); [i]Expression increased on splenic ILC3s under inflammatory conditions (130); [j]Expressed on splenic, but not intestinal ILC3s under inflammatory conditions (130, 133); [k]Expressed after activation (159, 160); expressed at steady state (161); [l]Molecule expressed on certain subsets (16); [m]Human peripheral blood ILCs heterogeneously express CD69 (162); [n]Human ILC2s express CD80/CD86 and HLA-DR (93); [o]Human ILC population resembling ILC3s (122); [p]Human splenic ILCs are $CD69^+$ (122).

cells, cNK cells become hyper-responsive toward MHC I-deficient target cells that are recognized via missing-self mechanism. This was attributed to the increased availability of IL-2 produced by activated $CD4^+$ T cells (59). Another report demonstrated in a genetic model of type 1 diabetes that the acute removal of T_{reg} cells leads to the accumulation of activated cNK cells in pancreatic islets (60). On the contrary, in this experimental setting, depletion of T_{reg} cells did not result in an increase of IL-2 secretion by $CD4^+$ T_H cells, but more likely increased the availability of IL-2 to cNK cells by decreasing IL-2 consumption by T_{reg} cells. Interestingly, the accumulating cNK cells express CD127 (61) and might therefore constitute an "ILC1-like" subset. These studies provide the first example of T_{reg} cell-dependent control of cNK cell and possibly ILC1 activity. Given the importance of IL-2 for the expansion of other ILC subsets (45, 62), T_{reg} cells might also be involved in controlling their activity. Taken together, these findings illustrate the reciprocal immuno-regulatory relationship between group 1 ILCs and T cells.

Group 2 ILCs

ILC2s are the most homogenous ILC subset albeit with a specific phenotypic signature in the lung and intestine (44, 63). They express CD127, CD90.2 (Thy1), various levels of CD25, and the IL-33-receptor subunit ST2 (**Table 1**). The development of ILC2s depends on the transcription factors, ROR-α, Gata3, and T cell factor (TCF)-1 (64–67). ILC2s in both humans and mice secrete T_H2-type cytokines IL-4, IL-5, and/or IL-13 in response to IL-9, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), as well as during pulmonary inflammation or infection with *Nippostrongylus brasiliensis*, a helminth controlled by T_H2-type cytokine responses (63, 68–78). In addition to ILC2s, another cell type, the multipotent progenitor type 2 (MPP^{type2}) is described. MPP^{type2} cells exhibit similar phenotypic and functional characteristics with ILC2s (79), but do not produce T_H2-type cytokines in response to IL-33 (80). The release of T_H2-type cytokines by ILC2s is not only involved in *N. brasiliensis* expulsion (81) but can also trigger airway inflammation and allergic responses in humans (82–84). Together, ILC2s share developmental and inducible cytokine signatures with T_H2 cells suggesting a role in type 2 immune responses.

Group 2 ILC–T Cell Interactions

Type 2 immune responses are severely impaired in IL-4-receptor-α-deficient ($Il4R\alpha^{-/-}$) and IL-4-deficient ($Il4^{-/-}$) mice indicating that IL-4 has a role in T_H2 cell differentiation (85, 86). Further, the accumulation of T_H2 cells after *N. brasiliensis*/ovalbumin challenge is dramatically reduced in IL-4 and IL-13-double-deficient ($Il4^{-/-}Il13^{-/-}$) mice as compared to wild type (*WT*) mice (87). T_H2 cell differentiation is most likely initiated by innate immune cells, which become activated in the early phase of immune responses. Beside basophils and mast cells (88–90), it is now well established that ILC2s can secrete IL-4 suggesting a role for these cells in the induction of T_H2 cell differentiation and type 2 immune responses. Indeed, several reports provide evidence that ILC2s and $CD4^+$ T cells cooperate at multiple levels (91–97). In mice, which either have dramatically reduced numbers or a complete lack of ILC2s, the generation of type 2 immune responses upon *N. brasiliensis* infection, challenge with house dust mite Ag or with protease-allergen papain is impaired indicating a contribution of ILC2s to T_H2 cell responses (91, 93, 95). The addition of ILC2s to cultures of naïve $CD4^+$ T cells promotes the differentiation into T_H2 cells, while inhibiting the differentiation into T_H1 cells even in the presence of IL-12, a cytokine that drives T_H1 differentiation (33, 34, 92). In line with this finding, type 2 cytokines are not detectable when T_H cells are co-cultured with ILC2s unable to secrete IL-4 (94). On the other hand, *in vivo* differentiation of T_H1/T_H17 cells occurs independently of ILC2s, since mice, which lack ILC2s, show normal responses when exposed to *Saccharopolyspora rectivirgula*, a bacterium inducing T_H1/T_H17 inflammatory responses (95). Together, there is evidence that ILC2-derived IL-4 contributes to type 2 cytokine production of T_H cells, although an IL-4-independent pathway for ILC2-driven type 2 immune responses may also occur (91). Beside the direct effect of ILC2s on T_H2 differentiation, T_H2-type cytokines secreted by ILC2s can also affect $CD4^+$ T cells indirectly via DCs. Evidence for this comes from the finding that ILC2-derived IL-13 promotes migration of DCs into lung-draining lymph nodes (LNs), where activated DCs induce the differentiation of $CD4^+$ T cells into T_H2 cells (91).

Interleukin-33, a pro-inflammatory cytokine expressed by a variety of cell types can trigger the generation of inducible regulatory T (iT_{reg}) cells (98) and the activation of ILC2s to produce type 2 cytokines and amphiregulin (AREG). AREG is an epithelial growth factor that promotes restoration of airway epithelial integrity following influenza virus-induced damage (63). Importantly, analysis of ILC2-depleted, influenza virus-infected mice revealed a strong reduction in AREG mRNA suggesting that ILC2s are the main source of AREG under such inflammatory conditions. In other inflammatory models, mast cells were thought to be the major source of AREG and importantly, in these models, AREG was found to be critical for efficient T_{reg} cell function (99). In view of their abundance in the skin, lung, and colon, their strong responsiveness to IL-33, and early inflammatory signals, AREG-secreting ILC2s may have a function in tissue repair and likely also in triggering T_{reg} cell responses.

Another mechanism through which ILC2s have an influence on $CD4^+$ T_H cells is by their ability to serve as APCs. Co-stimulatory signals via OX40 are crucial for effector/memory T cell responses and for initiating T_H2 differentiation (100, 101). OX40-ligand (OX40L) is detectable on ILC2s, and the production of T_H2-type cytokines in ILC2-T cell co-cultures is significantly inhibited when anti-OX40L antibodies (Abs) are added, suggesting that ILC2s promote T_H2-responses via OX40/OX40L interactions (94). Further evidence for cell–cell interactions between ILC2s and $CD4^+$ T cells is provided by the finding that human and mouse ILC2s express both inducible T cell co-stimulator (ICOS) and ICOS-ligand (ICOSL) (70, 102), a co-stimulatory receptor/ligand pair known for its function for survival, proliferation, and cytokine secretion of T_H cell subsets (103). Moreover, ILC2s can process Ags and present peptides on MHC II. They express the co-stimulatory molecules, CD80 and CD86, and induce proliferation of T_H2 cells, albeit to a lesser extent than

professional APCs (92, 93). Interestingly, the expression of MHC II is higher on LN-, spleen-, and Peyer's Patch (PP)-derived ILC2s than on peritoneal lavage-, bronchoalveolar lavage-, and lung-derived ILC2s. Therefore, lymphoid tissue-specific factors might be responsible for sustained MHC II expression.

Together with the finding that ILC2s can express MHC II and co-stimulatory molecules, the direct ILC2–T cell interaction not only promotes T_H responses but also extends to cytokine-mediated help from activated T_H cells for ILC2 effector functions. During the acute phase of *N. brasiliensis* infection, Rag2-deficient (*Rag2*$^{-/-}$) mice show a similar expansion of ILC2s as *WT* mice. However, adaptive immune cells are required for prolonged ILC2 expansion and complete clearance of the infection (70). In a papain-induced inflammation model, IL-9 production by ILC2s is severely reduced in *Rag2*$^{-/-}$ mice suggesting that cytokine secretion by ILC2s is also dependent on the adaptive immune system (68). *In vitro* co-culture of CD4$^+$ T cells and ILC2s results in the upregulation of IL-4 mRNA in ILC2s, suggesting that T_H cells induce type 2 cytokine production by ILC2s (94). Additionally, activated CD4$^+$ T cells in co-culture with ILC2s can directly induce ILC2 proliferation and IL-5/IL-13 secretion (92). This effect is partially impaired by adding anti-IL-2-neutralizing Abs but not by separating CD4$^+$ T cells from ILC2s in transwell assays, suggesting an IL-2-driven feedback mechanism from activated CD4$^+$ T cells to ILC2s (92). In line with this, treatment of mice with IL-2/anti-IL-2 complexes results in increased *in vivo* proliferation of ILC2s (62) and expansion of ILC2 progenitors in the bone marrow (BM) (45). IL-2 can also promote IL-9 release by ILC2s, whereas IL-33 induces the upregulation of the IL-2-receptor subunit CD25 on ILC2s (104). The induction of CD25 expression may help ILC2s to become more sensitive to T cell-derived IL-2. It is currently unclear to what extent ILC2s and T_{reg} cells, which express high levels of CD25, or other T_H subsets, compete for IL-2. Hence, the expression of CD25 by ILC2s may also reduce the availability of IL-2 for T cells. Based on these observations, we propose the following model (**Figure 1**): ILC2s can be rapidly activated by various alarm signals leading to the release of T_H2-type cytokines, which help to induce T_H2 cell responses and DC migration into LNs toward T cell zones. Further, activated ILC2s secrete AREG, and it remains to be investigated whether this can trigger T_{reg} cell responses. The cognate interaction between ILC2s and CD4$^+$ T cells via MHC II–Ag presentation, co-stimulatory signals, and cytokines helps to amplify both ILC2 and CD4$^+$ T cell responses.

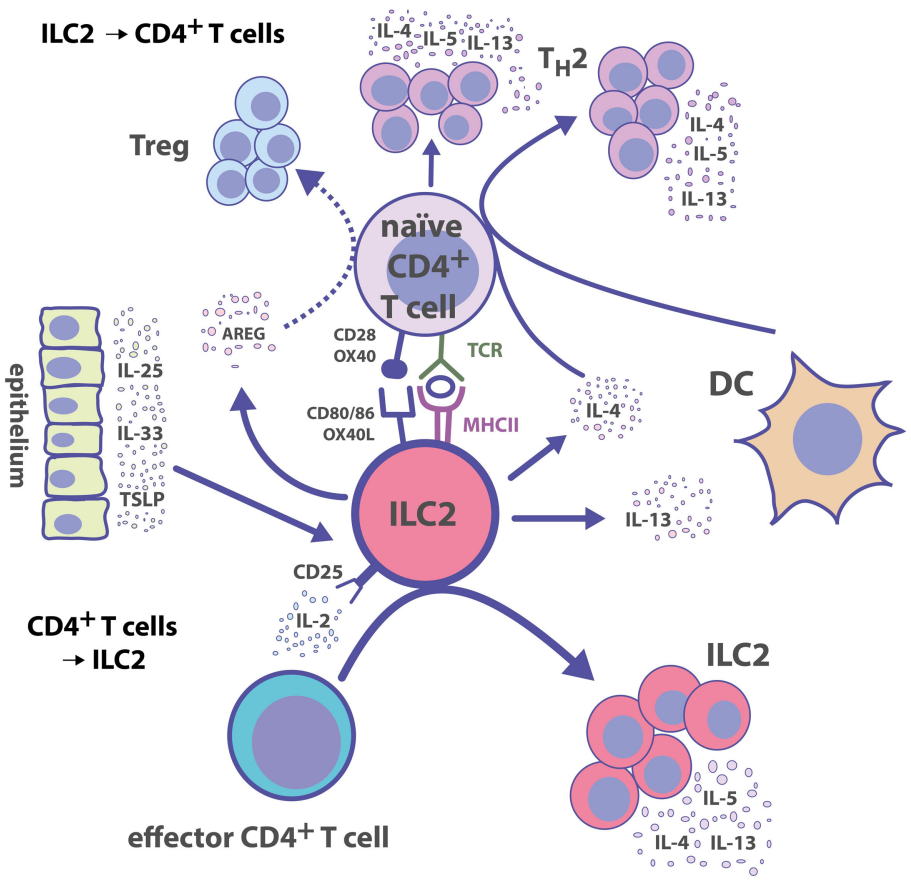

FIGURE 1 | Group 2 ILC–CD4$^+$ T cell interactions. ILC2s polarize CD4$^+$ T cell responses toward T_H2 immunity directly by presenting cognate Ag and by secreting T_H2-inducing cytokines. Reciprocally, activated CD4$^+$ T cells produce IL-2, which serves as a growth factor leading to the expansion of ILC2s.

Group 3 ILCs

All ILC3 subsets depend on the transcription factor RORγt for their development (105–107), and produce the T_H17-type cytokine IL-22 (107–111). IL-22 has a major role in protecting intestinal epithelial cells from bacterial infections and in promoting tissue repair through induction of epithelial cell proliferation and production of antimicrobial peptides (112). Group 3 ILCs can be phenotypically classified into a subset of fetal RORγt$^+$ CD127$^+$ CD117$^+$ LTi cells (106, 113–116), and adult NCR$^+$ or NCR$^-$RORγt$^+$ ILC3s (107, 108, 111, 117).

Group 3 ILC–T Cell Interactions

ILC3s can modulate T_H cell immune responses in several ways. One pathway involves the development of lymphoid tissue and T cell zone stroma. Already before birth, the cellular crosstalk of fetal lymphotoxin (LT)$\alpha_1\beta_2$-expressing LTi cells with mesenchymal stromal cells (MSCs) plays a pivotal role in the formation of LNs and PPs, in which immune responses are generated. Adult ILC3s retain the capacity to induce lymphoid tissue formation (118, 119). Following lymphocytic choriomeningitis virus (LCMV) infection in mice, the crosstalk between LT$\alpha_1\beta_2$-expressing ILC3s and T cell zone fibroblastic reticular cells helps to restore the disrupted T-zone compartment and hence the structure to generate proper immune responses (120). Similarly, LT$\alpha_1\beta_2^+$ ILC3s can restore lymphoid follicle organization in the colon of mice infected with *Citrobacter rodentium* (121). The interaction of ILC3s with MSCs is also reciprocal. In humans, the crosstalk between LT$\alpha_1\beta_2^+$ ILC3s and marginal reticular cells (MRCs), a subset of marginal zone stromal cells, induces the production of MRC-derived survival factors for ILC3s, such as IL-7 (122). A second pathway, by which ILC3s can modulate T_H cell immune responses, is through altering the recruitment of CD4$^+$ T_H cells. ILC3s are able to release soluble LTα_3, which promotes the homing of CD4$^+$ T_H cells to the gut lamina propria where they differentiate into functional T_H cell subsets (**Figure 2**) (123). In a model of airway inflammation, ILC3-derived IL-22 reduces CCL17 production by epithelial cells thereby limiting T_H2 cell recruitment and immune responses to allergens in the lung (124). These data show that ILC3s have an impact on generating functional T cell compartments and recruitment of CD4$^+$ T_H cells to mucosal sites.

In the adult spleen, ILC3s are localized in the marginal zone and around the central arterioles, and in LNs in proximity to

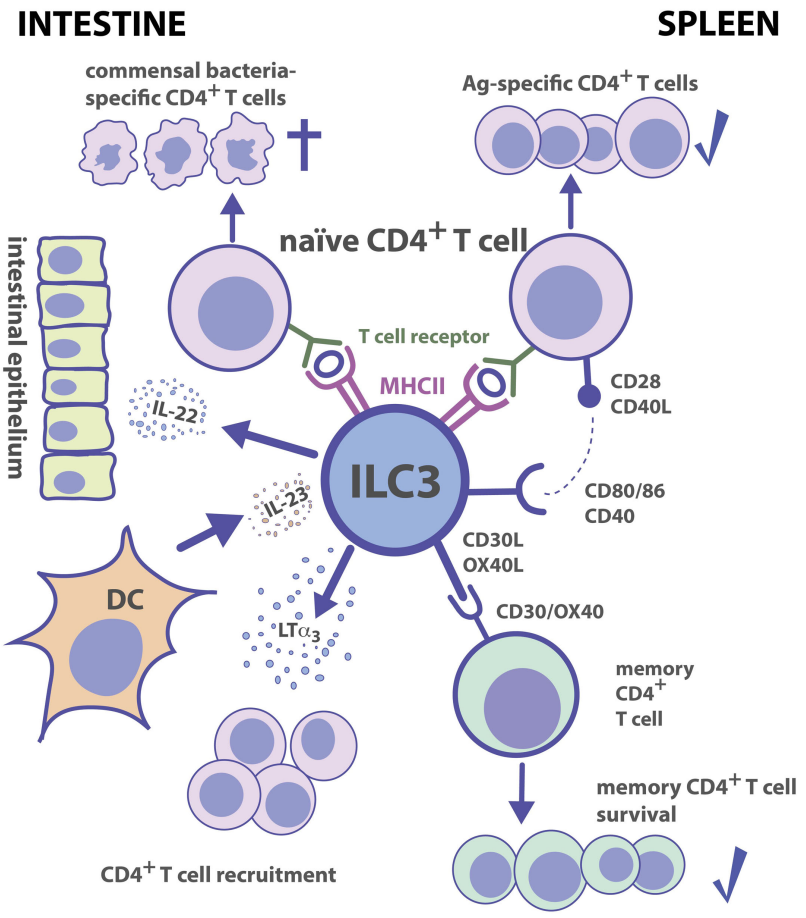

FIGURE 2 | Group 3 ILC–CD4$^+$ T cell interactions. Tissue localization greatly affects the outcome of Ag-dependent T cell–ILC3 interaction. Intestinal ILC3s maintain tolerance toward commensal microbiota, while splenic ILC3s are efficient in the induction of Ag-specific CD4$^+$ T cell responses and memory CD4$^+$ T cell survival.

high endothelial venules and interfollicular areas (122, 125–127). Because of the close association of splenic ILC3s to Ag-entry sites and T cells as well as their expression of the co-stimulatory molecules, CD30-ligand (CD30L) and OX40L, it has been assumed that they may directly interact with T cells during adaptive immune responses (125). Mice with a deficiency in CD30 and OX40 (*CD30*$^{-/-}$*OX40*$^{-/-}$ mice) lack proper memory Ab responses due to a failure in survival of primed CD4$^+$ T_H cells (128). *In vitro*, ILC3s can promote survival of memory CD4$^+$ T_H cells from *WT* but not from *CD30*$^{-/-}$*OX40*$^{-/-}$ mice suggesting that both CD30L and OX40L molecules expressed by ILC3s are essential for CD4$^+$ T_H memory responses (128). This possibility was supported by an *in vivo* study, which identified ILC3s as the key players in the maintenance of CD4$^+$ memory T_H cells (**Figure 2**) (129).

A third mechanism by which ILC3s interact with CD4$^+$ T_H cells is through receptors required for immune recognition. ILC3s isolated from various tissues of fetal, neonatal, and adult mice express MHC II and MHC II-associated gene transcripts (44, 113, 130–132). NCR$^-$ILC3s are able to internalize, process, and present foreign Ags to CD4$^+$ T_H cells (130, 131). Under non-inflammatory conditions, ILC3s express neither CD40 and CD80 nor CD86 (130, 131). However, following stimulation with IL-1β splenic but not intestinal, NCR$^-$ILC3s can upregulate co-stimulatory molecules (130). A recent study confirmed that even after toll-like receptor ligand (TLRL) or pro-inflammatory cytokine exposure, intestinal ILC3s do not upregulate co-stimulatory molecules (133). The finding that mLN-derived ILC3s are as well unable to express co-stimulatory molecules upon stimulation is likely due to the fact that ILC3s found in the mLNs are originally intestinal ILC3s, which were trafficking from the intestine to the mLNs (127). It is noteworthy that genome-wide transcriptional profiling of splenic ILC3s reveals an enrichment for genes involved in cell activation and immune responses (63). In contrast to splenic ILC3s, intestinal ILC3s express the activation marker, CD69 (44), a glycoprotein involved in establishing oral tolerance (134) and limiting dextran sodium sulfate (DSS)-induced inflammation (135). Moreover, ILC3s present in the small intestine express neuropilin-1 (Nrp1) (44), which promotes T_{reg} cell survival and functional activity (136–138). It is therefore conceivable that ILC3s exert tissue-specific immune functions with immunogenic versus tolerogenic activity in the spleen and intestine, respectively. This hypothesis is further supported by the notion that splenic NCR$^-$ILC3s promote CD4$^+$ T_H cell responses *in vitro* and *in vivo*, whereas intestinal ILC3s fail to efficiently stimulate CD4$^+$ T_H cells (**Figure 2**) (130). In mice, intestinal ILC3s express lower levels of MHC II as compared to ILC3s identified in other tissues (130, 131, 133). Together with the observation that intestinal ILC3s lack co-stimulatory molecules, this may contribute to maintaining intestinal T cell tolerance, similar to immature DCs expressing low surface levels of MHC II and co-stimulatory molecules (139).

Hepworth et al. reported the development of spontaneous intestinal inflammation in mice lacking MHC II exclusively on ILC3s (ILC3Δ^{MHCII} mice) and found a role for intestinal ILC3s in limiting commensal bacteria-specific pro-inflammatory colonic CD4$^+$ T_H cell responses through induction of PD (131, 133). Since other laboratories failed to detect spontaneous signs of inflammation in ILC3Δ^{MHCII} mice (130, 132), it is possible that the development of immunopathology is triggered by microbial co-factors. In the intestine, ILC3s can inhibit T_H17 cell-mediated inflammation through AHR signaling, release of IL-22, and by preventing the expansion of aberrant segmented filamentous bacteria (SFB) (140). In pediatric Crohn's disease (CD) patients, MHC II levels on intestinal ILC3s are significantly reduced, and such low expression correlates with increased frequencies of colonic T_H17 cells and circulating commensal bacteria-specific IgG (133). This study is the first to describe an association of ILC3-mediated Ag presentation and control of commensal bacteria-specific adaptive immunity in humans. It remains unclear which are the mechanisms that underlie loss of MHC II in CD patients and whether this is sufficient to trigger inflammatory bowel disease. Together, these findings suggest that intestinal ILC3s can inhibit expansion of T_H17 cells and immunopathology after exposure to pro-inflammatory stimuli.

Analogously to ILC2–T cell interactions, the crosstalk between ILC3s and CD4$^+$ T_H cells might be bidirectional and depends on cytokines. This is further supported by the findings that the presence of the adaptive immune system has an effect on the number and IL-22 production of intestinal ILC3s, most likely through competition for growth factors (141, 142). Human and activated mouse ILC3s produce IL-2 (19, 130), and conversely, TLR2-driven proliferation of human ILC3s is partially dependent on IL-2 (19). Availability of IL-2 alone or in combination with Pam3Cys promotes increased CD25 expression in human ILC3s suggesting that CD25 expression might help ILC3s to win the competition for IL-2 against T cells (19). Moreover, there is some evidence that mouse ILC3s have a higher capacity to bind IL-2 than activated CD4$^+$ T_H cells (133). Therefore, the availability of IL-2 can restrict ILC3 and T_H responses as a result of receptor density, efficiency of binding, and kinetics of IL-2 consumption.

Immune Homeostasis in the Gut: Tolerance Versus Inflammation

The critical question regarding maintenance of immune homeostasis is where, when, and how immune responses prevent tissue injury. The intestine is a prime example that has been extensively studied with respect to cellular networks and pathways patrolling tissue integrity and regulating inflammation. T_{reg} and T_H17 cells are the most abundant CD4$^+$ T_H cells in the intestinal mucosa under steady state (143–145). The balance between the two subsets is crucial for the outcome of mucosal immune responses (146). Commensal bacteria have a specific impact on the number of both T_H subsets (147) and on the capacity of ILC3s to regulate T_H subset responses (148). On the other hand, ILC3s contribute to maintenance of intestinal epithelial barrier function thereby limiting microbes entry and inflammatory T_H cell responses (108, 109, 117, 141, 148). Whereas under steady-state conditions, intestinal ILC3s produce high levels of IL-22, the production of IL-17 is rather low (44). Importantly, T_H17 cells are induced by SFB (149, 150) by a mechanism that requires SFB presentation by DCs (132, 151). In contrast, ILC3 presentation of Ag prevents amplification of SFB-independent T_H17 cells (132). In line with this, the

expansion of SFB and pathogenic T_H17 cells inversely correlates with the number of intestinal ILC3s (140). In an IL-17-dependent autoimmune mouse model, it was recently shown that SFB colonization was associated with enhanced auto-Ab titers (152). The increase in IL-17-producing cells, as observed in CD patients (153), is probably not sufficient *per se* to induce immunopathology. Specificity of inflammatory T_H cells, intestinal infections, pro-inflammatory bystander cells, and loss of functional T_{reg} cells might be required to trigger intestinal inflammation.

All these studies published in recent years raised the question of whether and how ILC–T cell interactions regulate pro- or anti-inflammatory responses in the gut. Since ILC3s can prevent dissemination of commensal bacteria in the gut and commensal bacteria-specific T_H cell responses (123, 131, 132, 148), they probably promote an immunological tolerogenic state in the gut. In addition, the production of GM-CSF by ILC3s has the potential to enhance iT_{reg} cell numbers and function thereby promoting intestinal homeostasis (154). In some colitis models, however, ILC3s were reported to enhance intestinal inflammation (13, 15), and pathogenic ILC1 numbers were increased in patients with CD (16, 41). The functional polarization toward IFNγ-producing ILC1s or IL-22-producing ILC3s appears to depend on tissue-specific and pro-inflammatory conditions. Environmental changes may immediately affect the ratio and/or polarization of ILC and T cell subsets. For example, induction of pro-inflammatory cytokines, such as IL-23, was shown to counteract the responsiveness toward IL-33, and the generation of iTregs in the intestine (98). As for T_H cell differentiation, it is likely that the amount of cytokines determines ILC cytokine polarization. Under homeostatic conditions, the intestine provides a microenvironment enriched of cytokines with inhibitory effects, such as TGF-β. At high dose, TGF-β inhibits T_H17 responses, whereas low-dose TGF-β promotes T_H17-differentiation (155–157). A similar impact of cytokine concentrations for immune homeostasis has also been discussed for IL-22 (158). Therefore, excessive release of cytokines by ILCs may contribute to immunopathology, whereas under steady-state conditions, ILCs rather promote epithelial tissue integrity and tolerogenic T cell responses. During inflammation, ILC3s can switch off RORγt expression, which may eventually be regained at later time points. The modulation of cytokine receptors during a critical time window of ILC activation and ILC-T cell interaction might also contribute to prevent excessive immunopathology. This has been shown for a number of receptors controlling growth and survival of both ILCs and T cells. Finally, the polarization toward protective versus inflammatory response in the gut likely requires a tight balance between temporal regulation, amount, and combination of cytokines co-expressed by individual ILCs.

Conclusion

Our understanding of immune homeostasis has been challenged by the notion that environmental factors, including commensal bacteria and nutritional components, as well as cholinergic and metabolic signals can regulate immune functions and pro-inflammatory processes. ILCs are important "early sentinel" cells, which connect innate and adaptive immunity by sensing environmental changes, such as infections and inflammation and by the release of immuno-regulatory cytokines. They not only contribute to T cell immune homeostasis by promoting T_H cell differentiation and effector functions but can also directly interact with $CD4^+$ T_H cells. Both ILC2s and ILC3s internalize and present Ag to T_H cells. Considering the fact that the number of ILCs in most tissues is rather low as compared to other immune cells, they appear to have a surprising *in vivo* impact on immune homeostasis. The localization of ILCs in relatively high density at Ag-entry sites and T cell areas as well as bystander effects on classical DCs might explain this effect. In addition, advances in two-photon microscopy have shown that several $CD4^+$ T_H cells are often clustering with the same APC, a fact that may increase local cytokine concentrations for optimal cell–cell interactions. The capacity to elicit cognate T_H cell proliferation or rather prevent T_H cell responses strongly depends on environmental factors and the nature of Ag, and it will be important to further investigate the mechanisms by which ILCs prevent or promote T cell responses in various tissues. For example, it will be interesting to unravel whether ILCs can express inhibitory receptors and/or collaborate with T_{reg} cells. Finally, there are clearly cytokine-driven reciprocal effects between ILCs and T cells, which might help to coordinate and/or limit immune responses. Taken together, a better understanding of the regulation of cytokine expression by ILCs and their interaction with T cells will help to develop new strategies to treat inflammatory diseases in humans.

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REVIEW
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The Interplay Between Monocytes/Macrophages and CD4+ T Cell Subsets in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by inflammation of the synovial lining (synovitis). The inflammation in the RA joint is associated with and driven by immune cell infiltration, synovial hyperproliferation, and excessive production of proinflammatory mediators, such as tumor necrosis factor α (TNFα), interferon γ (IFNγ), interleukin (IL)-1β, IL-6, and IL-17, eventually resulting in damage to the cartilage and underlying bone. The RA joint harbors a wide range of immune cell types, including monocytes, macrophages, and CD4+ T cells (both proinflammatory and regulatory). The interplay between CD14+ myeloid cells and CD4+ T cells can significantly influence CD4+ T cell function, and conversely, effector vs. regulatory CD4+ T cell subsets can exert profound effects on monocyte/macrophage function. In this review, we will discuss how the interplay between CD4+ T cells and monocytes/macrophages may contribute to the immunopathology of RA.

Keywords: rheumatoid arthritis, inflammation, immune regulation, cell polarization, myeloid cell, T helper cell, Treg

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RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory and debilitating disease, characterized by inflammation of the lining of the joint (synovitis), eventually leading to the destruction of cartilage and the underlying bone. RA affects between 0.5 and 1% of the Western adult population with a female:male ratio of 3:1. The exact etiology of RA is still unknown, but it is widely accepted that RA is a multifactorial disease with genetic, environmental (e.g., smoking), gender and age-associated factors contributing to the disease process (1–3).

Typical hallmarks of RA are pannus formation and synovial hyperplasia, caused by proliferating fibroblasts and infiltrating immune cells. These events promote leukocyte recruitment, immune cell activation, and production of inflammatory mediators and proteinases, all of which eventually contribute to joint damage. A wide range of immune cells has been detected in the RA joint, including CD4+ T cells, CD8+ T cells, B cells, NK cells, γδ T cells, mast cells and myeloid cells. Various soluble mediators produced by these immune cells have been shown to correlate with disease progression and/or severity in RA, e.g., rheumatoid factor, anti-citrullinated peptide antibodies, tumor necrosis factor (TNF)α, interleukin (IL)-6, IL-1, and IL-17A (2, 4). The importance of the immune system in disease pathogenesis is illustrated by the recent success of biologic therapies that target key inflammatory cytokines (e.g., TNFα blockade and anti-IL-6R therapy), immune molecules (e.g., CTLA4-Ig

leading to blockade of CD80/CD86-mediated costimulation), and immune cells (e.g., B cell depletion).

MONOCYTES AND MACROPHAGES IN RHEUMATOID ARTHRITIS

Monocytes/macrophages are a potent source of proinflammatory cytokines, in particular TNFα, IL-6 and IL-1, and matrix metalloproteinases (MMPs), leading to endothelial cell activation, acute phase reactions, and cartilage damage. These cells can also produce a wide range of chemokines, which help recruit additional leukocytes to the inflamed joint. In addition, monocytes have the ability to polarize $CD4^+$ T cells and can differentiate into osteoclasts, which may further contribute to their role in RA pathogenesis. As such, monocytes and macrophages are viewed as relevant therapeutic targets in RA (5–7).

Myeloid cells with a monocyte/macrophage phenotype (i.e., $CD14^+CD68^+$) are present in large numbers in the rheumatoid joint. Several studies have shown that these cells produce proinflammatory cytokines (8, 9) and have an activated phenotype with increased expression of HLA-DR (involved in antigen presentation to $CD4^+$ T cells), costimulatory molecules (e.g., CD80, CD86, and CD40), adhesion molecules (e.g., CD54), and some chemokine receptors (10–18). The importance of synovial $CD68^+$ macrophages in RA pathogenesis is underlined by the findings that the presence of these cells correlates with disease activity markers (19, 20) and that a change in their presence has been reported to be a reliable biomarker for response to treatment (21). An early, small study in patients with RA ($n = 10$) noted that synovial $CD68^+$ cells were reduced in the perivascular and connective tissue areas 12 weeks after treatment with gold (22). Sublining macrophage count was also shown to correlate significantly with radiologic outcome and radiologic progression in patients with RA ($n = 23$–27) (20). An elegant study by Haringman et al. investigating arthroscopic synovial tissue biopsies from patients with RA ($n = 88$) participating in various clinical trials, showed that a reduction in the number of synovial sublining $CD68^+$ macrophages correlated significantly with clinical improvement independently of the therapeutic strategy (23). Importantly, the number of sublining macrophages did not change after placebo or ineffective treatment, supporting its use as a predictive biomarker for response to treatment (21, 23).

A growing number of studies have reported on the frequencies and phenotype of peripheral blood monocyte subsets in RA. Phenotypically, peripheral $CD14^+$ monocytes in patients with RA show some signs of altered activation with studies reporting increased expression of CD14, FcγRs, CD54, CD11b, and/or HLA-DR (18, 24–27), although not all studies agree on increased HLA-DR expression (24, 27). Peripheral blood monocytes from RA patients are also reported to express increased levels of transmembrane TNF (tmTNF) (28). Human monocytes can be divided into subpopulations based on expression of CD14 [lipopolysaccharide (LPS) coreceptor] and CD16 (FcγRIII). $CD14^{++}CD16^-$ "classical" monocytes are the most prevalent subset, representing ~90% of blood monocytes in healthy individuals. CD16-expressing cells are less frequent among circulating monocytes but are expanded in infection and inflammatory conditions (29). $CD16^+$ monocytes can be further subdivided into $CD14^{++/bright}$ (also called "intermediate") and $CD14^{dim}$ ("non-classical" or "patrolling") subsets (29, 30). Earlier studies, which did not necessarily discriminate between $CD14^{++/bright}CD16^+$ and $CD14^{dim}CD16^+$ cells, showed an increase in the percentage of $CD14^+CD16^+$ monocytes in RA blood (31–33). $CD14^+CD16^+$ monocytes in RA express high levels of CCR1, CCR5, ICAM-1, or TLR2 (27, 32, 33). Higher frequencies of $CD14^+CD16^+$ blood monocytes were associated with clinical parameters of active disease (31, 33). More recent data suggest that only the frequency of $CD14^{++/bright}CD16^+$ intermediate cells is increased in peripheral blood of patients with chronic RA compared to sex- and age-matched healthy donors, while the frequency of non-classical $CD14^{dim}CD16^+$ monocytes does not differ between patients and controls (27, 34, 35). The frequency of $CD14^{++/bright}CD16^+$ cells at baseline was found to negatively correlate with response to methotrexate treatment (34). However, another study reported, using absolute cell counts, that both $CD14^{++/bright}CD16^+$ and $CD14^{dim}CD16^+$ monocyte populations are increased in RA blood (36). Ligation of CD16 using immune complexes or FcγRIII-specific mAb enhances proinflammatory TNFα production, suggesting that the expression of CD16 could be functional (32, 34, 37).

Recently, Krasselt et al. showed that $CD14^{bright}CD56^+$ monocytes, which are predominantly composed of classical $CD14^{bright}CD16^-$ cells, produce more TNFα, IL-10, and IL-23 in response to LPS and demonstrate increased spontaneous production of reactive oxygen intermediates [reactive oxygen species (ROS)] compared to $CD14^{bright}CD56^-$ monocytes (38). $CD14^{bright}CD56^+$ frequencies were positively correlated with age in healthy controls and were expanded in the blood of young RA patients compared to age-matched healthy controls. The $CD14^{bright}CD56^+$ subpopulation was reduced in a longitudinal study of 16 RA patients following anti-TNF treatment. Additional studies are required to further elucidate the role of $CD14^{bright}CD56^+$ monocytes in RA.

In RA synovial fluid, it appears to be the intermediate $CD14^{++/bright}CD16^+$ monocyte population that is increased in frequency compared to matched peripheral blood (31, 33, 35). One explanation for the increased frequency of CD16 expressing synovial monocytes is that there is a specific expansion of the $CD14^{++/bright}CD16^+$ monocyte population. Another non-mutually exclusive explanation is that there is *de novo* expression of CD16 triggered by the inflammatory milieu. It was shown that *in vitro* stimulation of healthy monocytes with recombinant transforming growth factor β (TGFβ) or RA synovial fluid induced elevated CD16 expression, an effect that was inhibited by TGFβ signaling blockade (35).

Table 1 summarizes the reported phenotypic features of $CD14^+$ cells derived from RA peripheral blood or synovial fluid, and cells with a macrophage phenotype in synovial tissue. It should be noted that studies on synovial fluid or synovial tissue generally involve the whole $CD14^+$ and/or $CD68^+$ population (which may contain monocytes and macrophages), rather than sorted subsets. Therefore, **Table 1** represents a summary of relevant literature reports on monocyte/macrophage cell phenotypes

TABLE 1 | Phenotypic features of monocytes/macrophages from RA peripheral blood, synovial fluid, and synovial tissue.

Compartment	Phenotype	Reference
(a) Peripheral blood	↑ HLA-DR, CD14, CD40, CD54, CD11b	(18, 24–26)
	↑ Fcγ receptors (including CD16)	(18, 24, 26, 27, 31–36)
	↑ CCR3, CCR4, CCR5	(15)
	↑ tmTNFα	(28)
	↑ Spontaneous IL-1β production	(25, 28)
	↑ Resistance to apoptosis	(28, 39)
(b) Synovial fluid	↑ HLA-DR, CD40, CD54, CD80, CD86, CD276	(11, 13, 14, 16–18, 35)
	↑ $CD16^+$	(18, 31, 33, 35)
	↓ CCR1, CCR2, CCR4, ↑CCR3, CCR5	(15)
	↑ Resistance to apoptosis	(39, 40)
(c) Synovial tissue	$HLA\text{-}DR^+$	(10, 14)
	$CD68^+$	(15, 19–23)
	$CD163^+$	(12)
	$IL\text{-}1^+$, $TNF\alpha^+$, $IL\text{-}6^+$, $GM\text{-}CSF^+$, $TGF\beta^+$	(8, 9, 14)

Arrows indicate phenotypic alterations observed in $CD14^+$ cells from the peripheral blood of rheumatoid arthritis (RA) patients in comparison to healthy donor peripheral blood $CD14^+$ cells (a), or in $CD14^+$ cells from RA synovial fluid compared to RA peripheral blood $CD14^+$ cells (b). Phenotypic markers that are expressed on cells with a macrophage phenotype from RA synovial tissue (c).

within different anatomical compartments rather than a direct comparison of these cells *between* different compartments.

EFFECT OF MONOCYTES/MACROPHAGES ON $CD4^+$ T CELL SUBSETS

In addition to the innate effector functions of monocytes/macrophages in terms of proinflammatory cytokine and chemokine production, their inflammatory role in RA pathogenesis may stem from their function as a bridge to the adaptive immune system. Colocalization of $CD14^+$ cells with clusters of $CD4^+$ effector T cells at sites of inflammation has been reported in inflamed rheumatoid synovium, as well as in inflamed tonsil, and psoriatic or atopic dermatitis skin (41, 42), suggesting that $CD4^+$ T cells and monocytes/macrophages can interact *in vivo* at sites of inflammation.

$CD4^+$ T Helper Cell Polarization by Monocytes/Macrophages

Dendritic cells (DCs) are classically considered to be the major drivers of $CD4^+$ T helper cell polarization; however, evidence is accumulating that monocytes/macrophages can also play a role in this process. Monocytes and/or macrophages can be major sources of IL-1β, IL-6, IL-12, and IL-23, cytokines known to be present in the RA joint (4, 8, 9, 43, 44). IL-12 is involved in driving $CD4^+$ T helper 1 (Th1) cell polarization, while IL-1β, IL-6, and IL-23 can drive and maintain Th17 polarization. Interferon γ $(IFN\gamma)^+CD4^+$ T cells (indicative of Th1 cells) and $IL\text{-}17^+$ $CD4^+$ T cells (indicative of Th17 cells) are readily detectable in the RA joint, in both the tissue and the fluid (45–47). Th1 cells were originally thought to be one of the major contributors in RA pathogenesis, based on their abundance in RA synovial fluid, their key role in certain experimental models of arthritis, as well as the inflammatory function of IFNγ particularly on macrophage activation. However, studies have shown that IFNγ may also have a protective, rather than an exacerbating role in RA (48–50), which may be due to its antagonistic effects on Th17 induction (51) or on VEGF production (46, 52), thereby possibly inhibiting angiogenesis.

In recent years, IL-17 and Th17 cells have gained attention as critical mediators in RA pathogenesis. IL-17 is a potent proinflammatory cytokine that works in synergy with TNFα to induce the inflammatory events and joint damage that are characteristic of RA (53, 54). The receptors for IL-17 (IL-17RA and IL-17RC) are expressed in RA synovium, including on $CD14^+$ monocytes/macrophages (55) and stimulation of RA synovium with IL-17 leads to production of IL-6, MMPs, and joint degradation (56–58). Blood $CD14^+$ monocytes can be potent inducers of human Th17 responses depending on their activation status. Human blood monocytes activated by peptidoglycan or LPS were shown to efficiently promote Th17 responses from cocultured naive $CD4^+$ T cells in the presence of anti-CD3 mAb (59). Our own lab found that following *in vitro* activation with LPS, peripheral blood $CD14^+$ monocytes from either healthy donors or RA patients promoted Th17 responses in an IL-1β- and TNFα-dependent manner (17, 60). It was also shown that human monocytes stimulated *in vitro* with heat-killed pneumococci triggered a Th17 response which was dependent on TLR2 signaling (61). In contrast, stimulation with live pneumococci led to a mixed Th1/Th17 response due to monocyte production of IL-12p40. In a non-infectious setting, peripheral blood monocytes from patients with type 1 diabetes spontaneously secreted the proinflammatory cytokines IL-1β and IL-6. These cells induced higher frequencies of Th17 cells from memory T cells *in vitro* compared with monocytes from healthy control subjects, which was reduced by a combination of an IL-6-blocking Ab and IL-1R antagonist (62). Finally, healthy peripheral blood monocytes that were treated with RA synovial fluid prior to coculture with anti-CD3/CD28-stimulated $CD4^+$ T cells were shown to promote Th17 differentiation, which was attributed to a TNFα-mediated increase in monocytic production of IL-6 and IL-1β (63).

Additional studies in mice and human show that monocytes/macrophages from the synovial fluid of the inflamed arthritic joint, which may contain extravasated monocytes as well as tissue-resident macrophages, can promote IL-17 production in $CD4^+$ T cells (17, 35, 64). These data suggest that newly recruited $CD4^+$ T cells in the rheumatoid joint might be steered toward a Th17 response by local monocytes/macrophages. The ensuing positive feedback loop between Th17 cells and monocytes/macrophages may then perpetuate inflammation (42). In our own work, the induction of Th17 responses by *in vivo* activated monocytes isolated from RA synovial fluid was found to be independent of TNFα, IL-1β, IL-6, and IL-23, and to involve cell contact (17). In line with our observations, Yoon et al. found increased Th17 responses when stimulated autologous peripheral blood memory $CD4^+$ T cells were cocultured with RA synovial

fluid monocytes, compared to responses elicited by circulating monocytes. In contrast to our finding that Th1 polarization was not strongly affected by the anatomical origin of monocytes (17), Yoon et al. found that intracellular IFNγ expression (and in most donors tested, the level of IFNγ in culture supernatants), was also significantly increased by coculture with synovial monocytes compared to blood monocytes (35). Thus, synovial monocytes/macrophages may promote both Th17 and Th1 responses.

It is currently unclear whether the capacity of monocytes to promote Th17 responses resides in a particular monocyte subset. Rossol et al. found that circulating intermediate $CD14^{++/bright}CD16^{+}$ monocytes in the presence of LPS promoted Th17 cell expansion from peripheral blood memory $CD4^{+}$ T cells *in vitro*, and that the frequency of $CD14^{++/bright}CD16^{+}$ monocytes in peripheral blood of RA patients correlated closely with *ex vivo* Th17 cell frequencies (27). Traunecker et al. reported that non-classical ($CD14^{dim}CD16^{+}$) monocytes from healthy donors when cocultured with autologous $CD4^{+}$ T cells and specific superantigens in the absence of pathogen-recognition receptor (PRR) stimuli were more efficient stimulators of IL-17-producing T cells; however, in the presence of PRR stimuli, Th17 expansion was mostly observed in cocultures with classical ($CD14^{++/bright}CD16^{-}$) or intermediate ($CD14^{++/bright}CD16^{+}$) monocytes (65). Blocking of LFA-1/ICAM-1 interaction increased the frequency of IL-17-producing T cells expanded from non-classical monocyte/$CD4^{+}$ T cell/superantigen cocultures. However, no significant differences in capacity to promote Th17 responses were observed in experiments assessing peripheral blood monocyte subsets from 11 RA patients. In contrast to the above studies, Smeekens et al. found that only $CD14^{++/bright}CD16^{-}$ classical monocytes and not $CD16^{+}$ monocytes could induce a protective Th17 response in response to *Candida albicans,* due to increased IL-1β and prostaglandin E2 production (66). It is crucial to achieve high degree purity separation for functional characterization of cell subpopulations and the use of magnetic bead separation to isolate $CD16^{+}$ vs. $CD16^{-}$ monocytes rather than high purity FACS-based cell sorting in the study by Smeekens et al. may therefore limit interpretation of the results. Nonetheless, a recent study by Liu et al. also found that after coculturing autologous memory $CD4^{+}$ T cells with FACS-purified monocyte subsets and anti-CD3, classical $CD14^{++/bright}CD16^{-}$ monocytes most potently expanded $IL\text{-}17^{+}$ memory $CD4^{+}$ T cells (67). Non-classical $CD14^{+}CD16^{++}$ monocytes were the strongest inducers of IFNγ expression in naive $CD4^{+}$ T cell cocultures. The above *in vitro* experiments employ different modes of T cell activation with or without PRR stimulation of monocytes, which may contribute to the heterogeneity of the results obtained. Activation of T cells via crosslinking of soluble anti-CD3 (67) triggers MHC-independent T cell activation, whereas stimulation via superantigen (65) crosslinks MHC on monocytes and TCR on T cells, more closely representing an MHC-restricted antigen-specific stimulation. Altering the activation state of monocyte subsets via PRR agonists (27, 65, 66) may also affect their capacity to polarize $CD4^{+}$ T cells. Any conclusions drawn from these and future studies regarding the contribution of different monocyte subsets to $CD4^{+}$ T cell polarization should take into account the choice of *in vitro* stimulation and ideally should be confirmed in multiple systems.

Monocytes/macrophages are also a major source of IL-15 (68), a pleiotropic cytokine which mediates several important proinflammatory effects on both monocytes/macrophages and $CD4^{+}$ T cells. The IL-15Rα is overexpressed on blood-derived lymphocytes and monocytes in RA patients (69), and IL-15 is found at high levels in RA SF (70). IL-15 plays an important role in regulating T cell migration and was shown *in vivo* to facilitate accumulation of adoptively transferred T cells in RA synovial tissues engrafted into immune deficient SCID mice (71). IL-15 also promotes TNFα production by synovial T cells (72), and IL-15-activated blood-derived or synovial T cells can induce TNFα in a macrophage cell line and in RA blood- or synovium-derived monocytes/macrophages in a cell-contact-dependent manner (72). IL-15 may also promote IL-17 production by synovial T cells (73), although in mice a fine-tuning effect of IL-15 on Th17 differentiation was reported (74). IL-18 is another proinflammatory cytokine expressed in RA synovium, most prominently in $CD68^{+}$ macrophages (75). IL-18 acts in synergy with other cytokines, including IL-12 and IL-15, to stimulate T cell production of IFNγ and synovial macrophage release of TNFα (75). In the RA joint, IL-18 also acts as a chemoattractant for synovial $CD4^{+}$ T cells (76) and monocytes (77). Together, these data indicate that production of IL-15 and IL-18 by monocytes and/or macrophages may also be relevant in driving or polarizing inflammatory $CD4^{+}$ T cell responses in the RA joint.

In addition to their role as antigen-presenting and cytokine-producing cells, macrophages can efficiently generate ROS, an important antimicrobial defense mechanism that occurs via activation of the phagocytic NADPH oxidase complex. Rats and mice with genetic variation in the neutrophil cytosolic factor *Ncf1* (encoding p47phox, an activating protein in this complex) demonstrate reduced capacity to exert oxidative burst and increased incidence and severity of T cell-dependent arthritis (78, 79). When efficient ROS production was restored specifically in macrophages, T cell-dependent arthritis development was ameliorated (80). *In vitro*, T cells from mice lacking functional macrophage *Ncf1* demonstrated increased proliferation and cytokine production in response to antigenic stimulation, as compared to T cells from mice with ROS-competent macrophages. Coculturing T cells from ROS-deficient mice with ROS-competent macrophages led to suppressed T cell IL-2 production and proliferative responses to antigen, suggesting that macrophage ROS production affects antigen presentation *in vitro*. However, irrespective of whether macrophages used in the coculture could produce ROS, IFNγ production was increased when $CD4^{+}$ T cells were derived from ROS-deficient mice, indicating that *in vivo* exposure to macrophage ROS production may suppress Th1 responses. In agreement with these findings, King et al. found that human T cells stimulated with anti-CD3/CD28 in the presence of ROS demonstrated an increase in type 2 cytokines with no alteration in type 1 cytokine production, even in culture conditions polarizing toward a Th1 phenotype, and which could be reversed by concomitant antioxidant exposure (81). Together, these findings suggest that ROS production by macrophages may have the potential to downregulate or modulate T helper cell responses.

It is well known that certain inbred mouse strains show a bias toward either Th1 or Th2 responses, e.g., C57BL/6 and BALB/c,

respectively. The bias in Th1- or Th2-like cytokine profiles is maintained in NUDE or SCID mice lacking an adaptive immune system, suggesting a role for the innate immune system in driving this polarization and giving rise to the concept of M1 and M2 macrophages (82). Studies of transgenic (Tg) mice have provided *in vivo* evidence that inflammatory macrophage subpopulations can polarize $CD4^+$ T cell responses in mouse models of arthritis. Li et al. crossed a *Floxed* STOP-human/mouse DR5 Tg mouse with the LysM.Cre mouse, to express the Tg human/mouse-chimeric death receptor 5 (DR5) restrictively in myeloid lineage cells. Treatment of these mice with an antihuman DR5 agonistic antibody led to targeted depletion of $CD11b^{high}Ly6C^+$ inflammatory macrophages and reduced development and severity of collagen-induced arthritis (83). Interestingly, the depletion of this macrophage subpopulation also significantly reduced protein levels of IL-6 and IL-17A in sera, reduced synovial *Il17a*, *Il6*, *Tnfa*, and *Il23a(p19)* mRNA expression and reduced the frequency of IL-$17A^+$ and $IFN\gamma^+CD4^+$ T cells while increasing $CD4^+Foxp3^+$ cells in draining lymph nodes. These data support the notion that macrophages may be important contributors to $CD4^+$ T cell polarization.

Dissecting the precise role of monocytes/macrophages in initiating adaptive immune responses can be challenging due to overlapping function and lineage marker expression with DCs. DCs are professional antigen-presenting cells that play a key role in promoting an effective immune response. A multitude of pattern recognition receptors allows DCs to sense invading pathogens and present both exogenous and endogenous antigens to naive and memory T cells. Different subsets of DC exist, including conventional or classical DC (cDC) type 1, cDC type 2, plasmacytoid DC, and monocyte-derived DC (mo-DC). cDC develop independently from monocytes and originate from a common DC progenitor which expresses flt3 (84). It can be difficult to distinguish monocytes or macrophages from mo-DCs or cDC type 2, due to overlap in certain markers (e.g., CD14, CD11b, CD11c, and CX3CR1) (85). CD64 has been identified as a marker that can be used to distinguish mo-DCs from cDCs in mice; however, these cells remain difficult to distinguish from cDCs in humans (86). Recently, researchers have taken a genetic approach to ablate monocytes and macrophages while sparing cDCs and lymphocytes by depleting M-CSF-R^+LysM^+ cells. Although neither monocytes nor macrophages were required to initiate immunity, when both cell types were depleted during infection with the intestinal pathogens *Citrobacter rodentium* or *Listeria monocytogenes*, $IFN\gamma^+CD4^+$ T cells were reduced in the lamina propria, demonstrating the capacity of monocytes/macrophages to influence T cell polarization (87). In contrast, the IL-17 response was not altered by monocyte and macrophage depletion but was significantly impaired upon selective depletion of cDCs, indicating that this cell population is necessary for mucosal Th17 responses. Macrophage depletion has also been shown to reduce type 2-dependent immune responses in the lung and gut. Depletion of $CD11b^+$ $F4/80^+$ macrophages led to a significant reduction in recruitment and cytokine expression of Th2 cells in affected tissues of three models of IL-13-dependent inflammation, fibrosis, and immunity, without any such reduction in the draining lymph nodes (88). Since $CD11b^+$ DCs might also be susceptible to depletion, studies of CD11c-DTR and CD11b/c-DTR double Tg mice confirmed that macrophages but not $CD11c^+$ DCs were critical for the maintenance of type 2-dependent responses. Reduced expression of the Th2 cell chemoattractants CCL1 and CCL22 may be one mechanism through which Th2 cell recruitment is impaired in a *Schistosoma mansoni* egg-induced lung granuloma model following macrophage depletion (88). Macrophage production of the chemokine CCL5 was recently shown to be important for the maintenance of stable tissue-resident memory IFNγ-producing $CD4^+$ T cell (T_{RM}) populations in memory lymphocyte clusters in a mouse model of genital herpes (89). Further studies are needed to investigate the presence of T_{RM} in RA synovial tissue and the requirement for monocytes/macrophages in maintaining these populations.

T Helper Cell Recruitment by Monocytes/Macrophages

As alluded to the previous section, monocytes/macrophages can play an important role in recruiting or maintaining $CD4^+$ T cells in the arthritic joint. The C–X–C motif chemokine receptor $CXCR6^+$ is abundantly expressed on type 1 polarized effector memory T cells in RA synovial fluid (90, 91). Expression of CXCR6 on synovial T cells is reported to coincide with elevated expression of CXCL16 (the ligand for CXCR6) by synovial macrophages, endothelial cells, and fibroblast-like synoviocytes (FLS) in hypertrophic RA synovium (90). Significantly increased levels of cleaved CXCL16 have been demonstrated in RA SF compared to control samples (90, 92), and RA synovial tissue macrophages express both CXCL16 and CXCR6 (92). *In vitro* migration experiments demonstrate that healthy PBMC or $CXCR6^+$ T cells isolated from RA SF can migrate in response to exogenous CXCL16 or CXCL16 present in SF (90, 92). Together, these data suggest that increased CXCL16 expression in RA synovium, either due to increased expression by macrophages (90) or due to increased influx of monocytes (93), promotes recruitment of $CXCR6^+$ T cells and may thereby contribute to synovial inflammation and immunopathology. CXCL16 expression can be differentially regulated by cytokines: the Th2-like cytokines IL-4, IL-10, and IL-13 suppress secretion of CXCL16 by monocytes/macrophages, while the Th1-associated cytokine IFNγ slightly enhances CXCL16 secretion (93). No modulation of CXCL16 levels was observed upon addition of IL-15, IL-18, or TNFα to monocytes/macrophages. In contrast, earlier work found that exposure of monocytes to TNFα upregulated transmembrane expression and secretion of CXCL16 (90) suggesting that a reduction in synovial TNFα levels might impact on recruitment of $CXCR6^+$ T cells to the joint. In a small study of three patients responding to anti-TNF treatment, *in situ* immunohistochemistry demonstrated significantly reduced synovial CXCL16 expression compared to the high expression levels observed pretreatment (90). This observation may be due to reduced monocyte numbers in the joint following treatment, since synovial cellularity is known to be reduced soon after anti-TNF infusion (94–96). Conversely, in three non-responder patients, CXCL16 expression remained high. In accordance with reduced CXCL16 expression in the joint following successful anti-TNF therapy, serum levels of CXCL16 were also decreased after anti-TNF treatment in two cohorts

of 23 and 44 RA patients, respectively (97, 98). Thus far, these studies suggest that monocyte infiltration and/or type 1 cytokine production in the RA joint may enhance local production of soluble CXCL16, which may exacerbate local inflammation via recruitment of CXCR6$^+$ T cells.

Another chemokine receptor interaction of increasing interest in RA is the C–C chemokine ligand CCL20 [also known as macrophage inflammatory protein-3 alpha (MIP-3α)] and its C–C chemokine receptor CCR6 [recently reviewed in Ref. (99)]. CCR6 expression is commonly associated with Th17 cells (100), but this receptor is also expressed on memory T cells (101), including CD4$^+$ T cells expressing IFNγ with or without IL-17 (102), as well as DC, B cells, and regulatory T cell (Treg) (99). In the SKG mouse model of T cell-mediated autoimmune arthritis, the CCL20–CCR6 axis is implicated in recruiting Th17 cells to the joint, via spontaneous CCL20 production from adherent FLS (103). The authors found that dispersed monocytes from mouse synovial tissue did not produce CCL20 unless stimulated. Others found that unstimulated FLS from RA patients did not produce CCL20, but that stimulation with either IL-1β or TNFα led to the production of CCL20 at levels sufficient to promote CCR6-specific recruitment of mononuclear cells, and which could be increased by IL-17 and decreased by IL-4 (104). In patients with juvenile idiopathic arthritis, CCL20 mRNA was shown to be constitutively expressed by synovial monocytic cells (105). In addition, *in vitro* exposure to plasmin, a component of the fibrinolytic cascade which is generated in inflamed tissues, can trigger production of CCL20 by human macrophages and lead to chemotactic migration of CCR6$^+$ Th17 cells *in vitro* (106). A role for plasmin in RA has been suggested by observations from collagen-induced arthritis (107) but has yet to be confirmed in the human setting. In summary, there is a potential role for monocytes in influencing the CCL20–CCR6 axis in RA.

Modulation of CD4$^+$ Treg Function by Monocytes/Macrophages

In addition to effects on T helper cell polarization, activated monocytes and macrophages can also positively or negatively influence the function of CD4$^+$ Tregs through production of soluble mediators. IL-1β has been shown by several groups to drive IL-17 expression by CD4$^+$CD25$^+$ Tregs [reviewed in Ref. (108)]. Excessive production of IL-6 *in vivo* inhibited inducible Treg generation from naive T cells but did not affect the development and function of natural Tregs (109). A recent study showed that IL-6 negatively affected FOXP3 protein expression by reducing expression of USP7 and disrupting USP7–FOXP3 association. USP7 is a deubiquitinase that prevents proteasomal degradation of FOXP3, thereby increasing FOXP3 expression levels and enhancing Treg function (110). TNFα has also been shown to decrease FOXP3 expression and reduce Treg function (111, 112). Additionally, anti-TNF therapy was shown to induce the differentiation of a suppressive CD4$^+$CD25^{hi}FOXP3$^+$CD62L$^-$ Treg subpopulation through conversion of CD4$^+$CD25$^-$ T cells in RA patients (113).

Contrastingly, TNFα has also been shown to boost Treg expansion and/or function (114, 115). Our lab recently showed that monocytes, activated *in vitro* with LPS or with cytokines known to be present in the RA joint, can induce expression of proinflammatory cytokines (IL-17 and IFNγ) in CD4$^+$CD25$^+$CD127^{low} Tregs in an IL-6-, TNFα-, and IL-1β-dependent manner (18). However, despite the increase in proinflammatory cytokine expression, these Tregs maintained and even enhanced their suppressive capacity, indicating that acquisition of proinflammatory cytokine expression does not necessarily imply loss of suppressive function (18). Conversely, it has been shown that TNFα and IL-6 can alter the susceptibility of effector T cells to Treg-mediated regulation, making them resistant to suppression (116–118).

Monocytes/macrophages can also boost Treg function directly by producing soluble mediators that are immunoregulatory in nature. A population of CD11b$^+$F4/80$^+$CD11c$^-$ macrophages has been identified in the lamina propria which can induce Foxp3$^+$ Treg differentiation through a mechanism dependent on IL-10, retinoic acid, and TGFβ in the local milieu (119). As discussed above, macrophage-derived ROS might play a role in modulating effector T cell responses (80); however, it has also been suggested that ROS can promote Treg-mediated immune regulation (120–122). Using both human and rat systems, macrophages were shown to suppress T cell responses by inducing FOXP3$^+$ Tregs in a ROS-dependent manner (120). This was confirmed using macrophages from patients with chronic granulomatous disease (CGD) that are defective in ROS production; CGD macrophages allowed significantly more T cell activation and expansion and induced fewer FOXP3$^+$ cells than did macrophages from control subjects. T cells primed by CGD macrophages showed reduced inhibition of responder T cell proliferation and IFNγ or IL-17 production than did cells primed by control macrophages. Similar results were observed using rats with defective ROS production due to a SNP in *Ncf1*.

It was shown that monocyte subsets and their cytokines may have differential effects on subsets of Treg cells. In both humans and mice, ~70% of Tregs express the transcription factor Helios (123). CD16$^+$ monocytes have been described to inhibit proliferation of Helios$^+$ Tregs through a mechanism dependent on IL-12. In contrast, Tregs lacking Helios expression were suppressed by CD16$^-$ monocytes via TNFα, while TNFα blockade specifically expanded the Helios$^-$ Treg subset (124).

Figure 1 summarizes data from existing reports on the proposed mechanisms via which monocytes/macrophages may affect the function of CD4$^+$ effector vs. Tregs subsets. It should be noted that much of the reported evidence comes from *in vitro* studies, which indicate the potential involvement of these mechanisms but do not demonstrate conclusively if or where these events occur at the site of inflammation.

EFFECTS OF CD4$^+$ T CELL SUBSETS ON MONOCYTE/MACROPHAGE ACTIVATION AND FUNCTION

Monocyte/Macrophage Activation and Polarization by CD4$^+$ Effector T Cells

Monocytes and macrophages are capable of responding to a wide range of stimuli and environmental cues, which in turn

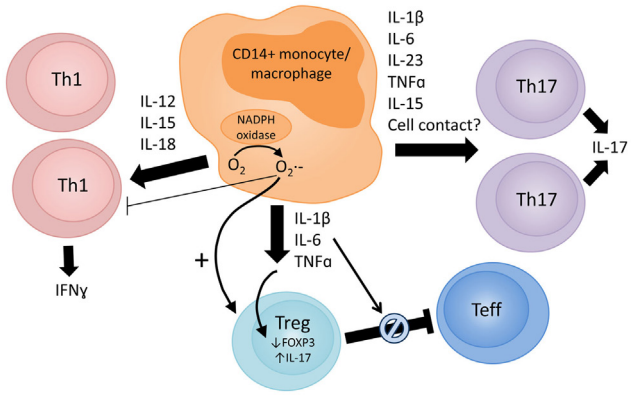

FIGURE 1 | Proposed mechanisms via which monocytes/macrophages in the inflamed RA joint can modulate CD4⁺ T cell responses. The arrow thickness reflects the evidence base to support the proposed mechanism. Monocyte/macrophage-derived cytokines, such as IL-1β, IL-6, TNFα, IL-12, IL-15, IL-18, and IL-23, are present in the RA joint (4, 8, 9, 43, 44, 70, 75). These cytokines can promote Th1 (IL-12, IL-15, and IL-18) and Th17 (IL-1β, IL-6, IL-15, IL-23, and TNFα) responses, and both Th1 and Th17 cells are detectable in synovial tissue and fluid (45–47). *In vitro* and *in vivo* data indicate that activated CD14⁺ blood monocytes or synovial monocytes/macrophages can potently induce Th17 (17, 35, 59–64) and/or Th1 (35, 61, 87) responses. Further studies are required to clarify whether different monocyte subsets preferentially promote specific Th responses. Activated monocytes/macrophages can also have profound effects on the phenotype and function of regulatory T cells (Tregs). Several groups have reported that IL-1β can drive IL-17 expression by CD4⁺CD25⁺ Tregs [reviewed in Ref. (108)], but these cells may retain suppressive capacity (18). Others have reported that TNFα and IL-6 lead to reduced FOXP3 expression in Tregs (110–112) or render effector T cells resistant to Treg-mediated suppression (116–118). Macrophage production of reactive oxygen species (ROS) may play a role in suppressing Th1 responses (80) and inducing FOXP3⁺ Tregs (120–122).

can determine their phenotype. Macrophages can be polarized into diverse subtypes often termed M1 and M2; however, these phenotypes represent extremes on a spectrum of functional states (125). A recent transcriptomics study provided elegant evidence to support the latter concept (126). In this study, gene expression profiling was performed on 299 macrophage samples stimulated with 28 different conditions. Monocytes were differentiated into macrophages using either GM-CSF or M-CSF. The macrophages were subsequently activated using typical M1 (e.g., IFNγ, LPS, and TNF) or M2 (e.g., IL-4 and IL-13) stimuli, and standardized microarrays were performed. Analysis of the transcriptomes using coregulation analysis generated two groups of samples positioned at either end of a bimodal axis, representing M1/M2 states. However, when the effect of other activation factors not associated with typical M1/M2 states was included in the analysis (e.g., high-density lipoprotein and free fatty acids), the spectrum expanded away from the axis to account for these dissimilar states. These data confirm that while it is possible to polarize macrophages into distinct M1/M2 populations, not all activators will generate populations that fit into or in between these states. It is therefore important to keep in mind that although in this review we refer to M1/M2 macrophages, a spectrum of activation outside of these phenotypes exists. Outside of stringent M1/M2 phenotypes, it has become increasingly difficult to determine the exact nature of macrophages within published work. A recent review has highlighted a lack of coherently used markers used to define macrophage populations within the field (127). The authors discuss that incomplete descriptions of how macrophages have been isolated, stimulated, and analyzed can lead to confusion between laboratories. Therefore, they propose a new nomenclature system, based on the macrophage activator and markers used to define the population. In this way, the anticipation is that we can better define and understand the distinct macrophage populations generated in different labs under different conditions and avoid confusion when attempting to place macrophages on a spectrum of activation.

Bearing in mind the limitations of the M1/M2 classification, cytokines that are typically associated with Th1 and Th2 cells have been used to polarize monocytes or macrophages *in vitro* with the Th1 cytokine IFNγ driving the M1 (classically activated) phenotype and the Th2 cytokines IL-4/IL-13 driving an M2 (alternatively activated) phenotype (128). Three further M2 subtypes have since been described; M2a, M2b, and M2c, all with different functional capabilities to those seen in an M1 phenotype (127, 129). M1 macrophages typically release high levels of proinflammatory cytokines, such as IL-1, IL-6, IL-12, and TNFα, have high production of reactive oxygen intermediates and metabolize arginine to nitric oxide. Typical M1 chemokines include CXCL5, CXCL8, CXCL9, CXCL10, and CXCL13. M2 macrophages are characterized by a switch in arginine metabolism from the iNOS pathway seen in M1 cells to the arginase pathway, releasing orthonine and polyamines. M2 cells release IL-10 and some IL-12 and express CD163, CD206, scavenger receptors A and B, and Dectin-1. M2 macrophages are generally considered to be involved in wound healing and to promote tissue remodeling through release of growth factors VEGF and TGFβ (129, 130).

In addition to Th1 and Th2 cytokines, it has been shown that the Th17-associated cytokine IL-17A has direct effects on macrophages leading to an increase in IL-1β, TNFα, and IL-6 (131, 132). Silencing of IL-17RA using siRNA reduced the upregulation of IL-1β, TNFα, and IL-6 by macrophages, thus showing that IL-17 signaling through IL-17RA can influence cytokine release of macrophages (132). IL-17 has been shown to be directly chemotactic for monocytes *in vitro* at concentrations found in RA synovial fluid, via ligation of IL-17RA and IL-17RC on monocytes and p38 MAPK activation (133). *In vivo*, human monocytes injected intravenously into SCID mice were recruited into subcutaneously implanted sponges which had been soaked with human IL-17 or the positive control CCL2 [also called monocyte chemoattractant protein-1 (MCP-1)] but not sponges soaked with IL-8 or IL-10 (133). Tissue-infiltrating Th17 cells (unlike Th1 and Th2 cells) also secrete CCL20 (45), which has been shown to be chemotactic for monocytes (134). IL-17 may also have indirect effects on monocyte chemotaxis through the induction of chemokine expression by other cell types present in the RA joint. *In vitro* addition of IL-17 to RA synovial fibroblasts or normal blood-derived macrophages effectively induced expression of CCL2 and CCL20 (135). However, *in vivo* only CCL2 was secreted following adenovirus-mediated intraperitoneal (i.p.) expression of IL-17. The increase in peritoneal CCL2

levels contributed to increased monocyte recruitment, which was reduced by i.p. injection of neutralizing anti-CCL2 (135). Local IL-17 expression in ankle joints was also associated with increased F4/80 staining and CCL2 levels. The IL-17-mediated induction of CCL2 appeared to involve the PI3K, ERK, and (at least in RA synovial fibroblasts) JNK pathways.

There is good evidence that TCR- or cytokine-activated T cells can activate monocytes, resulting in inflammatory cytokine and MMP production by monocytes in a cell-contact-dependent manner (39, 136–138). The $CD4^+CD45RO^+CCR7^-$ effector memory T cell subset of cytokine-activated T cells has been suggested to be a main driver of this stimulation (139). One study showed that distinct $CD4^+$ T cell subsets (Th1, Th2, or Th17) may differentially affect monocyte differentiation into distinct mo-DC subsets in a cell-contact- and cytokine-dependent manner (42). Cocultures of sorted Th1, Th2, and Th17 cells with isolated $CD14^+$ monocytes led to the generation of three distinct mo-DC subsets, as defined by typical DC markers. The monocytes cultured with Th1 cells formed DCs that secreted IL-12 and expressed CD86 and CD274 (DCth1), whereas those generated through culture with Th2 cells expressed increased levels of IL-10, CD275, and DC-SIGN (DCth2). Monocytes cultured with Th17 cells developed into DCs that secreted IL-1β, IL-6, and IL-23 but not IL-12 (DCth17). The DCs generated through coculture were then used as stimulators in a mixed leukocyte reaction; IFNγ and IL-17A were released from responding $CD4^+$ T cells when cultured with DCth1 and DCth17, respectively. These data are in line with the results described above on how different Th-related cytokines affect macrophage polarization.

$CD4^+$ T cells can also drive differentiation of monocytes into osteoclasts. Bone resorption by osteoclasts is of pathological significance in RA, causing "erosion sites" which can be used as a measure of disease severity and outcome. Studies have shown that $IFN\gamma^+$ human T cells cultured with peripheral blood monocytes in the presence of M-CSF can induce osteoclast formation via expression of the cytokine receptor activator of NF-κB ligand (RANKL) (140). IFNγ may, however, also disrupt the formation of osteoclasts by rapidly degrading the RANK adaptor protein TRAF6 (141), suggesting that the $IFN\gamma^+$ T cells can both contribute to and hinder the formation of osteoclasts. Th17 cells are often implicated in promoting osteoclastogenesis; Th17-associated cytokines were shown to upregulate RANKL on RA FLS and to directly induce monocyte-to-osteoclast differentiation (142, 143). In addition, RANKL-expressing Th17 cells were recently shown to convert mature osteoclasts to a bone resorptive state (144). T cells in the synovial fluid have been shown to express RANKL (145), and high levels of RANKL-expressing $CD3^+$ cells have been found in the synovial tissue of patients with RA (146), thus potentially contributing to osteoclast formation and therefore higher levels of bone resorption.

Together, these data indicate that prototypical T helper cell-associated cytokines can polarize, recruit, activate, or differentiate monocytes and/or macrophages. *In vivo*, the phenotype and function of monocytes and macrophages is likely to be dependent on many soluble factors and cellular interactions acting in concert.

Induction of Apoptosis in Monocytes and Macrophages by $CD4^+$ T Cells

In addition to activating monocytes or macrophages, several reports have shown that activated $CD4^+$ T cells can kill these cells (147, 148). Later studies assigned this killing to $CD4^+CD25^+$ Tregs (149, 150) as well as to activated effector T cells, defined as Treg-depleted $CD4^+CD25^-$ T cells or cloned antigen-specific $CD4^+$ T cells (39, 148). A recent study showed that $CD4^+$ (and $CD8^+$) T cells from the BAL fluid of C57BL/6 mice express FASL and that these T cell populations can induce apoptosis in autologous alveolar macrophages. Although apoptosis by $CD8^+$ T cells was more prevalent, killing by $CD4^+$ T cells was observed (151). Evidence from our lab has shown that activated $CD4^+CD25^-$ effector T (Teff) cells upregulate FASL, and upon coculture with human monocytes, activate, and then kill the monocytes in a FAS/FASL-dependent manner (39). Blocking the FAS/FASL interactions reduced monocyte apoptosis but did not affect the expression of FAS, CD14, or HLA-DR on the monocytes, indicating that the monocytes still became activated by the T cells. T cells may also kill monocytes/macrophages in a FAS-independent mechanism, as TRAIL and TWEAK death receptor pathways have been implicated in macrophage killing by $CD4^+$ T cells (152).

Modulation of Monocyte/Macrophage Function by $CD4^+$ Regulatory T Cells

Regulatory T cells are generally defined as $CD4^+CD25^+CD127^{low}$ $FOXP3^+$ cells. The suppressive effects of Tregs on immune cells have been documented widely, in particular on cells from the adaptive immune system ($CD4^+$ and $CD8^+$ T cells) [reviewed in Ref. (153–155)]. Tregs can employ several mechanisms of suppression including release of the inhibitory cytokines IL-10 and TGFβ, cytolysis via release of granzymes A and B, metabolic disruption via IL-2 consumption or through degradation of ATP to AMP/ADP, and eventually adenosine via the ectonucleotidases CD39 and CD73. There is ample evidence that Tregs can also interact directly with antigen-presenting cells, including monocytes and macrophages [reviewed in Ref. (156)].

Modulation of monocyte function *in vitro* has been shown by a series of experiments from our lab. The first study compared monocytes from the peripheral blood of healthy human donors cultured alone, or cocultured with autologous effector T cells or Tregs. Coculture with anti-CD3 mAb and effector T cells induced an activated phenotype in the monocytes, with increased levels of CD80, CD40, and HLA II compared to monocytes cultured alone (157). When monocytes were cocultured with Tregs, levels of CD40, CD80, and HLA II on monocytes were not increased compared to the monocyte only culture, while CD86 expression was significantly reduced. These monocytes were impaired in their ability to induce T cell proliferation in subsequent T cell stimulation assays. In a following study, we demonstrated that upon coculture with Tregs, monocytes expressed increased levels of the mannose receptor CD206 and hemoglobin/haptoglobin scavenger receptor CD163 (158), markers which are associated with M2-like macrophages (127, 129). In support of

these phenotypic changes, we found that phagocytosis of FITC-zymosan/latex beads was increased in Treg cocultured monocytes, although the number of monocytes that phagocytosed the beads did not change significantly (158). Furthermore, LPS-induced NFκB activation and secretion of TNFα and IL-6 were decreased in monocytes cocultured with Tregs, compared to those cultured alone or with effector T cells. Using flow cytometry, it was shown that the frequencies of LPS-induced IL-6 and TNFα expressing monocytes were also reduced upon coculture with Tregs as compared to monocytes cultured alone or with Teffs, indicating that the observed differences in cytokine secretion were due to changes within cytokine-expressing monocytes (39). This was not due to cell death, as in contrast to $CD4^+CD25^-$ Teff, Tregs did not kill the monocytes upon interaction (39). The decreased production of IL-6 and TNFα in response to LPS was still observed when monocytes were re-purified after coculture (158), indicating that Tregs imprint changes in the monocytes, suggestive of "trained immunity." Immune memory has traditionally been associated with cells of the adaptive immune system; recently, the term "trained immunity" has been used to define the memory capacity within innate immune cells (159). Induction of trained immunity in monocytes has been shown in studies whereby exposure to beta-glucan led to epigenetic modifications in monocytes, resulting in phenotypic changes and altered function (160). Further investigation is required to determine whether the effects of Tregs on monocytes are reflective of a state of "trained immunity" in these cells.

The modulation of monocytes by Tregs was shown to be dependent in part on soluble factors (IL-10 and IL-4/IL-13) as well as cell-contact (158). A different group showed that cocultures of human $CD14^+$ monocytes with sorted Tregs led to increased levels of secreted IL-10 and higher levels of B7-H4 receptor on the monocytes. These monocytes were less capable of stimulating a T cell proliferative response, which was due in part to the expression of IL-10 and B7-H4 (161). Notably, work from the same group showed that the expression of B7-H4 was a marker for immunosuppressive tumor-associated macrophages in ovarian carcinoma (162), and that the presence of Tregs and macrophage-associated B7-H4 at the tumor site was negatively associated with patient outcome (163, 164). Together, these data indicate that modulation of monocytes and macrophages by Tregs may have functional consequences in disease.

Modulation of macrophage function by Tregs has also been shown *in vivo*. Adoptive transfer of syngeneic $CD4^+CD25^+$ Tregs into the peritoneal cavity of SCID mice revealed both phenotypic and functional changes in peritoneal macrophages (165). In this study, SCID mice were adoptively transferred with either no T cells, $CD4^+CD25^+$ Tregs, $CD4^+CD25^-$ T cells, or both $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ T cells at a 1:1 ratio. The percentages of $F4/80^+$ macrophages expressing CD54, CD80, CD86, or $I\text{-}A^d$ were significantly decreased in SCID mice transferred with Tregs compared to the control mice and were increased in mice receiving $CD4^+CD25^-$ T cells. The latter increase was inhibited when both T cell types were cotransferred. The group also showed that macrophages from mice transferred with Tregs had an enhanced phagocytic capacity compared to those transferred with $CD4^+CD25^-$ T cells or no T cells. The enhanced phagocytic capacity was reversed by transfer with both cell types. Upon LPS stimulation, macrophages from SCID mice transferred with $CD4^+CD25^+$ Treg cells produced more IL-10 and less IL-12 than those from mice transferred with $CD4^+CD25^-$ T cells. The macrophages from mice receiving Tregs were also impaired in their antigen-presenting capacity and showed higher arginase activity and lower nitric oxide production compared to those from mice transferred with $CD4^+CD25^-$ T cells or no T cells. Together, this work suggests that Tregs modulate macrophages toward an "M2" phenotype, with the functional and phenotypic characteristics of the modulated cells closely resembling those seen in the previously mentioned *in vitro* studies of human Tregs and monocytes.

Figure 2 summarizes existing evidence on the cellular and molecular mechanisms via which $CD4^+$ effector vs. regulatory T cell subsets can affect the phenotype or function of monocytes and macrophages. As indicated in **Figure 1**, since much of the reported evidence comes from *in vitro* studies, some caution should be exerted when extrapolating these findings to an *in vivo* situation.

DYSREGULATED MONOCYTE/MACROPHAGE HOMEOSTASIS IN THE RHEUMATOID JOINT

The abundance of $CD14^+/CD68^+$ monocytes/macrophages in the rheumatoid joint suggests that these cells are recruited at a high rate, are long-lived or proliferative, and/or resistant to apoptosis. Evidence exists to support all these scenarios. Chemokines involved in monocyte recruitment, such as CCL2/MCP-1, CCL3/MIP-1α, and CCL5/RANTES, are readily detectable at the site of inflammation (96, 166), and blood monocytes from patients with RA express the corresponding chemokine receptors CCR1, CCR2, CCR3, and CCR5 (15). One study investigated the migration of labeled autologous $CD14^+$ blood monocytes, isolated by CliniMACS procedure, in RA patients using single photon emission computer tomography. A very small but specific fraction of 0.003% of re-infused monocytes was found to migrate to the inflamed joints, being detectable within 1 h after re-infusion (167). Interestingly, monocyte influx into the inflamed joint was not altered early after anti-TNF treatment (2 weeks post-treatment) even though disease activity was significantly reduced (168). The authors concluded that monocytes migrate continuously into the inflamed synovial tissue of RA, but at a slow macrophage-replacement rate, and that the rapid decrease in synovial macrophage numbers observed after anti-TNF treatment (23) cannot be explained by an immediate effect on monocyte influx.

Another contributing factor to the persistently high monocyte/macrophage presence in the rheumatoid joint is apoptosis resistance. Both RA peripheral blood monocytes and RA synovial monocytes/macrophages have been shown to be resistant to spontaneous cell death, agonistic Fas-antibody induced apoptosis, or responder T cell-mediated killing (28, 39, 40). Proposed underlying mechanisms include the enhanced expression of antiapoptotic molecules, such as FLIP and Mcl-1 in RA synovial

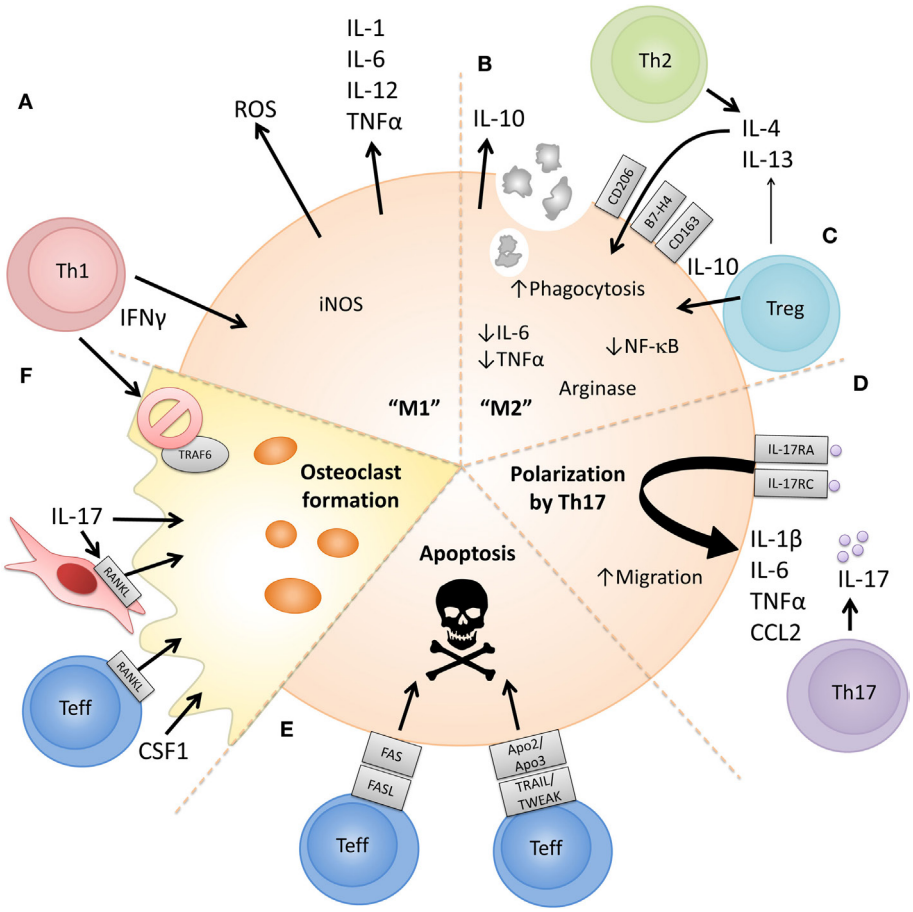

FIGURE 2 | Proposed cellular and molecular mechanisms via which CD4⁺ T cell subsets can polarize or modulate monocyte/macrophage function. **(A)** The prototypical Th1 cytokine IFNγ can polarize monocytes to an "M1" phenotype, promoting proinflammatory cytokine release, reactive oxygen species (ROS) production, and metabolism of arginine to nitric oxide [reviewed in Ref. (125, 127)]. **(B)** Prototypical Th2 cytokines IL-4 and IL-13 can polarize monocytes to an "M2" phenotype, characterized by expression of anti-inflammatory cytokines such as IL-10, expression of CD206, increased phagocytosis, and metabolism of arginine to orthonine and polyamine intermediates [reviewed in Ref. (125, 127)]. **(C)** CD4⁺CD25⁺ Tregs have also been shown to induce an alternatively activated or anti-inflammatory phenotype in monocytes in a cell-contact and soluble factor (IL-4, IL-13, and IL-10) dependent manner leading to reduced IL-6 and TNFα production, decreased NFκB activation and increased expression of IL-10, CD163, and B7-H4 (157, 158, 164, 165). **(D)** The prototypical Th17-associated cytokine IL-17 has been shown to increase levels of IL-1β, TNFα, and IL-6 released by macrophages (131, 132). IL-17 has also been suggested to be chemotactic for monocytes via ligation of IL-17RA/RC (133). **(E)** Evidence shows that activated CD4⁺CD25⁻ T cells can activate but also induce apoptosis in monocytes/macrophages. Both FAS/FASL- and TRAIL/TWEAK-dependent mechanisms have been proposed (39, 147, 148, 151, 152). **(F)** Activated RANKL-expressing Teff can drive osteoclast formation from monocytes when cultured in the presence of M-CSF (140). Th17-associated cytokines can upregulate RANKL on RA fibroblast-like synoviocytes and also directly induce monocyte-to-osteoclast differentiation (142, 143), while IFNγ has been shown to suppress osteoclast formation (141).

tissue (169, 170), the reduced expression of the proapoptotic Bcl-2 homology 3 (BH3)-only protein Bim (171), the increased production of TNFα and IL-1 which have antiapoptotic effects (172), and the increased presence of Tregs in the RA joint (173), which do not exert apoptotic effects on monocytes (39). Incubation with the anti-TNF drugs infliximab or adalimumab has been shown to increase apoptosis in healthy or RA peripheral blood monocytes, lending support to the role of TNF in regulating this process (28, 174). Conversely, defects in apoptosis pathway-associated molecules, such as occur in Fas-deficient (*lpr/lpr*) mice, lead to an increase in the number of circulating monocytes and an increase in the proinflammatory activity of peritoneal macrophages and the development of systemic autoimmune disease including lupus-like disease and inflammatory arthritis (175). A recent study showed that selective loss of Fas in myeloid cells was sufficient to induce SLE-like disease in mice (176). Together, these data illustrate that a dysregulation in monocyte/macrophage homeostasis may be an important contributing factor to chronic inflammatory joint disease.

In addition, recent advances in genetics have revealed an increasing number of susceptibility loci for RA (177), several of which may have relevance to monocyte/macrophage function or homeostasis, e.g., *CD40*, *TNFAIP3*, *IRAK1*, *TRAF1/TRAF6*, *IRF5* (178), and *RBPJ* (179). Future functional genomic studies may elucidate the exact role of these genes in RA and in monocytes/macrophages in particular.

Although knowledge regarding the phenotype and function of synovial macrophages is continuing to grow, it is much less clear what the origin of these cells is. Fate-mapping experiments in mice demonstrate that tissue-resident yolk sac-derived macrophages develop in a Myb-independent manner and can persist in adult mice independently of hematopoietic stem cells (180, 181). However, other work suggests that Myb-dependent fetal liver-derived monocytes also contribute to the pool of tissue-resident cells (182, 183). In addition, although self-renewing tissue-resident macrophage populations have been described (182–185), evidence suggests that recruited blood monocytes may play a role in replenishing the macrophage population during inflammation (186–188). The investigation of the origin and homeostasis of tissue macrophages remains an active field of research, and future work may be able to determine whether RA synovial macrophages are equivalent to these long-lived cells, or are continuously replaced by newly recruited peripheral monocytes.

TARGETING MONOCYTES/ MACROPHAGES IN RA

The findings discussed in this review indicate that selective targeting of monocytes/macrophages could have therapeutic benefit in RA [also reviewed in Ref. (5)]. This is supported by older data showing that treatment of severe RA with leukapheresis efficiently removed blood monocytes concomitant with a clinical response (189), and that depleting $CCR2^+$ monocytes using anti-CCR2 mAb could ameliorate collagen-induced arthritis, although the effects were dependent on the dose of mAb used (190). *In vitro*, selective elimination of human macrophages using toxin-conjugated Abs against CD64 resulted in reduced T cell proliferation and reduced TNFα production by synovial fluid mononuclear cells and synovial tissue explants (191). Bim-BH3 mimetic therapy could induce apoptosis in myeloid cells and suppress clinical severity of experimental arthritis (171). siRNA-based therapeutic approaches are also being developed in order to selectively target certain genes or pathways (192).

Additionally, given the importance of T cell-monocyte crosstalk in promoting inflammation (17, 137, 193), approaches that target T cell-monocyte interactions may have therapeutic benefit. Indeed, this may be one way via which drugs like abatacept (CTLA4-Ig) exert their clinical effect. Previously underappreciated mechanisms of existing therapeutics may contribute to their efficacy by (in)directly targeting monocytes/macrophages. For example, multiple studies have shown that blockade of IL-6 signaling via monoclonal antibody to the IL-6 receptor (tocilizumab) can boost Treg frequencies (194–198), but additional mechanisms of action may include reduction in $CD69^+CD14^+$ and $CD16^+CD14^+$ monocyte frequencies (197), induction of monocyte apoptosis, and inhibition of monocytic IL-6 mRNA expression (199). Despite the widespread uptake and high efficacy of TNFα inhibitor drugs in RA, the underlying mechanisms of action are not firmly established [reviewed in Ref. (200)]. TNF inhibitor drugs block signaling of the key monocyte/macrophage-derived cytokine TNFα but can also reduce production of other proinflammatory mediators, such as IL-1β, GM-CSF, IL-6 and IL-8, in synovial membrane (201–203). Furthermore, it has been reported that Treg function can be restored or enhanced following TNFα inhibitor therapy (111, 204), and we and others recently demonstrated that TNFα blockade in cocultures of antigen presenting cells and $CD4^+$ T cells favors development of an immunoregulatory phenotype in effector $CD4^+$ T cells by promoting expression of IL-10 (205, 206). We showed that following TNF blockade, a significant proportion of IL-17 expressing $CD4^+$ T cells coexpressed IL-10, which was biologically active and was able to modulate the phenotype of monocytes leading to reduced HLA-DR and CD40 expression and increased CD163 expression (205). Another postulated mechanism of anti-TNF drugs is via interaction with tmTNFα (28, 207). It was recently shown that production of cytokines and decoy receptors triggered by monocyte tmTNF crosslinking might provide a prognostic parameter for predicting therapeutic response to etanercept, suggesting a role for monocytic reverse signaling in the clinical efficacy of TNF inhibitors (208).

As previously discussed, monocytes/macrophages contribute to bone erosion in RA via differentiation into osteoclasts. Inhibiting osteoclastogenesis therefore presents a key target for therapeutic intervention, since the presence of bone erosions represents irreversible structural damage, associated with loss of joint function and poor quality of life. TNFα inhibitor drugs inhibit radiological progression, even in the absence of clinical response, via direct inhibitory effects on osteoclast differentiation and activity (209). Binding of CTLA4 to CD80/86 on monocytes provides a potent signal to inhibit differentiation into osteoclasts; accordingly abatacept was recently shown to inhibit osteoclastogenesis in human peripheral monocytes (210). A phase II clinical trial found that twice-yearly injections of the anti-RANKL drug denosumab inhibited progression of bone erosion in RA patients with active erosive disease but demonstrated no effect on disease activity (211). Similarly, RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis but display inflammation that is clinically and histologically similar to wild type (212). These data suggest that targeting osteoclastogenesis may have the potential to reduce structural joint damage, but that RANKL blockade alone may be ineffective at controlling the underlying inflammatory process. As an alternative strategy to inhibit osteoclastogenesis, targeting M-CSF receptor (M-CSFR, also known as CSF1R, c-FMS, and CD115) has been shown in various animal models of autoimmune arthritis to reduce both joint inflammation and bone destruction (213–216) However, in some models, while bone erosion is reduced, inflammation is unaffected by M-CSFR antibody blockade (215) or c-FMS kinase inhibition (217). Effective targeting of the M-CSFR pathway in RA may require blocking signaling of both its ligands, M-CSF and IL-34. Several clinical trials are currently investigating antibodies or small molecules targeting the M-CSFR pathway in RA and other indications [reviewed in Ref. (218)].

Epigenetic control of immune-mediated processes is a growing field of study with the potential to unveil mechanisms of immune regulation which may be amenable to therapeutic

intervention. Bromodomain and extraterminal (BET) inhibitors have recently emerged as a promising approach to treat cancer but are also being investigated in the context of inflammatory disease. Blocking the recruitment of BET proteins to acetylated histones inhibits BET-mediated transcriptional activity. BET inhibitors have been shown to suppress expression of proinflammatory cytokines and chemokines in LPS-stimulated bone marrow-derived macrophages *in vitro* (219, 220). *In vivo* BET inhibition resulted in reduced Th17 differentiation, suppression of established Th17 responses, and protection against pathology in collagen-induced arthritis (221). A recent study found that BET inhibition suppressed cytokine-induced transcription in primary human monocytes in a gene-specific manner, without affecting JAK-STAT signaling. Instead, BET inhibition reduced recruitment of transcriptional machinery to the CXCL10 promoter and an upstream enhancer (222). In future studies, global approaches, such as genome-wide profiling, may identify additional functional pathways that are amenable to BET-mediated transcriptional regulation in monocytes/macrophages.

Additional therapeutic approaches under development for RA include proteasome inhibitors, such as bortezomib (223). Conflicting data on the effects of bortezomib on bone resorption are reported in different animal models of RA (224, 225). In human cells, bortezomib appears to inhibit osteoclastogenesis (226, 227). Selective inhibition of a subunit of the immunoproteasome (a class of proteasome primarily found in monocytes and lymphocytes) was shown to inhibit production of IL-23, TNFα, and IL-6 by LPS-activated monocytes and to reduce IFNγ and IL-2 production from anti-CD3/CD28-activated T cells (228). These data were generated using cells from healthy donors, but similar results were observed using PBMC from three RA patients. In mouse models of RA, immunoproteasome inhibition ameliorated disease and also blocked IL-23 production from activated monocytes.

Protein kinase inhibitors are another class of small molecule therapeutics gaining attention for the treatment of immune-mediated diseases. Tofacitinib, the first Janus kinase (JAK) inhibitor to be developed for RA was approved by the FDA in 2012. To date, most studies have focused on the effects of JAK inhibition in T cells

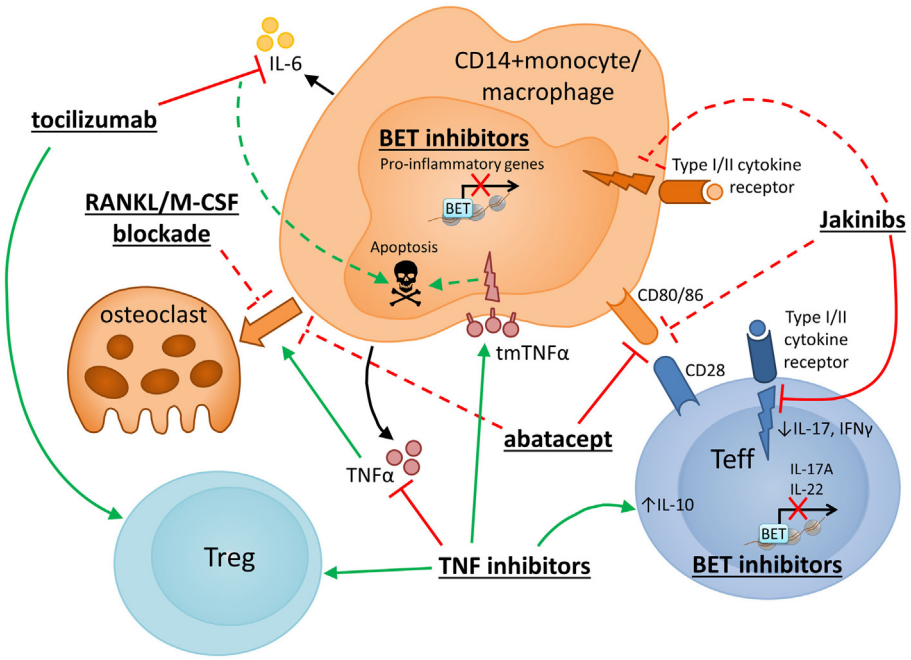

FIGURE 3 | Therapeutic strategies to target monocytes/macrophages in RA. Several novel strategies for drug intervention in RA, as well as currently used therapeutics, have potential mechanisms of action that target monocyte/macrophages or their interaction with CD4+ T cells. Selected therapies are presented with particular reference to their reported effects on monocyte/macrophage function and CD4+ T cell interactions. Red and green lines indicate inhibition and promotion, respectively, while dashed lines indicate novel or putative mechanisms that may require further confirmation. TNF inhibitor drugs block signaling of the key monocyte/macrophage-derived cytokine TNFα and have been shown to promote increased IL-10 expression in CD4+ T cells (205, 206), enhanced or restored Treg function (111, 204) and anti-osteoclast effects (209), and may also exert anti-inflammatory/proapoptotic effects on monocytes via "reverse signaling" through tmTNFα (28, 208). Blockade of IL-6 signaling (via monoclonal antibody to the IL-6 receptor, tocilizumab) reportedly boosts Treg frequencies (194–198) and has been suggested to inhibit monocyte IL-6 mRNA expression and induce monocyte apoptosis (199). Another widely used RA drug, abatacept (CTLA4-Ig), targets the interaction between monocytes and T cells, specifically impairing T cell costimulatory signals via CD80/CD86. Abatacept has also been reported to inhibit monocyte differentiation into osteoclasts (210). Several other approaches are currently under development to specifically target osteoclastogenesis, including blockade of RANKL (211) or M-CSF (218). Janus Kinase (JAK) inhibitors (Jakinibs) target JAK/STAT-mediated cytokine signaling in T cells and possibly also in macrophages (231, 232) and may reduce monocyte-derived DC costimulatory capacity (230). Bromodomain and extraterminal (BET) inhibitors are also under consideration for the treatment of inflammatory disease. Efficacy has been shown in collagen-induced arthritis where BET inhibition reduced Th17 responses (221). Reduced transcription of proinflammatory genes has been described in human monocytes (222) and mouse macrophages (219, 220) following *in vitro* exposure to BET inhibitors.

with relatively few data on the consequences in monocytes/macrophages. A recent report found that tofacitinib did not directly affect RA synovial monocytes, but that tofacitinib-exposed $CD4^+$ T cells demonstrated reduced proliferation, impaired IL-17 and IFNγ expression, and generated conditioned medium that when added to $CD14^+$ monocytes could inhibit IL-8 production (229). Others showed that tofacitinib reduced CD80/CD86 expression and therefore the T cell costimulatory capacity of human mo-DCs (230). In addition, tofacitinib and ruxolitinib, another JAK inhibitor drug, were shown to effectively suppress inflammatory responses of blood-derived and RA synovial macrophages (231). Tofacitinib also efficiently suppressed development of arthritis in a K/BxN serum transfer model. A cautionary observation was that JAK inhibition could enhance osteoclast differentiation *in vitro*. Also, in isolated mouse macrophages, both tofacitinib and ruxolitinib were found to block the IL-10-mediated feedback inhibition of cytokine transcription, thereby increasing LPS-induced cytokine production (232). These data indicate that JAK inhibitor drugs do have the capacity to modulate monocyte/macrophage function, but the exact cellular mechanisms that mediate the clinical efficacy of these therapeutics are still under investigation.

Figure 3 shows an overview of how drugs currently used to treat RA, as well as therapeutics under development, may target monocytes/macrophages or their interaction with $CD4^+$ T cells, to intervene in the underlying inflammatory and erosive disease processes.

SUMMARIZING CONCLUSION

In summary, there is strong evidence for a contributing role of both monocytes/macrophages and $CD4^+$ T cells in RA. In addition to directly promoting local inflammation by secreting proinflammatory mediators, synovial monocytes/macrophages secrete chemokines that can attract and maintain $CD4^+$ T cells in the joint. A growing evidence base suggests that activated (subsets of) monocytes can influence $CD4^+$ T helper cell polarization toward Th1/Th17. Through their cytokine production monocytes/macrophages may also impact on frequencies and function of regulatory $CD4^+$ T cells. Conversely, $CD4^+$ effector T cells can activate, polarize, as well as kill monocytes and macrophages and may influence monocyte chemotaxis, while $CD4^+$ Tregs can exert immunomodulatory effects on these cells, thereby enhancing their survival and inducing an anti-inflammatory state in monocytes/macrophages. To inform future research, refining the phenotypic characterization of monocyte/macrophage subpopulations by validating additional phenotypic markers would facilitate further investigation of their involvement in inflammatory conditions, such as RA. Given that the complexity of monocyte/macrophage phenotypes is not well understood or necessarily reflected in animal models or immortalized cell lines, studies of human primary monocytes and macrophages are essential to understand the contribution of these cells to RA pathogenesis. Improved knowledge regarding the origin of synovial monocyte/macrophages could lead to a better understanding of the roles of tissue-resident macrophages in the RA joint. Finally, further investigation into the direct interactions between tissue-resident $CD4^+$ T cell subsets and macrophages may elucidate how effector T cell responses are generated *in situ* as well as how macrophage responses can be regulated differentially by $CD4^+$ effector and Tregs. Several existing RA therapeutics are known to impact on the crosstalk between monocytes and $CD4^+$ T cells. An increased understanding of how the interactions between these cell types may contribute to immune pathology will benefit the development of new and improved therapeutic strategies.

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Mutual interaction of basophils and T cells in chronic inflammatory diseases

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Basophils are, together with mast cells, typical innate effector cells of allergen-induced IgE-dependent allergic diseases. Both cell types express the high-affinity receptor for IgE (FcεR1), release histamine, inflammatory mediators, and cytokines following FcεR1 cross-linking. Basophils are rare granulocytes in blood, lymphoid, and non-lymphoid tissues, and the difficulties to detect and isolate these cells has hampered the study of their biology and the understanding of their possible role in pathology. Furthermore, the existence of other FcεR1-expressing cells, including professional Ag-presenting dendritic cells, generated some controversy regarding the ability of basophils to express MHC Class II molecules, present Ag and drive naïve T cell differentiation into Th2 cells. The focus of this review is to present the recent advances on the interactions between basophils and peripheral blood and tissue memory Th1, Th2, and Th17 cells, as well as their potential role in IgE-independent non-allergic chronic inflammatory disorders, including human inflammatory bowel diseases. Basophils interactions with the innate players of IgE-dependent allergic inflammation, particularly innate lymphoid cells, will also be considered. The previously unrecognized function for basophils in skewing adaptive immune responses opens novel perspectives for the understanding of their contribution to the pathogenesis of inflammatory diseases.

Keywords: basophils, memory T cells, Th17 cells, Th2 cells, inflammatory bowel diseases

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Basophils in Type 2 Immune Responses

Basophils represent <1% of all blood leukocytes. Together with mast cells, they are regarded as typical effector cells of IgE-dependent allergic inflammation (1, 2). Because both types of cells express high levels of the high-affinity receptor for IgE (FcεR1), and rapidly release histamine and inflammatory mediators upon cross-linking of FcεR1 by IgE-allergen complexes, basophils were long considered as redundant granulocytes lacking unique functions (3). Until recently, the investigations of the functional properties of basophils have been hampered by the difficulty of detecting and purifying these rare cells in blood and tissues from mice and humans (4). Despite the development of transgenic mice (IL-4-eGFP, Basoph8) that allow the tracking or transient depletion of basophils *in vivo*, conflicting results were generated regarding the antigen-presenting function of basophils and their ability to trump DCs in the priming of Th2 responses (5–8). However, it was reported that cooperation between highly purified murine basophils and DCs, isolated from blood or lungs, is required to induce *in vitro* Th2 cell differentiation (9, 10). In this context, DCs ensure naïve T cell proliferation and basophils provide IL-4 to drive Th2 polarization. As a consequence,

a careful exclusion of trace amounts of MHC class $II^+Fc\varepsilon R1^+$ DCs from basophils preparation is mandatory to draw valid conclusions about any yet unrecognized *in vitro* function of these rare cells, especially in human studies (11).

Despite their low frequency in blood, increased numbers of basophils were detected in the tissues of several IgE-dependent allergic diseases that include allergic rhinitis, atopic dermatitis, and asthma (2, 12). How basophils are attracted to the site of allergen challenge remains to be clarified. Histamine and PGD2, which are produced by mast cells, as well as IL-3 secreted by activated T cells have been proposed to play crucial roles in the recruitment of basophils to tissues, since basophils express histamine receptors, CRTH2 (i.e., PGD2 receptor) and IL-3 receptors (13–15). In tissues, basophils may interact with resident memory T cells, as demonstrated by two-photon microscopy (8). Hence, prolonged contacts between basophils and T cells occurred in the inflamed lungs but not in the mediastinal lymph nodes (LNs) of parasite-infected mice. The same study also revealed that activated T cells induced IL-4 secretion by basophils in affected lungs. Optimal IL-4 production by basophils required a direct cell/cell contact, as well as the presence of IL-3, a cytokine that promotes expansion and survival of basophils. Conversely, *in vitro* interactions between pulmonary basophils and lung $CD4^+$ T cell promoted IL-4-dependent T cell survival and amplified release of Th2 cytokines, without inducing memory T cell expansion (9). In experimental asthma, transfer of lung basophils worsens ongoing Th2 responses by increasing airway inflammation and local IL-4 and IL-13 expression (9). Furthermore, human basophils increase IL-4 expression in effector memory T cells *in vitro*. Notably, responding T cells mainly included $CRTH2^+$ cells, corroborating the *in vivo* and *in vitro* data seen in mice. In addition to Th2 cells that produce IL-4, IL-5, and IL-13, double IL-4- and IL-17-expressing memory $CD4^+$ T cells were detected in severe asthmatic patients and in lungs of mice developing experimental asthma (16). In contrast, human basophils were not reported to promote the generation of Th2/Th17 double positive cells. The basophil enhancing effect on memory Th2 responses was partially contact dependent, but did not involve OX40/OX40L, CTLA4/B7family, and CD2/LFA3 interactions. Because IL-3-stimulated basophils express RANKL and activated Th cells may express RANK (17, 18), we speculate that RANK–RANKL pairs of molecules may represent other potential candidates involved in basophil-T cell interactions.

Cellular aggregates made of basophils and memory $CD4^+$ T cells were detected in the dermis of patients with atopic dermatitis and in a mouse model of TSLP-mediated contact dermatitis, underlying the key role of basophils in T cell-mediated skin allergic disorders (19). However, basophils may regulate type 2 inflammatory responses by interacting with cells other than T cells. Indeed, innate immune cells, such as macrophages, innate lymphoid type 2 (ILC2), and eosinophils, are major contributors of allergic lung and skin inflammatory responses (20, 21). Moreover, keratinocyte-derived TSLP activates basophils that results in local recruitment, activation, and proliferation of ILC2, which is mediated by basophil-derived IL-4 (19). This mechanism initiates experimental atopic dermatitis and appears essential for the development of food allergy induced by the application of food antigen to inflamed skin (22). Furthermore, the interactions of activated basophils with ILC2, fibroblasts, and/or endothelial cells regulate recruitment of eosinophils in experimental models of contact dermatitis and allergic asthma (21, 23).

Taken collectively, these finding indicate that, although basophils do not appear to initiate IgE-dependent allergic disease, the interaction between basophil and memory T cell in inflamed tissues may be bidirectional, thus contributing to the exacerbation of chronic allergic airway inflammation at late stages. In support of this, asthmatic patients are successfully treated by administration of Omalizumab, a monoclonal antibody to IgE that prevents IgE binding to $Fc\varepsilon R1$ and regulates basophil homeostasis (24). It has been suggested that its therapeutic efficacy largely result from its effect on basophils, since these cells have a much shorter life than mast cells. In fact, the improved clinical outcome of allergic patients following Omalizumab therapy was associated with a reduction in circulating basophil numbers (24).

Basophils in IgE-Independent Th Responses

Basophils were historically associated exclusively with IgE-dependent allergic disorders. However, TSLP-activated basophils induce and perpetuate experimental eosinophilic esophagitis (EoE), which may be triggered in the absence of IgE and mast cells (25). Notably, the circulating number of basophils is increased in patients with EoE. Similarly, increased numbers of basophils are observed in blood of patients with chronic inflammatory bowel diseases (IBD) (26). Basophilia is found in both Crohn's disease (CD) and ulcerative colitis (UC), the two main chronic relapsing IBD types that are associated with Th17/Th1 and Th17 or Th2 cells, respectively (27). Strikingly, increased percentages of basophils, but not of mast cells are found in the inflamed relative to the non-inflamed colonic mucosa in both CD and UC patients (26). In contrast, basophils are not detected in the intestinal mucosa of non-IBD individuals. The accumulation of basophils in the inflamed colons of IBD patients suggests that these rare cells contribute to disease pathogenesis by influencing pathogenic T cell responses in tissues.

Indeed, similar to their ability to amplify human memory Th2 responses, basophils promote memory Th17 responses *in vitro* (11). Blood basophils as well as basophils isolated from inflamed colonic mucosa or the mesenteric LNs of IBD patients favor the emergence of memory $IL\text{-}17^+$, $IL\text{-}17^+/IFN\text{-}\gamma^+$ but not $IFN\text{-}\gamma^+$ single positive Th cells (26). Activation of $CD4^+$ T lymphocytes generates functionally distinct antigen-experienced T cells, namely, effector memory $CD62L^{low}CCR7^-$ (T_{EM}) that migrate to peripheral tissues and central memory $CD62L^{high}CCR7^+$ (T_{CM}) T cells, which retain the ability to enter LNs (28). Thus, basophils activated by either IL-3, IL-33, or TSLP increase Th17 and Th17/Th1 responses by IL-2-stimulated T_{EM} in the absence of APC, as well as by TCR-stimulated T_{CM} isolated from blood, which mimic memory T cell activation in mucosa and lymphoid tissues, respectively (11). More specifically, basophils promote cytokine production by autologous T_{EM} cells in a contact-independent manner that involves the ERK1/2-pathway. Basophil-derived histamine partially increases IL-17 expression through H_2 and H_4, but not H_1 receptors.

Interestingly, histamine alone cannot replace basophils in their pro-Th17 activity.

Basophils also enhance IL-22 production by T_{EM} cells but not T_{CM} (11). However, Sharma et al. (29, 30) reported that human basophils lack the ability to drive IL-22 or IL-17 memory $CD4^+$ T cell responses. The reasons for the apparent discrepancies might at least result from the analysis of unfractionated stimulated $CD45RO^+CD25^-$ $CD4^+$ T cells, which mainly comprises T_{CM}, in co-culture with IL-3-activated basophils. Also, the purity of basophils was $94 \pm 5\%$ in the latter studies (29, 30), in contrast to >99% purity in the former studies (11, 26), further highlighting the importance of assessing highly purified basophil preparations.

Lymphoid tissues represent the major site of memory T cells in the body relative to circulating pool (31). It is therefore essential, whenever feasible in humans, to assess the functions of T cells that are found in tissues. Basophils enhance Th17 and Th17/Th1 responses by T_{CM} and T_{EM} CD4 T cells isolated from mLNs of IBD patients (26). Furthermore, a small number of tissue memory T_{EM} express CCR7 in inflamed tissues and $CCR7^+$ T_{EM} cells are prone to exit the tissues and re-circulate (32, 33). Notably, $CCR7^+$ T_{EM} cells are the preferential targets of basophils for enhancing Th17 responses (26). A recent study demonstrates that CCR7 expression controls intestinal Th17 and Th1 balance in a model of TNF-α driven Crohn's-like ileitis (34). The CCR7-deficient mice or mice treated with anti-CCR7 mAb develop an exacerbated ileo-colitis, which is associated with retention of Th effectors in intestinal and extra-intestinal tissues, suggesting that recirculation of $CCR7^+$ T_{EM} contribute to intestinal homeostasis.

Immunoregulatory Activity of Basophils

Although basophils clearly amplify allergic skin and airway inflammation, several studies showed that these rare cells might also exert anti-inflammatory activities in the context of autoimmune disease, contact dermatitis, as well as colitis. In a mouse model of arthritis, IL-4 production by IL-33-activated basophils was found to enhance expression of the inhibitory Fcγ Receptor (FcγRIIb) on inflammatory macrophages and mediates the immunosuppressive response elicited by injection of intravenous immunoglobulin (IVIG) (35). A recent report indicates that upon IL-33 and IgE triggering, human basophils down-modulate monocyte activation *in vitro* (36). Furthermore, basophil-derived IL-4 significantly attenuates the intensity of skin inflammation by mediating the differentiation of inflammatory monocytes into alternatively activated M2 macrophages that are endowed with an immunosuppressive and anti-inflammatory activity (37).

Basophils display immunoregulatory activity by enhancing the suppressive activity of FOXP3-expressing regulatory T cells (Treg) *in vivo* and *in vitro* (38). In a mouse model of contact hypersensitivity, basophils mediate UVB-induced immune suppression by increasing Treg function and this immunosuppressive effect is reduced in amphiregulin (AREG)-deficient mice (39). Indeed, basophils express AREG, an epidermal cell growth factor-like cytokine that enhances Treg function (40). Finally, the *in vivo* role of basophils in pathologies affecting the gastrointestinal tract remains to be clarified. A direct pathogenic role has been proposed for murine basophils in experimental EoE, as well as in allergen-induced colitis in a humanized mice model (25, 41). On the other hand, basophils depletion aggravates colitis induced by adoptive transfer of T cells in lymphopenic mice (42).

Concluding Remarks

Overall, the *in vivo* function of mucosal or lymphoid human basophils warrants further investigations in allergic diseases, CD, and UC. Basophils accumulate in inflamed tissues in IgE-dependent, as well as IgE-independent inflammatory disorders whereby they may directly interact with memory T cells to augment Th2, Th17, and Th17/Th1 effector responses. The accumulation of basophils in tissues indicates that they may contribute to the aggravation and flare up of the disease. Conversely, their increased numbers may as well reflect a negative regulatory feedback mechanism to dampen inflammation. Nonetheless, basophils represent an attractive therapeutic target for patients with chronic inflammatory disorders. This opens therapeutic avenues by targeting basophils and histamine using the clinically safe non-degranulating anti-IgE human monoclonal antibody (Omalizumab) (43) or selective anti-histamine receptor drugs (44).

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How neutrophils shape adaptive immune responses

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Neutrophils are classically considered as cells pivotal for the first line of defense against invading pathogens. In recent years, evidence has accumulated that they are also important in the orchestration of adaptive immunity. Neutrophils rapidly migrate in high numbers to sites of inflammation (e.g., infection, tissue damage, and cancer) and are subsequently able to migrate to draining lymph nodes (LNs). Both at the site of inflammation as well as in the LNs, neutrophils can engage with lymphocytes and antigen-presenting cells. This crosstalk occurs either directly via cell–cell contact or via mediators, such as proteases, cytokines, and radical oxygen species. In this review, we will discuss the current knowledge regarding locations and mechanisms of interaction between neutrophils and lymphocytes in the context of homeostasis and various pathological conditions. In addition, we will highlight the complexity of the microenvironment that is involved in the generation of suppressive or stimulatory neutrophil phenotypes.

Keywords: inflammation, immune-regulation, neutrophil, myeloid-derived suppressor cells, immune-paralysis, neutrophil phenotypes, T-cells

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Introduction

Neutrophils are particularly known for their potent anti-microbial functions (1). This notion is enforced by various congenital neutrophil deficiencies, which show marked clinical phenotypes characterized by enhanced susceptibility to bacterial and fungal infections (2, 3). Infections, sterile inflammation, and other non-chronic challenges to the immune system are characterized by a rapid influx of neutrophils into the affected tissue (4). These neutrophils respond to chemo-attractants and adhesion molecules expressed on endothelial cells, and their main function is to clear infections and/or debris. In addition, they influence inflammatory responses through interactions with various cells of the immune system, such as antigen-presenting cells (APCs) and lymphocytes (5, 6). This has been observed in both murine models and in *ex vivo* studies with isolated cells from humans. Although neutrophils have long been considered to be composed of a homogenous population, an increasing body of literature supports the presence of multiple neutrophil phenotypes in cancer and inflammation (7–10). This heterogeneity can be induced by specific differentiation programs in the bone marrow or orchestrated by extracellular signals derived from inflammatory tissue (e.g., cytokines, bioactive lipids, or chemokines) (11, 12). The contribution of distinct neutrophil populations to immune suppression has not been resolved. In addition, in murine models and some human studies, clear distinctions were suggested between neutrophils and granulocytic myeloid-derived suppressor cells (G-MDSCs). These issues have been reviewed in detail (13, 14). This review will focus on the location and relevant diseases in which both (suppressive) neutrophils and G-MDSCs modulate

adaptive immune responses and the mechanisms behind this process.

Location of the Interaction Between Neutrophils and Lymphocytes

Site of Inflammation – Bystander Response

The early phase of infection is characterized by an influx of neutrophils and monocytes, which precedes the development of an antigen-specific response. Simultaneously, small numbers of T-cells are recruited into the infected tissue. Some of these T-cells can be activated and proliferate "in situ" in response to antigen presentation by myeloid cells (15). In addition, inflammatory cytokines cause proliferation and activation of non-specific T-cells in the profoundly pro-inflammatory microenvironment. This process has been coined the "bystander response" and was first seen in viral infections (16). It has recently been suggested that this bystander response contributes to early pathogen control in mice by enabling bystander memory T-cells to recognize and eliminate micro-organisms, such as *Listeria monocytogenes* infected cells in a NKG2D-dependent manner (17).

It is conceivable that a large and uncontrolled bystander response might predispose for auto-immunity and self-reactivity through the proliferation of self-reactive T-cells (18). It is tempting to speculate that neutrophils are involved to limit and control this bystander T-cell response as the timing of massive neutrophil tissue infiltration and the bystander response coincide.

Lymph Nodes and Primary Lymphatic Tissue

Neutrophils are found both in LNs and spleen particularly under inflammatory conditions (19–24). Dynamic imaging studies have shown that neutrophils are recruited to and form swarms in infected LNs in mice (25, 26). In addition, neutrophil migration to afferent LNs in response to tissue inflammation has been shown in various murine models (19–24).

There are two possible routes for neutrophils to enter LNs, via blood vessels or via afferent lymphatics (**Figure 1**). The first route requires exiting the circulation via high endothelial venules (HEVs). This mechanism is controversial, as human neutrophils seem to lack the expression of CCR7, a receptor for CCL21 and required for lymphocyte exiting through HEVs (27). Nonetheless, it has been shown in a murine model of ovalbumin-induced inflammation that neutrophil homing to LNs via the HEV takes place and requires integrins αMβ2 (MAC-1), αLβ2 (LFA-1), and L- and P-selectin (19). In LN-draining inflammatory tissue, additional chemokines and cytokines could orchestrate the attraction of neutrophils via HEVs. This has also been shown in tumor-draining LNs, when the tumor was subjected to photodynamic therapy. This treatment induces additional sterile inflammation. In this model, neutrophils are recruited to tumor-draining LNs via the HEV in an IL-17-dependent manner (20).

Neutrophil migration to LNs via afferent lymphatics has been observed in various murine models of infections, vaccinations, and cancer and seems to depend on MAC-1 and CXCR4 expression on neutrophils (19, 22) (see **Figure 1**). The area in the LN that is occupied by neutrophils will determine which cells they encounter, and how they can influence subsequent immune responses. Neutrophils have been reported to occupy the medullary region and interfollicular zone (23). Neutrophils migrate to these areas in the LN during infection with *Staphylococcus aureus*. There they exhibit short- and long-term interactions with B-cells, thereby inhibiting production of antibodies, and thus humoral responses (23). Furthermore, neutrophil B-cell interactions have also been observed in primary lymphoid organs in various mouse models. In the marginal zone of the spleen, neutrophils were observed to contribute to antibody production and class switching by activating B-cells by producing BAFF, APRIL, and IL-21 (24). In this study, evidence was provided for the existence of a similar population of neutrophils that modulate B-cell responses in humans. However, this remains controversial as in a subsequent study, no splenic neutrophil–B cell interactions could be observed in humans (28). It seems firmly established, at least in various murine models that neutrophils enter primary and secondary lymphatic sites during the immune response evoked by various inflammatory stimuli. Apart from regulating immunity in inflamed tissue, they may play a role in regulating immune responses at these privileged immune sites (see below).

The Role of Neutrophils in Controlling Immune Responses Evoked by Bacterial and Viral Infections, Sterile Inflammation and Cancer

Viral Infections

Acute viral infections, such as influenza, are ideal models to study cellular kinetics during the immune responses and the putative modulating effects of neutrophils hereon. Influenza and RSV infections are characterized by an early large influx of neutrophils in the lung tissue followed several days later by a virus specific $CD8^+$ T-cell response (29–31). Neutrophils might facilitate the development of this antigen-specific response as they are able to serve as APCs in influenza infection in mice (31, 32). Such antigen presentation by influenza-infected neutrophils has been demonstrated and was found to be mediated by MHC-I and co-stimulatory molecules CD80 and CD86, which leads to induction and activation of anti-viral responses of $CD8^+$ T-cells (32). On the other hand, it is tempting to speculate that neutrophils may also inhibit T-cell responses in viral infections by inhibiting T-cell proliferation and inducing T-cell apoptosis. The mechanisms of this suppression will be discussed below and involve reactive oxygen species (ROS), arginase-I (ARG), and PD-L1. This has also been found in other inflammatory scenarios (33–35). The role of immune suppression by neutrophils *in vivo* in murine models of viral infections has not been adequately experimentally verified, but may be deduced from the fact that pathology in mice is T-cell dependent and that depleting neutrophils often results in an exaggerated response and pathology (31, 36). In chronic viral infections, such as human hepatitis B virus (HBV), it has recently been shown that recruitment of neutrophils to the liver limits immune pathology through inhibiting bystander and HBV specific T-cells in an arginase-dependent way, thus protecting the host from immune-mediated damage (37). These

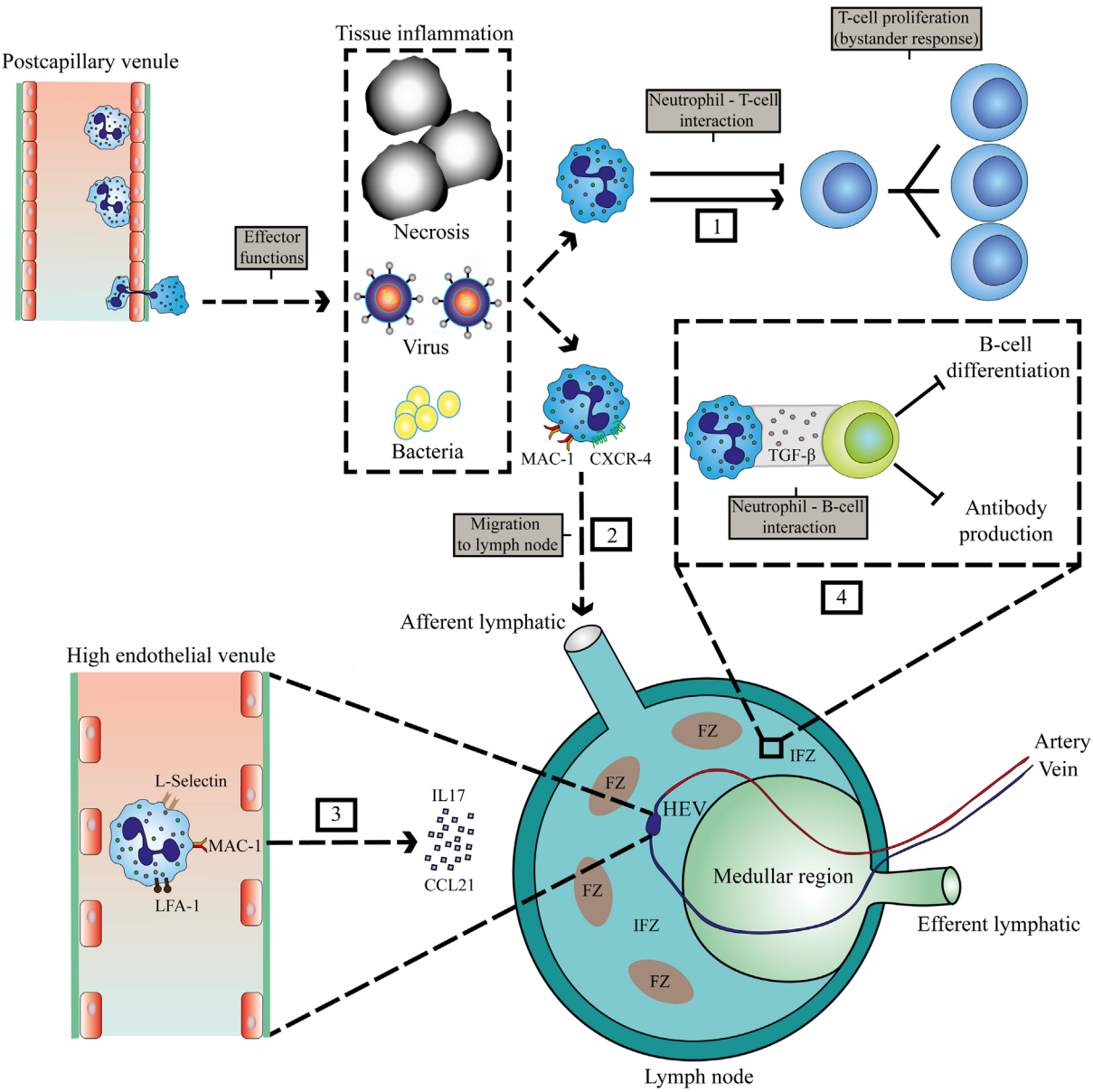

FIGURE 1 | Localization of interaction between neutrophils and lymphocytes at sites of inflammation and in lymphoid tissue. Invasion of pathogens or inflammation due to necrosis and cancer leads to extravasation of neutrophils. (1) Interaction of neutrophils with T-cells in the peripheral tissue. (2) MAC-1 and CXCR4-dependent migration of neutrophils to LNs via afferent lymphatics during inflammation (22). (3) IL17 and CCL21-mediated migration of neutrophils via HEVs to the LN that requires MAC-1, LFA-1, and L-selectin (19, 20). (4) Inhibition of humoral responses by neutrophils in the IFZ and medullar region (23). HEV, high endothelial venule; FZ, follicular zone; IFZ, interfollicular zone.

neutrophils were isolated from the PBMC fraction and were termed G-MDSC (13).

Bacterial Infections

As in viral infections, bacterial infections are associated with a large recruitment of neutrophils. The pivotal difference with viral infections is that phagocytosis and killing of bacterial targets by phagocytes is the principle mechanism of pathogen eradication (1). Ineffective killing of phagocytosed bacteria results in intracellular (phagosomal) survival and can lead to pathogen shuttling to distant sites and LNs (38, 39). Recently, it has been shown that neutrophils from mice infected with *S. aureus* migrated to the draining LNs and limited humoral responses through direct cellular interactions with B-cells. These direct cellular interactions were also found for neutrophil–T-cell interactions in humans (40). Kamenyeva et al. suggested that suppression of antibody production by neutrophils *ex vivo* was dependent on TGF-β (23). However, the

contribution of the reduced humoral response to pathogen load was not assessed.

As mentioned above, in the early course of infection, large numbers of neutrophils are recruited to the affected tissue where modulation of T-cell responses most likely occurs with early recruited T-cells. These early lymphocytes mainly belong to the family of γδ-T-cells (41). These γδ-T-cells are thought to play a role in early pathogen clearance through production of cytokines and their crosstalk with innate immune cells (41). Neutrophils play an important role in the initiation of these γδ-T-cell responses. Phagocytosis of bacteria enables neutrophils to activate γδ-T-cells and induce their proliferation (42). This is dependent on (1) the microbial metabolite (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), which neutrophils release after phagocytosis of bacteria and (2) the presence of monocytes for cellular contact-induced activation (42). However, other human studies have revealed the ability of neutrophils to suppress γδ-T-cells activation, possibly providing a negative-feedback mechanism (43, 44).

The early instruction of γδ-T-cells in humans by neutrophils parallels the role of these innate immune cells in the early instruction of T-cell responses in mice. In a murine model of *Legionella pneumophila*, neutrophils from pulmonary tissue are pivotal for the development of a TH1 response. In this model which resembles human disease, neutrophils were depleted by neutrophil-specific antibody Ly-6G, and this led to more TH2 skewing and more disease (45).

T-cell instruction, activation, and proliferation mostly require antigen-presenting cells, such as dendritic cells, B-cells, and macrophages. Neutrophils have been shown to both negatively and positively affect antigen presentation by these APCs under different conditions. This has extensively been reviewed previously (46, 47). Neutrophils may simply affect the amount of available antigen by phagocytosis, and thus limit antigen presentation by professional APCs (48). Alternatively, neutrophils might function as APCs themselves (49, 50). This possibility is supported by several studies showing the expression of MHCII and co-stimulatory molecules on neutrophils under different clinical conditions (51–53).

Disseminated Bacterial Infections (Sepsis)

Severe bacterial infections can result in systemic dissemination of bacteria that can lead to severe clinical conditions, such as sepsis and septic shock. These conditions are characterized by severe systemic inflammation, which can result in severe inflammatory damage to the host when not properly controlled. Immune inhibitory mechanisms have evolved in order to prevent this exaggerated inflammatory response (54). The specific role of neutrophils in this immune suppression has not been adequately studied. This is a challenging research question as depletion or inhibition of neutrophil functions with the purpose of studying their anti-inflammatory role has profound impact on bacterial clearance. Identification of suppressive mechanisms that do not influence pathogen clearance and neutrophil-specific murine knockout models may aid in answering this question.

In humans, evidence has accumulated that neutrophils might contribute to the immune suppression seen in sepsis. Neutrophils in septic-shock patients express ARG and suppress T-cell functions, probably through depletion of L-arginine as detailed below (55). Immune suppression in sepsis can be at least in part attributed to the PD-1/PD-L1 axis that is involved in control of apoptosis in T-cells (56). Interestingly, expression of PD-L1 on tissue neutrophils has also been shown during chronic inflammation (57). The expression of PD-L1 on human neutrophils was found to be induced by the TH1 cytokine, interferon-γ, *in vitro* (35).

Sterile Inflammation/Vaccination

Neutrophils also play a role in the fine tuning of inflammation under sterile conditions. Many studies have been performed in ovalbumine (OVA)-induced immune responses in murine models. The OVA models are used as vaccination and allergy models and are useful to study the development of adaptive immune responses. The role of neutrophils in the OVA model follows the above-described findings in microbial models. The cells seem to effectively cross-prime $CD8^+$ T-cells in an MHCI-dependent manner (58). They function as APCs or influence the capacity to present antigens by professional APCs (59, 60). For instance, dendritic cells have been shown to take up antigens acquired from phagocytosed apoptotic neutrophils (61).

These examples show that neutrophils can increase antigen presentation as APC or by delivering antigen to APCs. On the other hand, there are reports that neutrophils decrease the level of antigen presentation by APCs through an unknown mechanism during brief cellular interactions (48). These findings show that it is difficult to predict in which circumstances neutrophils stimulate or suppress antigen presentation even when very similar and well-controlled models are used.

Cancer

There is a large body of literature, which shows a heterogeneous population of myeloid cells characterized by their potential to inhibit adaptive immunity, and thus anti-tumor immune responses (14). These myeloid-derived suppressor cells consist of mononuclear cells and neutrophils in different stages of maturation. G-MDSCs facilitate tumor growth in various murine models through suppression of $CD8^+$ responses and production of cytokines (62–64). In addition, in human cancer patients, G-MDSCs and suppressive neutrophils are isolated from the peripheral blood (65, 66). Although the distinction between neutrophils and G-MDSCs is not clear, the modulating role of these cells in the immune responses induced by tumors has become an accepted paradigm, and is extensively reviewed elsewhere (13, 67). Neutrophils are involved in both pro- or anti-tumor immune responses. Importantly, they have recently been shown to promote metastasis (7, 68). Their anti-tumor effects are mediated by their direct antibody-dependent cytotoxicity and their production of pro-inflammatory cytokines near and inside the tumor (69). These properties will not be discussed in this short review.

The pro-tumor effects of neutrophils are mediated by different mechanisms. First, neutrophils play an essential role in angiogenesis through expression of matrix metallo-proteases, such as MMP9 (70, 71). Second, in multiple murine models they inhibit anti-tumor $CD8^+$ T-cell responses through mechanisms described below. The suppression of anti-tumor T-cell responses by neutrophils was recently shown to be pivotal in tumor

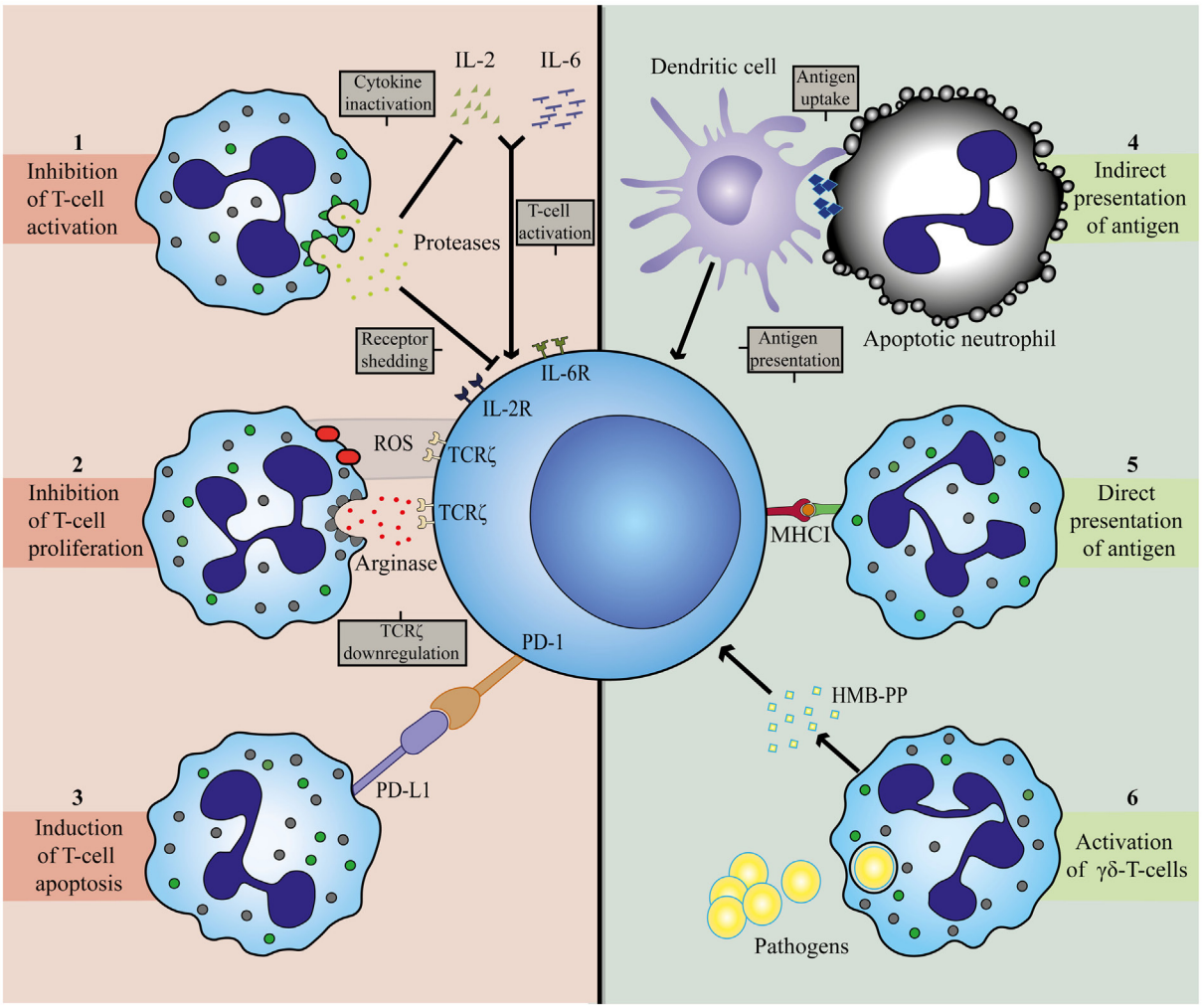

FIGURE 2 | Mechanisms involved in T-cell inhibition (left panel) and activation (right panel) by neutrophils. Neutrophils can establish T-cell inhibition by (1) degranulation of granular constituents. The serine proteases elastase and cathepsin G inactivate T-cell stimulating cytokines, IL-2 and IL-6, and catalyze shedding of cytokine receptors for IL-2 and IL-6 on T-cells (72, 73). (2) Production of ROS and release of arginase. Both agents can result in downregulation of TCRζ on T-cells, thereby arresting the cell in the G0-G1 phase (40, 74–78). (3) Expression of PD-L1. Upregulation of this ligand is associated with interferon-dependent PD1-mediated T-cell apoptosis (35, 56). T-cell activation by neutrophils is attained by (4) indirect antigen presentation. Dendritic cells take up antigens from apoptotic neutrophils and serve as APC for T-cells (61). (5) Direct antigen presentation. Neutrophils posses the capacity to cross-prime $CD8^+$ T-cells directly in a MHCI-dependent manner (32). (6) Release of microbial metabolites (HMB-PP). Neutrophils release bacterial products after ingestion to activate γδ-T-cells (42).

metastasis in a murine model of breast cancer (7). In this study, γδ-T-cells facilitated neutrophil recruitment to the tumor via an IL-17 and G-CSF mediated pathway. The microenvironmental cues for the switch in neutrophil phenotype from pro- to anti-tumor are slowly being unraveled. In a model of lung cancer, TGF-β induces or recruits neutrophils with a pro-tumor phenotype (termed N1), whereas blocking TGF-β induces an anti-tumor neutrophil phenotype (termed N2) (11).

Mechanisms of T-Cell Suppression by Neutrophils and G-MDSCs

Despite the evidence that neutrophils can stimulate T-cell responses, most studies point toward a direct suppressive role of these cells on different T-cell responses in various disease models as described above. The mechanisms of suppression have been reviewed in detail elsewhere and are summarized in **Figure 2** (13). Most of the mechanisms that neutrophils employ to suppress T-cell functions are closely related to their anti-microbial functions, i.e., the same or similar mediators are used. Two of the most frequently reported mechanisms are via ARG and ROS.

ARG is found in the gelatinase containing granules of neutrophils and is thought to contribute to antifungal immunity trough depletion of L-arginine (79, 80). Depletion of L-arginine also results in a cell cycle arrest in activated T-cells in the G0-G1 phase, which limits T-cell proliferation (74). This is thought to occur through downregulation of TCRζ (75, 76). It seems that the expression of TCRζ requires L-arginine for adequate expression and functionality

(81). In addition, L-arginine is required for dephosphorylation of cofilin. Cofilin is pivotal for the stability of the immunological synapse. Therefore, depletion of L-arginine by ARG impairs the formation and stability of an immunological synapse, which is required for T-cell activation and proliferation (82, 83).

ROS are an intricate part of neutrophil anti-microbial defense and the lack of ability to produce ROS as, seen in chronic granulomatous disease, is characterized by severe infections (84–86). One of the products of NADPH oxidase activation is hydrogenperoxide (H_2O_2). H_2O_2 can suppress T-cell proliferation and activation through various mechanisms. It induces apoptosis, decreases Nf-κB activation, and downregulates TCRζ (77, 78). In addition, T-cell suppression by ROS is also accompanied by the oxidation of cofilin (77, 87). Cofilin can, therefore, be influenced by both ARG and ROS: both mechanisms being employed by neutrophils. Therefore, the T-cell suppression by disruption of cofilin might prove to be a useful therapeutic target.

Interestingly, regulatory T-cells are resistant to oxidative stress (88). This suggests that regulatory T-cells are less sensitive to suppression than other T-cells, thus enhancing the overall suppressive effect of H_2O_2.

Suppression of T-cell activation and proliferation requires high concentrations of H_2O_2 (33, 87). This amount of ROS might only be reached in inflammatory tissue with massive neutrophil influx. A more elegant way of suppression via H_2O_2 is through cell-cell contact between neutrophils and T-cells. Such a direct mechanism for delivery of ROS in an immunological synapse has been identified (29). Here, neutrophil-T-cell contacts were mediated by MAC-1.

Another important suppressive pathway requires similar cell-cell contacts: inhibition via T-cell PD-1 by PD-L1, a potent inducer of apoptosis in T-cells 1, expressed on neutrophils during sepsis (25). The underlying mechanism of PD-L1 expression is an interferon dependent process (35). The PD-1/PD-L1 axis is thought to be an important mechanism in the immune suppression found in sepsis patients by inducing lymphocyte apoptosis and monocyte dysfunction (56). Blocking this axis after the induction of sepsis by administering a PD-1 blocking antibody improves survival in mice by increasing pathogen clearance (89). This suppressive mechanism might be protective in tissues with severe inflammatory infiltrates, but may be detrimental as immune suppression aggravates sepsis. At this moment, one can only speculate regarding the role of PD-L1 on neutrophils in this immune suppressed state.

Finally, neutrophils can modulate T-cells by degranulating granular constituents, such as neutrophil elastase. These proteases are able to cleave and inactivate essential cytokines, such as IL-2 and receptors, such as the IL-2 and IL-6 receptor on T-cells (72, 73).

Conclusion

The studies mentioned in this review have led to the consensus that neutrophils are capable of modulating adaptive immune responses through interactions with T- and B-cells and possibly APCs. The mechanistic studies in mice have been corroborated with human *ex vivo* data. These studies show that neutrophils are capable of directly interacting with lymphocytes and modulating their responses at local sites of inflammation as well as in draining LNs. One of the key remaining issues is the question whether human neutrophils show functional plasticity as has been suggested by us and others (13, 90). This plasticity can occur at different levels: (1) the existence of functional subsets, which are intrinsically different or (2) the transdifferentiation into suppressive neutrophils or even into an APC type of hybrid cell (90–92). The microenvironmental cues mediating the switch from classical neutrophils to suppressive neutrophils have barely been studied although TGF-β seems to play an important role in microbial and tumor models (11, 23).

In conclusion, murine and human studies to date show that neutrophils are potent modulators of immunity. The first step of establishing a strategy to target immune modulatory neutrophils without influencing their essential anti-microbial functions is finding relevant human diseases in which this modulation plays a pivotal role (93). The unraveling of microenvironmental cues mediating the recruitment of and/or "switching" into suppressive neutrophils in such diseases is essential in understanding and targeting of this pathway.

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frontiers in Immunology

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Human immunodeficiencies related to defective APC/T cell interaction

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The primary event for initiating adaptive immune responses is the encounter between T lymphocytes and antigen presenting cells (APCs) in the T cell area of secondary lymphoid organs and the formation of highly organized intercellular junctions referred to as immune synapses (IS). *In vivo* live-cell imaging of APC–T cell interactions combined to functional studies unveiled that T cell fate is dictated, in large part, by the stability of the initial contact. Immune cell interaction is equally important during delivery of T cell help to B cells and for the killing of target cells by cytotoxic T cells and NK cells. The critical role of contact dynamics and synapse stability on the immune response is well illustrated by human immune deficiencies in which disease pathogenesis is linked to altered adhesion or defective cross-talk between the synaptic partners. The Wiskott–Aldrich syndrome (WAS) is a severe primary immunodeficiency caused by mutations in the Wiskott–Aldrich syndrome protein (WASp), a scaffold that promotes actin polymerization and links TCR stimulation to T cell activation. Absence or mutations in WASp affects intercellular APC–T cell communications by interfering with multiple mechanisms on both sides of the IS. The warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome is caused by mutations in CXCR4, a chemokine receptor that in mutant form leads to impairment of APC–T cell interactions. Present evidences suggest that other recently characterized primary immune deficiencies caused by mutation in genes linked to actin cytoskeletal reorganization, such as WIP and DOCK8, may also depend on altered synapse stability. Here, we will discuss in details the mechanisms of disturbed APC–T cell interactions in WAS and WHIM. Moreover, we will summarize the evidence pointing to a compromised conjugate formation in WIP, DOCK8, and X-linked lymphoproliferative syndrome.

Keywords: immune synapse, immunodeficiencies, actin cytoskeleton, chemokines, T cell activation

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Introduction

Cells of the immune system communicate with one another by physical contacts and by soluble signals that may act on the interacting cells or at a distance. The formation of intercellular junctions is essential to bring receptor–ligand couples close enough to trigger downstream signaling and to transmit activatory/inhibitory signals between the two cells. Tight membrane apposition is also a prerequisite to allow focused delivery of soluble factors in a spatially confined fashion, ensuring specificity and effectiveness during killing of targets or polarized secretion of soluble mediators. Immune cell interaction is supported by several interconnected systems that assist the various

stages of contact formation from initial scouting to adhesion and stabilization of the junction. The importance of such pathways is underscored by human pathologies caused by mutations in genes controlling these systems.

APC–T Cell Encounter in the T Cell Area of Lymph Nodes

The first challenge for a T cell entering the T cell area of a lymph node is to find its cognate antigen on the surface of a dendritic cell (DC). This process is aided by the strategic distribution of DCs in an extensive network and by chemokine cues that guide motility and positioning in lymph nodes. *In vivo* imaging experiments have shown that lymphocytes entering the T-cell zones move randomly over densely packed networks of DCs and fibroblastic reticular cells (FRCs) (1, 2). This motility is driven by CCR7-binding chemokines. Besides CCL21, other chemokines produced in lymph nodes may coordinate specific encounters between cells. Thus, CCL3 and CCL4 seem to be involved in recruitment of naïve $CD8^+$ T cells, which can upregulate CCR5 expression during inflammation, to sites where they can receive help from $CD4^+$ T cells (3). CXCR3 expression on $CD4^+$T cells is important for the interaction with antigen bearing DCs and for the global intranodal positioning of T cells (4). Moreover, the same chemokine receptor selectively controls repositioning of memory T cells within lymph nodes during a recall response (5).

Interaction of the TCR with cognate antigen results in the activation of phospholipase C-γ and Ca^{2+} influx via calcium release activated channels (CRAC) Orai1/CRACM1 in the plasma membrane (6, 7). Among the other effects, Ca^{2+} influx induces ATP synthesis and release (8) that, in turns, induces P2X4/P2X7-mediated calcium waves in the neighboring lymphocytes and acts as a paracrine signaling molecule that regulates T cell motility during immune responses (9). ATP-induced Ca^{2+} waves induce a "stop" not only in cells that have already found their antigenic partners but also in lymphocytes that may be potentially triggered within the tissue. Several studies have indeed observed that in the lymph node microenvironment there is a significant drop in the velocity of polyclonal T cells during antigenic stimulation of TCR-specific cells (10, 11). The reduced motility of T lymphocytes in a tissue where antigenic recognition is occurring may be strategic for a better scanning of resident DCs and, in this perspective, extracellular ATP may alter the equilibrium between adhesive and chemoattractant forces operating in lymph nodes during T cell priming and thus modify T cell activation. Interestingly, destabilization of T–DCs conjugates *in vivo* by regulatory T cells is, in part, due to high levels of expression of CD39 and CD73, two cell surface ecto-enzymes that hydrolyze extracellular ATP to ADP, AMP and adenosine that, acting through the A2A receptor, prevents activation and proliferation of $CD4^+$ T cells (12, 13).

The Duration of APC–T Cell Contacts and the Consequences for T Cell Activation

The dynamics of cellular contacts and the functional consequences of short and prolonged cellular interactions in terms of T cell activation have been investigated mostly in the context of naïve T cells priming by DCs. *In vitro* studies showed that T cells remain stably attached to DCs in conditions that lead to T cell activation, whereas short intermittent contacts dominate when DCs are immature and unable to induce activation. With the limits of an *in vitro* analysis, these findings provided one of the first correlations between contact duration and function (14). An opposite result, i.e., short contacts may be enough to trigger naïve T cell activation, was obtained when analyzing cells in a collagen 3D matrix, suggesting that the requirements for T cell activation may depend on the context (15). Direct imaging of the immune response in lymph nodes revealed the presence of both sequential, brief, T–DC contacts (kynapses) and long antigen-specific contacts (synapses) (16). Different phases of short- and long-lasting antigen presenting cell (APC)–T contacts alternates during initial priming and longer arrest of T cells on the APC surface predominates in conditions of full T cell activation (17–19). This concept was later refined by studies showing that the affinity of the pMHC for the TCR critically determines contact duration. High-affinity antigens induce a complete T cell stop, whereas low-affinity antigens cause only T cell deceleration (20–22). Interestingly, the presence of bystander cells, such as regulatory T cells, modifies contact dynamics hampering the formation of stable contacts (12, 20). The state of T cell activation is a further critical parameter that determines contact dynamics. Naïve T cells stop and form mostly synapses upon antigen recognition, whereas previously activated T cells can collect activatory signals from kinapses (23). It has also emerged that kinapses may lead to T cell activation when antigen density is high enough to allow integration of signals over multiple serial encounters (24).

Molecular Structure of the Immune Synapse

Ex-vivo analysis of single T cells engaged in contact with APCs has been instrumental to understand the subcellular reorganization occurring in T cells during activation (**Figure 1**). Because of some analogies with the mode of intercellular communication used by neurons, the specialized structure formed between a T cell and an antigen presenting B cell was named as "immune synapse" (25, 26). The immune synapse (IS) was initially described as a central area containing signaling components such as the TCR and PKC-θ kinase (cSMAC), a peripheral ring containing adhesive molecules (pSMAC), and a distal region rich in actin (dSMAC). Subsequent studies using planar bilayer as surrogate APCs allowed the quantitative and dynamic analysis of synapse formation in T cells and to assess the contribution of single receptors (MHC peptide complexes, adhesion, and co-stimulatory molecules) on the reorganization of signaling platforms, cytoskeletal remodeling, and polarized vesicular trafficking (**Figure 1**). These studies revealed that microclusters (MCs) of 10–20 TCRs molecules forms in the dSMAC and are translocated into the cSMAC, where the signaling activity of the TCR extinguishes [(27–30) and reviewed in Ref. (31)]. An essential component to coordinate TCR signaling is the actin cytoskeleton. This is needed to support early events of T cell activation, such as clustering of TCRs in MCs, recruitment of

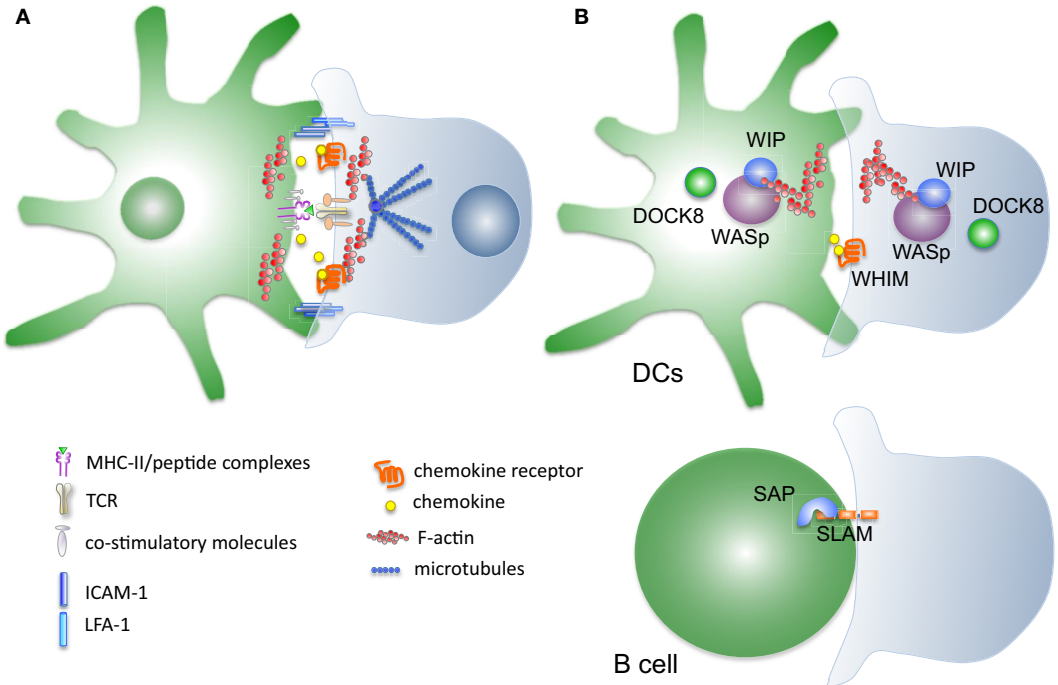

FIGURE 1 | Mutations in proteins controlling synapse stability linked to development of primary immune deficiencies. **(A)** Schematic representation of the molecules implicated DC–T cells cross-talk during synapse formation. Several interconnected systems, including membrane receptors and cytosolic proteins, contribute to form and stabilize the interaction between the two cell types. **(B)** Disease-causing mutations targeting proteins that control synapse stability. Wiskott–Aldrich syndrome protein WASp, causative of WAS syndrome, is a key node controlling actin polymerization in immune cells. Mutations in WASp have been associated to several defects in synapse formation on both the T-cell and the DC side. Most recently discovered mutations in DOCK8 and WIP cytoskeletal regulators cause immunodeficiency syndromes whose cellular basis include disturbance of the intercellular interactions. SAP, an adaptor for SLAM receptors mutated in XLP syndrome, is affecting selectively the stability of the B–T synapse.

signaling complexes to MC, and later mobility of signaling platforms. In turn, recruited signaling molecules, such as the adaptor LAT, serve as platforms to dock cytoskeletal regulatory proteins, such as Vav and Wiskott–Aldrich syndrome protein (WASp), necessary to sustain T cell activation (30, 32). Most recently, a novel view of the actin cytoskeleton as a global regulator of the cytoplasm poroelasticity and consequently of T cell signaling is emerging (33).

Interconnected to the role of actin cytoskeleton, the integrin LFA-1, acts at several levels in the IS. First of all, lateral movements of LFA-1, ensured by linkage with the underlying actin cytoskeleton, are essential to ensure correct T cell activation (31, 34). Besides its function in supporting synaptic architecture, LFA-1 is also an important co-stimulatory molecule during T cell activation by increasing the sensitivity for antigen by 100-fold. Mechanistically, LFA-1 engagement is known to enhance activation of early TCR signaling molecules and to promote later events of T cell proliferation and cytokine production (35, 36). LFA-1 plays a role also at earlier stages of synapse formation. During scanning in search of matching TCR/pMHC, the initial adhesive interactions between T cells and APC are mediated by LFA-1 and ICAM-1,3 on T cells and APCs, respectively (37). The functional relevance of LFA-1 on contact duration has been addressed by *in vivo* studies that correlated contact duration with acquisition of effector functions. Expression of the LFA-1 ligand ICAM-1 is required to sustain long antigen-specific DC–T contacts, whereas short interactions can still occur in the absence of ICAM-1. Importantly, T cells primed by ICAM-1 deficient DCs undergo early events of activation but fail to differentiate into effective memory CD8 T cells (38, 39). A mirroring finding in CD4 T cells lacking LFA-1 support is the importance of the LFA-1/ICAM-1 adhesion module to achieve optimal T cell priming *in vivo* (38). Interference with positive regulator of integrin activation yielded similar results. For instance, deletion of Talin in T cells leads to unstable contacts with APCs and failure to undergo full T cell activation (40, 41).

Soluble immunotransmitters like chemokines play also an important role in IS stabilization and T cell proliferation (42, 43). When approaching an APC, T cells emit CCR5 (or CXCR4)-enriched protrusions that indent the APC surface; this situation resembles the concentration of chemokine receptors at the leading edge of chemoattractant-stimulated T cells (44). These interactions culminate in the formation of a stable synapse, whereas CCR5 and CXCR4 are stably concentrated. Chemokine release at the immunological synapse and chemokine receptor recruitment into this region result in prolonged T-cell–APC interaction, and facilitate T cell activation by reinforcing T cell–APC pair attraction and delivering co-stimulatory signals (43). Interestingly,

chemokine recognition in the context of the immunological synapse induces a $G_{q/11}$-mediated CCR5 signaling, suggesting that chemokine receptor signaling pathways are modified by TCR triggering (43). Notably, coupling of G_q to the chemokine receptors delays their internalization, explaining the accumulation of CCR5 and CXCR4 at the T cell immunological synapse. In this scenario, chemokine receptors prolong the duration of T cell–APC interaction and facilitate T cell activation by increasing LFA-1 affinity (45), reinforcing T cell–APC pair attraction and avoiding pre-mature splitting due to other chemoattractant sources. On the basis of their actions, a dual role for chemokines in T-cell activation has been proposed, while the presence of chemoattractant forces when T cells are searching for the right partner may indeed prevent T cell–APC pairing, production of chemokines by the APCs, and subsequent accumulation and trapping of G_q-coupled chemokine receptors at the IS, may represent a strategy to reinforce T-cell–APC interaction and facilitate T-cell activation (46, 47).

The Wiskott–Aldrich Syndrome

Cytoskeletal remodeling is a highly dynamic process that ensures spatio-temporal coordination of diverse functions, such as mechanical support to the cell cortex, migration, phagocytosis, intercellular interactions, and subcellular distribution of signaling molecules and vesicles flow. Actin dynamics are tightly controlled by several different nucleation-promoting factors in turn activated by multiple complex pathways. Formation of branched actin networks is regulated by the Arp2/3 complex that is induced by the VCA domain contained in the WASp family of actin regulatory proteins. The eight members of the family (N-WASp, WAVE 1–3, WASH, JMY, and WHAM (48)] have different activation modes and control differential functions in various tissues. WASp, the founding member of the family, is expressed exclusively in the hematopoietic lineage and it was first discovered because loss-of-function mutations in its coding gene are associated with the X-linked immunodeficiency Wiskott–Aldrich syndrome (WAS) (49). The disease is characterized by multiple clinical manifestations, including susceptibility to infections, hemorrhages and eczema, and multiple forms of autoimmune disorders (50).

Wiskott–Aldrich syndrome protein mutations impact on disparate cellular functions in different hematopoietic lineages (51, 52). T cells were the first lineage recognized as being heavily affected by WASp mutations. A detailed review on the role of WASp in T cells has been recently published (53). Here, we will recall the main features of WASp deficient T cells and present the emerging defects in WASp null APCs.

Initial studies identified defects in TCR signaling and activation of IL-2 in T cells from WAS patients (54–56). WASp null T cells, similarly to cells of other hematopoietic lineages, also present with alteration of motility (57, 58). Later studies helped to better define how WASp controls selectively multiple sequential events in T cell activation. WASp is recruited to sites of early TCR receptor signaling in multimeric complex together with LAT, SLAP-76, Nck, and the cdc24 GEF Vav (32, 59, 60). At the synaptic interface, binding of activated cdc42, PIP2, and phosphorylation of tyrosine 291 by Src family kinases cooperate to release the auto-inhibited conformation of WASp, exposing the VCA domain and inducing acting nucleating activity [reviewed in Ref. (61)]. Genetic deletion of WASp in T cells causes alterations in the early dynamic events of stabilization of the synapse. Upon TCR triggering cycles of stable symmetric synapse structure alternates to phases of T cell motility when the synaptic structure is lost. WASp is required to reform the synaptic structure after these periodic breaking rather than for the initial synapse formation (62). This is in line with the finding that T cells derived from WASp patients, despite normal conjugate formation, fail to spatially organize signaling in the cSMAC and to polarize the microtubules organizing center (63). Downstream events of T cell activation, such as calcium fluxes, IL-2 production, and T cell proliferation, are also affected by WASp deficiency both in mouse models and in patient's-derived cells (64–68). The exact role of WASp-mediated F-actin dynamics in regulating synaptic structure and downstream signaling is still not fully resolved. A recent study proposes that WASp controls selectively a small fraction of synaptic F-actin required to sustain PLC-γ activation and calcium ion elevation, thereby linking the control of early events to later T cell activation (69). It is also emerging that WASp can have actin-independent activities in T cells, functioning as a transcription factor to regulate transcription of cytokine genes (70). Thus, WASp plays a central role in controlling multiple integrated functions that link TCR signaling to full T cell activation. Moreover, its role varies depending on the T cell subset, reflecting the existence of cell type-specific modes of actin regulation besides common shared mechanism (53).

Regulated cytoskeletal remodeling is needed also to support the function of APCs during synapse formation and maintenance. DCs are active player in synapse formation by virtue of their membrane protrusions that facilitate scanning of the T cell repertoire and interaction with T cells (14, 71, 72). This flypaper membrane activity of DCs is regulated by members of the Rho family of small GTPase and by actin regulatory proteins. Genetic deletion of an upstream regulator of cytoskeletal remodeling, the Rho GTPases Rac, inhibits dendrites extension, resulting in reduced DC–T contact time and inefficient priming (71). In WAS, loss of proper actin cytoskeletal rearrangement hampers the function of DCs at several levels. Defects in adhesion to ICAM-1, polarization and responses to chemokine gradients (73, 74) render DCs unable to properly migrate from site of antigen acquisition in the periphery to lymph nodes. Failure to properly initiate adaptive immunity by WASp deficient DCs arises from defects that go beyond the capacity to properly home to lymph nodes (75). Delivery of the model antigen DEC205-OVA to resident DCs resulted in poor activation of antigen-specific $CD8^+$ T cell in WASp null recipient. Further experiments to dissect the individual contribution of migration, antigen processing and DC–T cell interaction *in vivo* demonstrated that WASp null DCs fail to efficiently prime naïve CD8 T cells even when the migratory defect is compensated (75). Imaging of DC–T cell contacts *in vitro* and by two-photon microscopy *in vivo* indeed showed that WASp null DCs fail to form stable and long-lasting interactions with antigen-specific T cells (75). Interestingly, T cells primed by WASp null DCs can enter the cell cycle but fail to accumulate, similarly to what happens when priming is promoted by Cdc42 knock down DCs, an upstream regulator of WASp (76). A similar defect in the stability

of DC–T cell contacts and in the capacity to support formation of an organized synaptic structure was seen also using $CD4^+$ T cells (77). Taken together, these data indicate that defective cytoskeletal organization in WAS DCs affects two key steps during priming, i.e., migration to lymph nodes and formation of stable DC–T cell contacts and T cell activation once in lymph nodes. Thus, not only presentation of antigens that are taken up in the periphery and transported to secondary lymphoid organs but also presentation of blood born antigens by lymph node resident DCs is likely to be compromised in WAS. The impact of DCs to the overall immune deficiency is demonstrated by the fact that rescue of DCs functions upon gene therapy is capable to improve T cell priming (78).

It is also emerging that plasmacytoid DCs and myeloid cells present with defects in innate immunity pathways in WAS (79). The role that an altered cytokine secretion profile may have on synapse stability and signaling at the IS is an intriguing aspect that is currently being investigated by our group.

Wiskott–Aldrich syndrome patient experience recurrent autoimmune manifestations, whose cellular basis are not yet fully understood (80). Functional defects in regulatory T cells are likely to contribute to loss of peripheral tolerance (81–83). DC–T cell interactions are critical for the establishment of peripheral T tolerance besides initiation of adaptive immunity (17, 84). It is interesting to speculate that besides cell intrinsic Tregs abnormalities, defective interaction with APC may contribute to loss of peripheral tolerance in WAS.

Other Actin-Related Immune Deficiencies

Recently, a new cytoskeletal-related immunodeficiency caused by mutation in the WASp interacting protein WIP has been identified (85). WIP controls WASp activity in at least three different ways: regulating its stability, controlling its activation by Cdc42, and bringing WASp to sites of active polymerization (86). Indeed, a stop codon mutation in the WIP sequence that silenced protein expression resulted in almost undetectable WASp level, and clinical features similar to WAS (85). At the cellular level, WIP was shown to control podosomes assembly and cell migration in DCs (87, 88). In addition, WIP binds to actin and controls cytoskeletal integrity independently of WASp. The WASp-independent actin regulation exerted by WIP is essential for T cell homing to infected tissue (89). A further interesting function that has been attributed to WIP, independently of its binding to WASp, is the control of lytic granule secretion in NK cells. The failure in cytolytic activity of WIP null NK cells is due to lack of transport and polarization of granules at the IS (90). The role of WIP in controlling IS formation in T cells and DCs has not yet been addressed. However, it is likely that priming will not be efficient because of defects on both sides of the IS, thus explaining the poor immune responses (91).

A further example of immunodeficiency arising form cytoskeletal abnormalities that affect synapse formation is DOCK8 deficiency. DOCK8 is a GTP-exchange factor for Rho and Rac GTPases that controls conversion of extracellular signals into activation of actin regulatory proteins. Mutations in DOCK8 were found to be the genetic basis of a combined immunodeficiency characterized by increased susceptibility to skin viral infections, hyper IgE syndrome, T cell lymphopenia, and impaired antibody response (92). At the cellular level, DOCK8 was shown to be required for the accumulation of the integrin ICAM-1 at the B cell synapse and its mutation compromise synaptic architecture and B cell functions (93). Marginal zone B cells are highly reduced in DOCK8, similarly to what has been observed in WAS (94). DOCK8 mutant T cells were also shown to have defective LFA-1 polarization in synapse, resulting in decreased T cell proliferation and survival (95). The DC compartment is also affected in a way reminiscent of defects observed in WAS, such as defective homing to lymph nodes and reduced T cell priming activity (96). Although the direct role of DOCK8 in controlling the stability of the DC-T synapse has not been addressed, it is reasonable to predict that alterations in contact duration may contribute to disease pathology.

The WHIM Syndrome

As discussed above, chemokines and their receptors have a dual role in localization of T cells and APCs within secondary lymphoid organs, as well as in enhancing the strength of the T–APC interaction. Intriguingly, the relevance of the chemokine–chemokine receptor axis in promoting stable synapses has been further emphasized by recent studies on the rare immune deficiency warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM). A chemokine-mediated regulation of the duration of T–APC interactions was shown to contribute to the cellular basis of T cell-dependent response defects in this disease (47). The WHIM syndrome is an inherited immunodeficiency that features a wide range of symptoms, including recurring infections, human papillomavirus (HPV)-induced warts, reduced long-term immunoglobulin G (IgG) titers, myelokathexis, and leukopenia (97–100). The syndrome is associated with dominant mutations in the chemokine receptor CXCR4 that lead to truncation of its carboxy-terminal domain. This leads to a defect in the ability of the receptor to internalize after binding its cognate ligand, CXCL12. As a consequence, immune cells bearing the WHIM-mutant receptor display increased signaling and enhanced migration after stimulation by chemokine (98, 101–103). Historically, this enhanced functionality of the mutant CXCR4 has provided a mechanistic explanation for the abnormal retention of neutrophils in the bone marrow (myelokathexis), as demonstrated by experiments in a human-to-mouse *in vivo* xenograft model and in a zebrafish model (101, 104). Yet, symptoms of WHIM syndrome patients, such as the inability to successfully mount responses to a recurring pathogen and the decreased capacity to produce hypermutated IgG signify that antigen-specific memory responses, antibody class-switching and affinity maturation are defective in these individuals (99). The finding that CXCR4, along with other chemokines, is utilized in the organization of lymphoid organ follicles enabled the speculation that possible aberrations in lymphoid organ architecture could be the cause of the above adaptive immunity defects in WHIM (99). Reports of disrupted lymph node spatial organization in a recent mouse knock-in model of WHIM support this hypothesis (105). Nonetheless, the generation of antigen-specific memory, Ig class switching and affinity maturation do depend on the formation of successful T cell–APC interactions via immunological synapses (106). Recent

work has identified that T cells from WHIM patients, or indeed healthy donor T cells transfected with the dominant, WHIM-mutant CXCR4, form less stable conjugates with superantigen-pulsed B cells. Importantly, this only occurs in the presence of competing migratory ("go") signals from exogenous CXCL12, which appear to affect the mutant but not the wild-type receptor. A very similar impairment of T–APC immunological synapse stability occurs between antigen-specific WHIM-mutant T cells and antigen-loaded DCs in *ex vivo* lymph node slice cultures derived from a retrogenic model of WHIM, imaged via 2-photon microscopy (47). While both wild-type and WHIM-mutant CXCR4 are recruited to the immunological synapse, exogenous CXCL12, which is present in lymph nodes (107), is able to "distract" only the hyperfunctional WHIM-mutant CXCR4 away from the synapse. Indeed, wild-type CXCR4 is unable to impair immunological synapse formation (108) and has no effect on T cell activation (109, 110). Intriguingly, however, the hyperfunctional WHIM-mutant CXCR4 appears to exceed a threshold that favors motility over formation of stable immunological synapses, resulting in aberrant T cell activation (47). Further molecular studies will tell us more about the regulation of T–APC interactions. Nonetheless, the finding that many of the WHIM defects are reversible using a pharmacological inhibitor of CXCR4 is an interesting demonstration of how chemokines and their receptors, in specific circumstances, have the ability to affect T cell function.

Synaptic Defects in Patients with X-Linked Lymphoproliferative Disease

X-linked lymphoproliferative disease (XLP) is caused by loss-of-function mutations in signaling lymphocyte activation molecule-associated protein (SAP), an adaptor linking SLAM family receptors to downstream signaling. The protein is primarily expressed in T cells, NK cells, and B cells. XLP patients are subjected to severe Epstein–Barr viral infections and develop lymphomas and lymphoproliferative disorders (111). At the cellular level, the disease is characterized by a defect in germinal center formation and consequently poor humoral response, abnormalities in NKT cell development, NK cell cytotoxicity, and cytokine production (112). In the context of this review, it is interesting to discuss the evidences pointing to disturbed B–T cell interaction to explain poor germinal center formation. Upon initial activation of T cells by DCs in lymph nodes, the second circuit of immune cell interaction includes motile but prolonged interactions between activated B cells and T cells at the border between the follicle and the T cell zone, followed by translocation of T cells in the germinal center to sustain the germinal center reaction. Follicular helper T cells, specialized in this process, express high levels of SAP and SLAM (113). *In vivo* imaging of B–T interactions during T cell-dependent B cell-activation revealed that SAP-deficient T cells are intrinsically unable to form stable contact with B cells. Interestingly, this defect is selective for B–T cell interaction, as DC–T cell interactions proceed normally. These data show that SAP-associated family members controls, selectively, adhesive mechanism required to stabilize T cell–B cell conjugates required to deliver to B cells the signals supporting full B cell proliferation (112, 114). Further insight into the role of SAP and SLAM receptor in assembling B–T synapse comes from the finding that the SLAM receptor Ly108 is a potent negative regulator of T–B cell adhesion, counteracted by SAP, that act by recruiting the phosphatase SHP-1 at the synapse (115).

SAP functions also in controlling adhesion during cytolysis. SAP-deficient cytotoxic T lymphocytes fail to assemble a proper synaptic structure during conjugation to target cells, with altered polarization of perforin granules and lipid raft at the contact site (116, 117). In line with this observation, SAP-deficient NKT cells fail to polarize the microtubule-organizing center toward the target cell, resulting in reduced killing ability (118).

Conclusion

A class of primary immunodeficiency is caused by pathogenic mutations in genes controlling immune cell trafficking and cellular interactions dynamics (**Figure 1**). The cellular basis of these diseases has been increasingly investigated helping to improve patients management. Moreover, analysis of these naturally arising mutant cells revealed important insights into basic functioning of the immune system. As a prominent example, WASp mutant cells have been instrumental in understanding actin-mediated signal transduction during TCR triggering and to unveil the importance of an intact actin cytoskeleton in APCs. Characterization of mutant T cells in less common immunodeficiency like WHIM, WIP, DOCK8, and SAP is still at early stages and it will help to dissect subtle details of immune cell interaction regulation. Further analysis is needed to understand the reciprocal contribution of alterations on both sides of the IS to gain an integrated view of the parameters that control, in normal and pathological conditions, the transfer of information between APC and T cells during priming of adaptive immune responses.

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frontiers in Immunology

MINI REVIEW
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Modulation of APC function and anti-tumor immunity by anti-cancer drugs

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Professional antigen-presenting cells (APCs), such as dendritic cells (DCs), are central to the initiation and regulation of anti-cancer immunity. However, in the immunosuppressive environment within a tumor APCs may antagonize anti-tumor immunity by inducing regulatory T cells (Tregs) or anergy of effector T cells due to lack of efficient costimulation. Hence, in an optimal setting, anti-cancer drugs have the power to reduce tumor size and thereby may induce the release of tumor antigens and, at the same time, modulate APC function toward efficient priming of antigen-specific effector T cells. Selected cytotoxic agents may revert APC dysfunction either by directly maturing DCs or through induction of immunogenic tumor cell death. Furthermore, specific cytotoxic agents may support adaptive immunity by selectively depleting regulatory subsets, such as Tregs or myeloid-derived suppressor cells. Perspectively, this will allow developing effective combination strategies with novel immunotherapies to exert complementary pressure on tumors via direct toxicity as well as immune activation. We, here, review our current knowledge on the capacity of anti-cancer drugs to modulate APC functions to promote durable anti-cancer immune responses.

Keywords: chemotherapy, dendritic cells, anti-tumor immunity, antigen-presenting cell, tumor-induced T cell dysfunction, immunogenic cell death

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DCs are Central to the Initiation and Regulation of Anti-Cancer Immunity

Both the induction of endogenous anti-tumor immune responses and the successful implementation of immunotherapy protocols rely on adequate activation of adaptive immunity by antigen-presenting cells (APCs). Although innate and adaptive immune cells act in concert to fight cancer cells, T cells play a superior role in inducing and maintaining sustained anti-tumor immune responses (1–4). Professional APCs include B-cells, macrophages, dendritic cells (DCs), and skin-resident Langerhans cells. Among these, DCs are by far the most potent activators of adaptive immunity owing to their unique capacity to induce primary T cell responses (5, 6).

To initiate T cell responses in the tumor setting, DCs must first recognize tumor cells as "abnormal" cells. Tumor cells differ from normal cells due to expression of altered-self or neo-antigens that arise as a consequence of genetic instability and high mutation rates in transformed cells (7, 8). Efficient activation of naïve and central memory T cells requires at least three signals delivered by APCs (9, 10). Along with the uptake of tumor-associated antigens and presentation in the context of MHC molecules, professional APCs are further required to provide lymphocyte costimulation, such as through expression of the B7 molecules (CD80 and CD86) or CD40. Expression of T cell-directing cytokines and additional costimulatory surface receptors by DCs subsequently provides the impulse for appropriate

$CD4^+$ T helper cell polarization (11). Importantly, DC maturation is regulated by the type, duration, and timing of danger/stress signals, such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), both of which trigger DC-intrinsic pattern-recognition receptors (12–14). Consequently, these signals determine the quality and quantity of costimulation provided by DCs and thus have the power to define the outcome of T cell immunity.

Tumor cells may undergo cell death due to hypoxia and nutrient deprivation (15) resulting in the release of host-derived DAMPs as an indicator of a dying or "stressed" cell. Thus, on theoretical grounds, tumors may provide the basic elements needed for the initiation of successful anti-tumor immune responses. However, it is well established that the immune-suppressive microenvironment of progressive tumors may severely interfere with both APC and T cell activation, thereby limiting the induction of endogenous anti-tumor immunity and the success of immunotherapies. Importantly, numerous tumor-mediated mechanisms may induce DC dysfunction, leading to immature or semi-mature DCs that are incapable of providing the necessary activation signals (16, 17). Consequently, under these circumstances, antigen presentation will typically provoke T cell tolerance including T cell anergy, peripheral T cell clonal deletion, or the induction of regulatory T cells (Tregs) (18–21).

In light of the recent success of novel immunotherapy approaches, future therapeutic efforts will ultimately focus on the development of effective combination strategies that exert complementary pressure on tumors via immune activation and additional direct toxicity. Of note, accumulating evidence reveals unrecognized immune-modulatory features of chemo- and radiotherapy (22, 23). In addition to reducing the primary tumor burden and thereby, at least in part, reverting the immunosuppressive microenvironment, specific compound classes can also induce DC maturation, enhance antigen cross-presentation, selectively eliminate immunosuppressive cells, or induce immunogenic cell death (ICD) (24, 25). These mechanisms may provide the basis for initiation of anti-tumor immunity and therefore support the successful implementation of T cell-mediated immunotherapy protocols, such as blockade of inhibitory receptors. Consequently, chemo-immunotherapy protocols are being evaluated in numerous clinical studies with promising therapeutic activity (26).

A major drawback of cytotoxic anti-cancer agents is their possible interference with T cell activation and clonal expansion (27, 28). A precise definition of the immune-modulating properties of cytotoxic therapies is, therefore, crucial for optimizing chemo-immunotherapy regimens. Here, we review the current knowledge on the capacity of anti-cancer drugs to modulate the phenotype and function of APCs, and in particular, the impact of these agents on T cell effector functions.

Modulation of APC Function by Anti-Cancer Chemotherapeutics

Direct DC Maturation by Cytotoxic Anti-Cancer Agents

A lack of T cell effector function is mostly caused by inefficient expression of costimulatory molecules on tumor-associated DCs or by dysregulation of DC maturation pathways (29). Therefore, understanding how cytotoxic agents influence DC maturation is vital for designing effective chemo-immunotherapy protocols. An improved DC phenotype associated with T cell activation *in vitro* was reported after treatment of immature DCs with the topoisomerase I inhibitor topotecan. In contrast, treatment of lipopolysaccharide-matured DCs with topotecan resulted in decreased allogeneic T cell responses accompanied with a shift toward T_H2 responses and increased IL-10 in co-cultures (30). Therefore, topoisomerase inhibitors might down-modulate responses of previously activated tumor-resident DCs, suggesting limited suitability for T cell immunotherapy combinations. Studies on other topoisomerase I inhibitors report conflicting results on DC maturation (31, 32). Also, using a DC-based reporter system, nine of 12 investigated compounds were identified as DC stimulatory (33). The effects of topoisomerase inhibitors on the induction of anti-tumor immunity as well as T cell activation and expansion *in vivo*, therefore, require further investigations.

Liu and colleagues revealed mechanistic insights into the molecular events associated with chemotherapy-induced DC maturation (32). Expression of the cell cycle regulator $p21^{waf1/cip1}$ in human DCs was associated with a favorable DC phenotype and was shown to be upregulated by cytotoxic agents. $p21^{waf1/cip1}$ expression correlated with enhanced expression of CD83 and CD86 in response to the anti-malaria agent artesunate and several anti-cancer compounds including camptothecin, lenalidomide, and docetaxel (32). When considering the "two-signal model" in innate immune activation (34), it seems plausible that p21, which generally indicates intrinsic cell stress, is activated in response to cytotoxicity. This "cell stress" or "abnormal condition" in the innate cell itself may deliver the necessary secondary signal for complete activation of innate immune cells and therefore result in enhanced DC maturation.

Along the same line, microtubule-destabilizing agents (MDAs), such as the *Vinca*-alkaloids, dolastatins, or maytansines, can directly affect DC maturation. Early studies indicated that microtubule disruption by colchicine, vinblastine, and vincristine induced marked expression of IL-1 in monocytes (35). Interestingly, rupture of the actin filaments by cytochalasins could not recapitulate this effect. Broad immune-stimulatory effects on murine DCs upon colchicine or vinblastine treatment, including expression of further pro-inflammatory cytokines and enhanced cross-presentation, have subsequently been confirmed by Takashima and colleagues (33, 36, 37). In extension of those data, we were able to demonstrate that two further families of MDAs, the dolastatins and maytansines, potently induced DC maturation. Importantly, DC pre-treatment with these agents induced profound T cell immunity, while treatment of tumor-bearing mice synergized with blockade of CTLA-4 and PD-1. Mechanistically, tumor rejection could be explained by enhanced infiltration of lymphocytes into the tumors and a shift toward an increased effector T cell to Treg ratio (38–40). Experiments to elucidate DC signaling pathways induced by MDAs are currently ongoing.

In stark contrast, we did not detect significant changes in DC phenotype or cytokine expression upon exposure to microtubule-stabilizing agents (MSA), such as the taxane-family of compounds (38).

Various studies have reported modulation of DC phenotype upon exposure to low, non-cytotoxic, concentrations of paclitaxel and other chemotherapeutics (41–44). However, these studies mostly evaluated DC function and phenotypic maturation in the context of paclitaxel pre-treated tumor cells or in combination with lipopolysaccharide treatment. Direct effects of paclitaxel and other MSAs on DCs were generally very moderate and thus are largely consistent with our data. The stimulatory effects of paclitaxel on tumor-associated macrophages, which subsequently may lead to activation of intra-tumoral immune cells, such as DCs, NK cells, and cytotoxic T lymphocytes, are comprehensively reviewed elsewhere (45).

Antibody-drug conjugates (ADCs) are emerging as powerful treatment strategies with outstanding target specificity and high therapeutic activity in cancer patients. The immune-modulatory capacities of dolastatins and maytansines are of particular clinical interest as their synthetic analogs, i.e., monomethyl auristatin E (MMAE) and DM1, are used as cytotoxic payloads of ADCs (46). Importantly, in tumor-bearing mice, DC activation upon treatment with such ADCs is equally potent as observed after administration of the respective free compound. Upon internalization, the cytotoxic payload is released into the tumor cell cytoplasm but may also diffuse into the surrounding microenvironment (47). Notably, the latter may induce maturation of tumor-resident DCs. We detected increased CD8 and CD4 T cell infiltrates, activation of APCs and T cells as well as reduced Treg frequencies in patients treated with the MMAE-carrying ADC Brentuximab Vedotin (BV) (39). Furthermore, induction of long-lasting tumor-specific T cells was detected in relapsed lymphoma patients responding to BV (with or without donor lymphocyte infusions) post-allogeneic HSCT (48, 49).

DC Stimulation via Immunogenic Cell Death

Apoptotic cell death was historically considered to be non-immunogenic. However, some types of cell death have been demonstrated to induce an immune response against antigens released from dying cells, commonly referred to as immunogenic cell death (ICD). Immunogenic signals released by dying tumor cells can induce antigen uptake as well as antigen processing and presentation by the APC. Although cytotoxic anti-cancer therapies generally induce apoptosis, ICD is only induced in treatment with some of these agents, particularly, anthracyclines (50), oxaliplatin (51), and cyclophosphamide (52) as well as for irradiation (50). ICD is characterized by the induction of endoplasmic reticulum (ER) stress and autophagy, which is in distinction to non-immunogenic apoptosis (53, 54). Hallmarks of ICD include the pre-apoptotic exposure of calreticulin (CRT) on the cell surface, the secretion of adenosine triphophosphate (ATP), and the post-apoptotic release of the chromatin-binding protein high-mobility group box 1 (HMGB1) (50, 55, 56). Importantly, the suppression of each of these signals abolishes the immunogenicity of cell death, demonstrating the non-redundancy of each of these pathways (50, 55, 56).

Cytotoxic agents that trigger ICD are also efficient inducers of CRT cell-surface exposure. CRT is under normal circumstances located at the membrane of the ER. Following the induction of an ER stress response, CRT translocates to the cell surface where it serves as an established "eat me signal" for apoptotic cells (50, 54). This occurs well before the induction of apoptotic changes, such as the surface exposure of phosphatidylserine. Binding of CRT to CD91 on phagocytes induces phagocytosis and macropinocytosis leading to the efficient clearance of these cells (57). CRT is also detectable on the surface of viable cells; however, the expression of the surface molecule CD47 and its binding to SIRP-α on phagocytes efficiently inhibits the uptake of viable cells (57). The induction of CRT surface expression on tumor cells by cytotoxic agents efficiently mediates their uptake by DCs (50). Importantly, CRT-CD91 interaction leads to signaling through NF-κB in DCs and to the release of inflammatory cytokines, in particular TNF, IL-6, IL-1β, and IL-12. This cytokine milieu induced by CRT exposure leads to Th17 priming in an immunosuppressive, TGF-β containing, microenvironment (58).

Adenosine triphophosphate release by dying tumor cells manifests the second hallmark of ICD and is one of the most prominent "find-me" signals for myeloid cells. Upon treatment with selected cytotoxic agents, tumor cells secrete ATP in an autophagy-dependent fashion (53, 59). ATP induces recruitment of myeloid cells into the tumor upon its binding to P2Y2 receptors (55). In the second step, ATP facilitates myeloid cells to differentiate into inflammatory DCs. Furthermore, ATP activates P2RX7 receptors on DCs, which activates the NLRP3 inflammasome leading to IL-1β release. Of note, IL-1β then is required for the priming of $CD8^+$ T cells (60). Importantly, priming of T cells appears to occur predominantly in the tumor microenvironment as no significant abrogation in T cell priming is maintained in a mouse model upon surgical removal of draining lymph nodes (55).

High-mobility group box 1 is a chromatin-binding factor found within the nucleus that can be released by injured cells as they undergo primary or secondary necrosis and thereby induces inflammation (61). Treatment of tumor cells by ICD-inducing cytotoxic compounds leads to a post-apoptotic release of HMGB1, which is recognized by TLR4 on DCs. TLR4 controls the tumor antigen processing and is, therefore, indispensable for efficient cross-presentation of tumor cell antigens by DCs (56). In the absence of TLR4 stimulation, phagosomes fuse with lysosomes, which results in degradation of dying cells in the lysosomal compartment, and consequently inefficient antigen presentation (56, 62). In esophageal squamous cell carcinoma, levels of HMGB1 within the tumor microenvironment were significantly upregulated upon preoperative chemoradiotherapy and patients with a high HMGB1 levels showed a better overall survival compared to those with weak HMGB1 expression (63). These findings underline the clinical relevance of HMGB1 expression. However, the inter-patient variability of HMGB1 expression remains poorly understood. HMGB1 can also bind the T cell immunoglobulin- and mucin-domain containing molecule (Tim-3) that is preferentially expressed on tumor-infiltrating DCs (64). Galectin-9 independent ligation of Tim-3 with HMGB1 leads to a negative regulation of nucleic acid-mediated innate immune responses. It is, therefore, reasonable to hypothesize that the balance between a positive signal through TLR4 ligation and a negative signal through Tim-3 ligation might regulate the ICD induced activation of tumor-resident DCs.

While the described mechanisms leading to ICD are able to induce efficient antigen presentation and cytokine secretion, costimulatory molecules might not be upregulated in most of these ICD-inducing chemotherapeutic regiments (50, 55, 56),

leaving a gap for more efficient selection of chemotherapeutics or the additional administration of adjuvants.

Cytotoxic Agents Targeting Tumor-Resident Immunosuppressive Cells

Regulatory T cells interfere with anti-tumor immune responses by several mechanisms [reviewed in Ref. (65)]. For example, Tregs may inhibit DC maturation via CTLA-4/CD80/CD86 interaction and induce expression of the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO) (66, 67). Several cytotoxic agents are able to target Tregs and thereby promote adaptive anti-tumor immunity. One of the first drugs reported to interfere with Tregs was cyclophosphamide. At low doses, cyclophosphamide depletes Tregs and inhibits their effector functions and homeostatic proliferation, as demonstrated in mouse models and patients (68–71). Consequently, low-dose cyclophosphamide treatment promotes tumor-specific immune responses when combined with different vaccination strategies, including DC-derived exosomes (DEX) (72) and oncolytic adenovirus (73). Yet, the mechanisms underlying this synergy need to be further elucidated. However, it is reasonable, that in the absence of Tregs, $CD4^+$ T cells might activate tumor-resident DCs through CD40L–CD40 interaction, which then can efficiently present tumor antigen and promote T cell activation (74, 75). Additional chemotherapeutic agents targeting Tregs include paclitaxel, which selectively induces apoptosis of Tregs by upregulation of the cell death receptor Fas (76), and low-dose temozolomide, which reduces Treg numbers through poorly understood molecular mechanisms (77, 78).

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells located in the tumor microenvironment and lymphatic organs. These cells can inhibit innate and adaptive immune responses [reviewed in Ref. (79)]. Several chemotherapeutic drugs can promote anti-tumor immunity by either inducing apoptosis of MDSCs or inducing their differentiation into mature myeloid cells with features of DCs or macrophages. 5-Fluorouracil (5-FU) and gemcitabine substantially reduce the numbers of MDSCs by induction of apoptosis (80, 81). In addition, both 5-FU and gemcitabine induce activation of NLRP3 in dying MDSCs following release of cathepsin B from lysosomes. Active NLRP3 triggers secretion of IL-1β, which may induce IL-17 production by T cells, resulting in priming of Th17 cells, neoangiogenesis, and promotion of tumor growth (82). As 5-FU and gemcitabine, however, do not induce ICD, the measured IL-1β concentrations appear to be low compared to the IL-1β secretion triggered by ICD-inducing chemotherapeutics (80). Importantly, at low concentrations, IL-1β does not support the priming of $CD8^+$ T cells and the detrimental effects of IL-1β prevail. Therefore, the therapeutic potential of 5-FU and gemcitabine treatment could be enhanced by co-administration of IL-1β inhibitors. Importantly, 5-FU-induced depletion of MDSCs acts synergistically with Treg depletion induced by low-dose cyclophosphamide treatment, enhancing T cell functions and anti-tumor responses (80). A similar selective depletion of MDSCs and subsequent enhancement of T cell immunity was seen during treatment with doxorubicin or 5-azacytidine (83, 84).

In contrast, non-toxic, low doses of paclitaxel stimulate the differentiation of MDSCs into functional DCs expressing MHCII and costimulatory molecules (85, 86). These functional DCs have lost their suppressive capacity and contribute to the induction of T cell responses. Similarly, docetaxel treatment polarizes MDSCs toward an M1 phenotype with loss of suppressive effects, higher levels of MHCII and CD80 expression, and a shift from IL-10 to IL-12 secretion (87).

Concluding Remarks

The cytotoxic agents that have been used for several decades for anti-cancer therapy were originally selected for their ability to kill tumor cells. Some, but not all, of these reagents are now known to stimulate anti-tumor immunity, which contributes to their therapeutic effect. A detailed characterization of the immune-stimulatory effects of currently used chemotherapeutic agents should guide the way for rational combinations with immunotherapeutic approaches, which should stimulate anti-tumor immune responses in a synergistic fashion. Cytotoxic agents that directly induce DC maturation or ICD are ideal candidates for combining with inhibitors of immune checkpoints such as PD-1 or CTLA-4, which may result in a long-lasting population of effector memory $CD8^+$ T cells (38, 39). In addition, the selective depletion of immune-inhibitory subsets, such as Tregs and MDSCs induced by chemotherapeutic agents, may complement active vaccination strategies and/or checkpoint blockade, by strengthening effector T cell populations (72, 73). The findings discussed here provide the basis for the further development of rational immunotherapeutic protocols in the near future.

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REVIEW
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Regulation of T cell immunity in atopic dermatitis by microbes: the Yin and Yang of cutaneous inflammation

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Atopic dermatitis (AD) is a chronic inflammatory skin disease predominantly mediated by T helper cells. While numerous adaptive immune mechanisms in AD pathophysiology have been elucidated in detail, deciphering the impact of innate immunity in AD pathogenesis has made substantial progress in recent years and is currently a fast evolving field. As innate and adaptive immunity are intimately linked, cross-talks between these two branches of the immune system are critically influencing the resulting immune response and disease. Innate immune recognition of the cutaneous microbiota was identified to substantially contribute to immune homeostasis and shaping of protective adaptive immunity in the absence of inflammation. Disturbances in the composition of the skin microbiome with reduced microbial diversity and overabundance of *Staphylococcus spp*. have been shown to be associated with AD inflammation. Distinct *Staphylococcus aureus* associated microbial associated molecular patterns (MAMPs) binding to TLR2 heterodimers could be identified to initiate long-lasting cutaneous inflammation driven by T helper cells and consecutively local immune suppression by induction of myeloid-derived suppressor cells further favoring secondary skin infections as often seen in AD patients. Moreover dissecting cellular and molecular mechanisms in cutaneous innate immune sensing in AD pathogenesis paved the way for exploiting regulatory and anti-inflammatory pathways to attenuate skin inflammation. Activation of the innate immune system by MAMPs of non-pathogenic bacteria on AD skin alleviated cutaneous inflammation. The induction of tolerogenic dendritic cells, interleukin-10 expression and regulatory Tr1 cells were shown to mediate this beneficial effect. Thus, activation of innate immunity by MAMPs of non-pathogenic bacteria for induction of regulatory T cell phenotypes seems to be a promising strategy for treatment of inflammatory skin disorders such as AD. These new findings demonstrate how detailed analyses identify partly opposing consequences of microbe sensing by the innate immune system and how these mechanisms translate into AD pathogenesis as well as new therapeutic strategies.

Keywords: atopic dermatitis, bacteria, dendritic cells, microbiota, non-pathogenic, T cell, tolerance, skin

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Introduction

The skin is the body's outer interface organ forming a multi-layered barrier between the environment and the individual. To maintain integrity of the host and protect from potentially detrimental influences by pathogens or toxic substances, effective defense mechanisms have evolved. In addition, the skin is colonized by a multitude of bacteria forming the cutaneous microbiome

that provides essential functions for skin homeostasis. Thus anti-bacterial immune responses must be tightly controlled to allow pathogen defense but also to preserve the composition of the microbiota in the absence of inflammation. Innate and adaptive immunity interact and orchestrate different qualities of immune responses. A breakdown in the symbiotic relationship between the cutaneous microbiota and its host has been identified and is associated with skin inflammation in atopic dermatitis (AD) (1). Deciphering mechanisms of the innate immune system which promote skin inflammation in AD patients and identifying counter regulatory pathways that limit inflammation by shaping T helper cell responses have not only broadened our understanding of disease pathology but also opened up new therapeutic perspectives.

Atopic Dermatitis Pathogenesis: In the Beginning, It is All About T Cells

Atopic dermatitis is a frequent inflammatory skin disease affecting up to 10–20% of the children and approximately 3% of adults in western countries (2). Innate (mast cells, eosinophils, basophils, different types of dendritic cells (DC), innate lymphocytes, and myeloid-derived suppressor cells) and adaptive immune cells (B cells and T cells) are believed to contribute to the complex immune network underlying cutaneous inflammation in AD. Few of the experimental setups and studies trying to disclose a role for these different cell types for AD allow defining one of these cell types as causal for cutaneous inflammation with the exception of $CD4^+$ T helper cells which have been shown in several studies, clinical trials, and mechanistic analyses to drive AD (3–5). Consequently, a closer look at the state of the art in regard to T cells in AD is essential to create a modern concept of AD pathogenesis and to develop new therapeutic strategies.

In AD, a dense infiltrate of activated CD4+ T cells can be detected in the dermis especially in acute lesions (**Figure 1**) (6). To better understand the initiation of AD, analyses of atopy patch test lesions has contributed substantially. Analyses of cytokine expression revealed that T helper cells of early lesions produce IL-4, IL-5, and IL-13, hallmark cytokines of Th2 cells (7). Thus, the concept that was developed within the last 20 years was based on the interpretation that Th2 cytokines in the skin promote cutaneous inflammation in AD. As examples of Th2-associated pathology, the Th2-induced isotype switch in B cells leading to the production of IgE is frequently cited and IL-5 promoting maturation and survival of eosinophils are highlighted to play a role in some types of AD and other atopic diseases (8, 9). Mechanistic analyses have helped to understand how these Th2 cells are recruited to the skin also disclosing possible targets of new therapeutic strategies: cutaneous leukocyte antigen (CLA) as adhesion molecule allowing Th2 cells to roll on the luminal side of the high endothelial venules proved to be a good marker for T cells prone to migrate to the skin (10, 11). CCR4 and CCR10 were shown to be prominent chemokine receptors allowing T cells to migrate through endothelia in the skin upon binding with the respective chemokines such as CCL17, CCL22, and CCL27 (11–13), and the chemokines binding CCR4 are among the best biomarkers for AD inflammation (14). Once

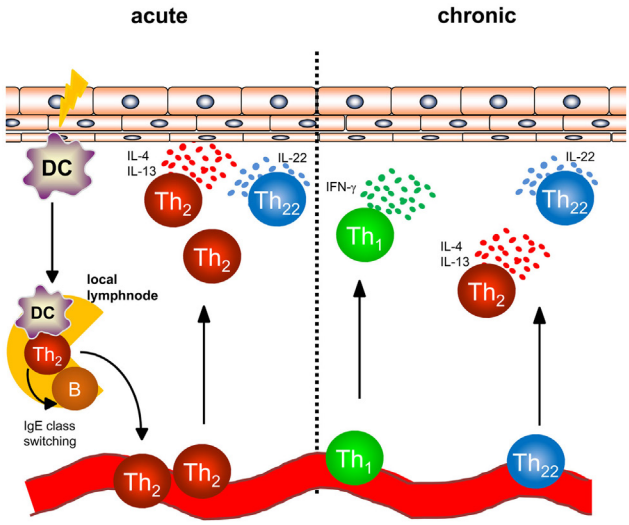

FIGURE 1 | Overview of T helper cell populations in acute and chronic dermatitis. In acute atopic dermatitis activated skin-resident DC migrate to local lymphnodes to prime naïve T helper cells and polarize them into a Th2 phenotype. Th2 cells induce IgE class switching in B cells accounting for enhanced IgE levels regularly found in atopic dermatitis patients. Th2 cells are recruited back to the skin and induce cutaneous inflammation by effector cytokines accompanied by Th22 cells. In chronic atopic dermatitis, Th1 cells are increasingly part of the skin infiltrate consisting of Th1, Th2, and Th22 cells.

Th2 cells are recruited to AD skin, Th2 cell activation leads to the accumulation of high levels of Th2 cytokines. Interestingly, IL-4 orchestrates monocytes and DC to produce high amounts of CCR4-binding chemokines further amplifying Th2 cell recruitment to the skin (15–17). Besides the effect on these immune cells, Th2 cytokines also fundamentally influence keratinocytes and how the epidermis responds to different stimuli. Th2 cytokines suppress the expression of terminal differentiation proteins of keratinocytes (filaggrin, loricrin and involucrin) therefore destabilizing cutaneous barrier function. In addition, Th2 cytokines have been shown to suppress the upregulation of antimicrobial peptides (AMP) in the skin upon stimulation such as beta defensin (HBD)-2, HBD-3, and LL-37 in keratinocytes. This failure to secrete AMPs to reach levels found in psoriasis has been argued to be the underlying mechanism of the susceptibility to bacterial and viral infections found in AD (18–20). Bacterial colonization on AD skin may be further supported by IL-4-mediated enhanced expression of fibronectin and fibrinogen acting as adhesion molecules for *Staphylococcus aureus* (21, 22). The Th1 cell and Th17 cell subsets are known for their potent anti-infectious properties controlling for intracellular and extracellular bacterial and fungal infections (23). Thus, demonstrating that IL-4 potently suppresses Th1 and Th17 cell immunity (24–28) further emphasized that AD skin is fundamentally more susceptible to cutaneous colonization and infection than normal or psoriasis skin. Most recent analyses even demonstrated that IL-4 reduces the Th17 inducing and maintaining cytokine IL-23 in antigen presenting cells both *in vitro* and *in vivo* in humans (28). These findings highlight that analyzing the

recruitment, persistence, and function of different Th cell subtypes into AD skin is of pivotal importance for better understanding AD and for disclosing the impact of bacteria for AD inflammation, its prevention, and resolution.

Th17 cells were characterized by the production of IL-17 and IL-22 (29, 30). Following Th17 characterization, screening analyses were carried out for different diseases and tissues to better understand the Th17 cell function. Immunohistochemical studies revealed IL-17 production in acute AD lesions and confirmatory studies showed correlation of AD severity with the number of IL-17-producing T cells in peripheral blood and acute lesions (31, 32). Further characterization of IL-17-producing T cells in acute AD lesions revealed that IL-17 was produced by newly described subsets of Th2/IL-17$^+$ and Th0/IL-17$^+$ cells (33). Interestingly, IL-17 production by these subsets required stimulation by staphylococcal superantigens indicating interdependence of bacterial products and IL-17 in AD skin. It is still not understood why despite Th2 cytokines such as IL-4 suppressing IL-17 and IL-23, IL-17-producing cells are still detected in AD and whether IL-17 contributes to AD initiation or represents an epiphenomenon of cutaneous colonization and infection with bacteria in AD (27, 28, 33). Thus, the role of IL-17 in AD needs further clarification and new drugs being available targeting IL-17 and IL-17R for the treatment of psoriasis such as secukinumab will soon shed light into the hitherto unknown role of IL-17 for AD. As microbiota also induce or condition for IL-17 production, defining the role of IL-17 for skin homeostasis, defense, and inflammation requires functional analyses, disclosure of the cellular network, and spatiotemporal differentiation.

More recently, another unique subset of T helper cells enriched in inflamed human skin producing IL-22 in the absence of IL-17 was identified and characterized (34). These Th22 cells express the skin homing chemokine receptors CCR4 and CCR10 like Th2 cells and are distinct from Th17 cells as shown by transcriptome analyses (34, 35). Accumulation of Th22 cells was demonstrated in acute and chronic lesions of AD (36, 37) as were IL-22-producing CD8+ T cells (33, 36). IL-22 binds to a complex of IL-22R1 and IL-10R2 for induction of downstream signaling (38). IL-22R1 is not expressed on hematopoietic cells but rather can be detected on tissue-resident cells including keratinocytes (39). Functional consequences of IL-22 production are dependent on the target organ and the presence or absence of other cytokines, such as IL-17 or TNFα leading to either protective immune responses or inflammation (38, 40). IL-22 acting on keratinocytes has been reported to downregulate filaggrin expression and to affect expression of profilaggrin processing enzymes leading to further impairment of the epithelial barrier (41). Furthermore, IL-22 was reported to inhibit terminal differentiation of keratinocytes and to induce epidermal hyperplasia which is prominently seen in chronic AD (34). Thus IL-22-producing T cells may well play a crucial role in the pathogenesis of AD (42), however, the exact role cannot be defined based on the data available. Functions enhancing processes of both inflammation and regeneration describe Th22 cells as a Janus-like player for AD.

Atopic Dermatitis Pathogenesis: Chronic and Self-Perpetuating Inflammation Through Bacterial Exposure

While acute flares of AD are characterized by an infiltrate consisting of Th2 and Th22 cells, Th1 cells can be detected in chronic lesions of AD (**Figure 1**) in addition to Th2 and Th22 cells as early as 48 hours following elicitation of dermatitis (7, 37, 43). Th1 cells are characterized by the transcription factor T-bet and the secretion of the inflammatory cytokine IFN-γ (44). In regard to the pathogenesis of chronic AD, IFN-γ was postulated to contribute to skin hypertrophy in chronic AD (45). IFN-γ strongly induces IL-22R1 on keratinocytes allowing IFN-γ and IL-22 to act together for induction of epidermal hyperplasia (39).

Th1 cells can be polarized from naïve T helper cells by DC secreting large amounts of IL-12p70 (46, 47). As DC are at the interface of innate and adaptive immunity and build a dense network of immune sentinels in the skin, innate immune signals activating skin-resident DC were postulated to contribute to enhanced priming of Th1 cells in AD leading to chronic skin inflammation (48, 49). *S. aureus* colonization found in the majority of AD skin lesions and very early during lesion development has been shown to contribute to the release of pharmacological relevant amounts of Toll-like receptor (TLR2) agonists such as lipoteichoic acid (50). Binding of *S. aureus*-derived lipoteichoic acid to TLR2 on DC *in vitro* leads to DC maturation and production of the pro-inflammatory cytokines IL-12p70 and IL-23 resulting in enhanced Th1 and Th17 priming (51). Amplification of Th1 polarization is further achieved by the presence of the Th2 cytokine IL-4 acting on DC resulting in enhanced IL-12p70 production during T cell priming (52–54). As IL-4 is abundantly present in the skin of acute flares of AD as is *S. aureus*, combinatorial activation of DC by IL-4 and TLR2-ligands is a constant feature in AD skin. Based on this combined stimulation of cutaneous DC, a profound shaping of consecutive immune response can be anticipated (55). Indeed, TLR2 activation together with IL-4R signaling transfers acute Th2-driven dermatitis (48 h before dermatitis resolution) into long-lasting skin inflammation (14 days) with enhanced expression of the Th1 cytokine IFN-γ (56). A key role could be ascribed to the Th2 cytokine IL-4 mediating suppression of the anti-inflammatory cytokine IL-10 while enhancing IL-12 (**Figure 2**). Administration of exogenous IL-10 reverted chronic skin inflammation induced by TLR2 ligands and IL-4 indicating a crucial role for sustained production of IL-10 in response to cutaneous exposure to microbes. In conclusion, innate immune signals derived from *S. aureus* colonization or infection play a pivotal role in the transition of acute dermatitis into chronic skin inflammation and disease exacerbation highlighting a crucial role for microbes in the pathogenesis of AD (56). Furthermore, these mechanistic studies in AD models also revealed that IL-4 is responsible for the transition of early self-limiting AD into chronic self-perpetuating AD as found in most of AD patients. These findings demonstrate that Th2 cytokines such as IL-4 and IL-13 are not only responsible for early AD lesions but also for the development and maintenance of chronic AD. This also explains why targeting AD by the recently described antibody dupilumab blocking both IL-4 and IL-13 is highly effective in AD patients.

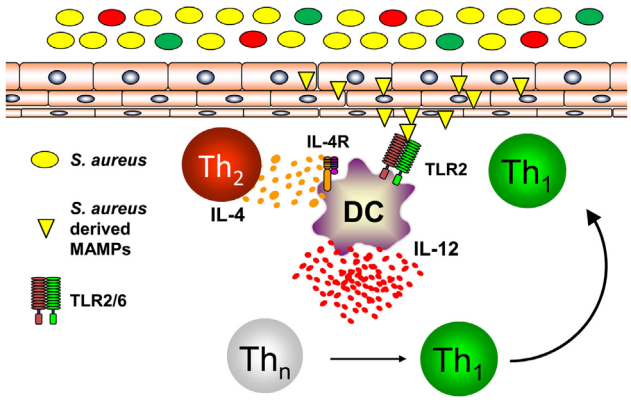

FIGURE 2 | Dual activation of skin-resident DC by IL-4 and TLR2 ligands promotes IL-12 expression and Th1 polarization. In atopic dermatitis skin, Th2 cells secreting IL-4 are abundantly present. Skin-resident DC are activated by *S. aureus* derived TLR2 ligands (lipoproteins, lipoteichoic acid) in an IL-4 rich environment leading to DC maturation and enforced IL-12 secretion by combinatorial activation of TLR2- and IL-4R-signaling while IL-10 production is markedly attenuated. As a consequence in the local lymph nodes, naïve T helper cells are preferentially polarized into a Th1 phenotype promoting long-lasting cutaneous inflammation after homing to atopic dermatitis skin.

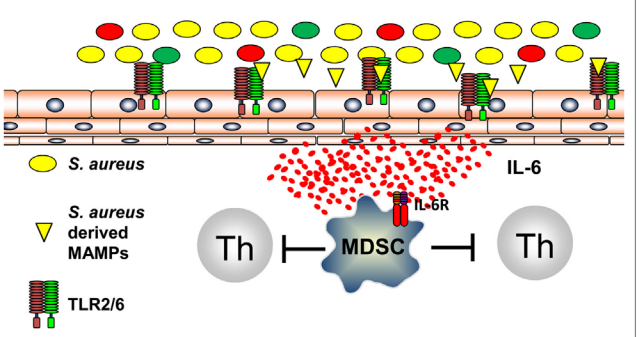

FIGURE 3 | *S. aureus*-derived lipoproteins induce MDSC. TLR2/6 heterodimers are expressed on skin-resident cells. Binding of diacylated bacterial lipoproteins induces IL-6 secretion leading to accumulation and activation of MDSC in the skin. T cell activation is vigorously suppressed by a NO-dependent mechanism.

Dupilumab is expected to be the first biologic being launched for AD treatment after having shown efficacy in several clinical trials (57). However, these analyses clearly highlight an intrinsic failure to terminate IL-4- and TLR2-driven chronic self-perpetuating AD as prominent feature of AD pathogenesis.

It can be anticipated that all types of inflammation, including those of anti-infectious immune responses, induce immune pathways that are capable to terminate inflammation and to prevent exaggerated and self-perpetuating potentially harmful courses of inflammation. Recently, it could be shown that innate immune recognition of *S. aureus* induces, following TLR2-induced inflammation, immune suppression by induction of myeloid-derived suppressor cells (MDSC) in a mouse model of AD (58). *S. aureus*-derived lipoproteins binding exclusively to the TLR2/6 heterodimer on skin-resident cells trigger IL-6 secretion that leads to activation and recruitment of $CD11b^+$ $Gr1^+$ MDSC into the skin (**Figure 3**). These $CD11b^+$ $Gr1^+$ MDSC efficiently suppressed T cell recall responses in the skin by an iNOS-dependent pathway. These MDSC, however, fail to suppress AD inflammation, but rather inhibit anti-infectious immune responses allowing *S. aureus* to further spread and drive AD and possibly also herpes viruses to mount a dangerous AD complication, eczema herpeticum (58). These investigations show that innate immune recognition of pathogenic bacteria on the skin induces an anti-inflammatory pathway presumably to limit damage induced by inflammatory responses. Distinct temporal and spatial distributions may coordinate these different and counter regulatory immune responses and understanding underlying mechanisms of attempts to prevent or resolve inflammation are important areas of research to identify new targets of treatments. Some of these mechanisms may be identified by the analyses that focus on the skin microbiome and immune consequences derived of the communication between the microbiome, the skin, and the cutaneous immune system.

Skin Microbiome

The skin is constantly colonized by myriads of bacteria with approximately 10^6 bacteria homing per square centimeter resulting in a total of 10^{10} bacteria covering the whole skin (59). The composition and distribution of the cutaneous microbiota have been deciphered in yet unknown details using deep sequencing techniques (60, 61). These techniques revealed a much more diverse microbiota as previously anticipated and detected using culture-based methods (61). The majority of bacteria identified by 16S ribosomal RNA sequencing can be assigned to four major phyla: Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria (62). Importantly, 16S rRNA sequencing also detected Gram-negative species at dry skin areas, which were previously seen as contaminants but not residents using culture-based techniques (63).

Examining different skin sites, it was found that the skin microbiota differs between topographical locations (60). The colonization of bacteria on the different areas of human skin is largely dependent on the physiology of the respective skin site. Whether the area is predominantly "moist," "dry" or "sebaceous" impacts the respective microbiota to a large extent. At moist areas, *Staphylococcus spp.* and *Corynebacterium spp.* are the most abundant bacterial communities detected (59, 60). The highest diversity of phylotypes can be observed at dry skin areas like forearms, buttocks, and part of the hands with multiple species from the phyla Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria being detected.

In contrast, sebaceous sites have the lowest diversity of phylotypes with a predominance of *Propionibacterium spp.* confirming culture-based approaches showing that *Propionibacterium spp*. are commensals in areas rich in the "pilosebaceous units" consisting of hair, hair follicle, and sebaceous gland (60).

Comparing interpersonal and intrapersonal variations of the skin microbiota at distinct topographical sites, it was shown that interpersonal variations are less than anticipated indicating that the colonized niche and its physiological features are a much stronger determinant for composition of the bacterial colonization than genetic variations of the individuals investigated (59, 62). In addition, the microbiota of one individual of, e.g., some sebaceous sites that share the same ecological features display a large similarity, further supporting the concept of cutaneous habitats determining the compositions of microbes by the cutaneous milieu.

Microbial colonization of the skin is established early in life. With birth, transition of the newborn's surfaces derived from a sterile uterine milieu then exposed to a microbial-rich environment occurs. Thus, immediately after birth the skin of the newborn is being colonized (64). The mode of delivery has been shown to play a major impact on the composition of the skin microbiota of the newborn. Following vaginal delivery, the skin microbiota resembles the mother's vaginal microbiota. In contrast, following cesarean delivery, the skin microbiota of the mother and the skin microbiota of the newborn show striking differences (64). The impact of these observations on the composition of the individual microbiota and the infant's health is largely unknown, but consequences for immune responses have been postulated. Interestingly, the composition of the skin microbiota of 1 to 3-month-old children did not differ in regard to mode of delivery (65), whereas other studies demonstrated a striking stability of individual microbiota over time. Indeed, the microbiome of adults is maintained over time whereas the microbiome of children shows less stability and even an increase in microbial diversity over time (65). The consequences of individual microbiota developing at different time points for immune responses, immune tolerance development, and the "readiness" for defense are still a matter of debate. Whether disturbances and dysbiosis during the process of microbiota development influence susceptibility to skin diseases associated with dyscolonization of the skin remains to be established.

Functional Consequences of the Skin Microbiome

The studies of the intestinal and the cutaneous microbiome are for the most part descriptive in nature and associations of certain microbiota or of compositions within detected microbiota with diseases are increasingly being detected. However, functional consequences still need to be proven, underlying mechanisms of diseases need firm linkage to microbial exposure, and pathways of translating surface exposure to microbes into immune responses and memory still need to be established. Only few mechanisms of how the skin microbiome or specific microorganisms contribute to cutaneous immune homeostasis have been identified so far. Importantly, functional studies need to also involve pre-clinical analyses in disease models. However, animals with fur may not follow the same rules and algorithms as human skin in the absence of fur.

Much attention has been given to consequences of cutaneous exposure to *staphylococci* with the assumption of *Staphylococcus epidermidis* being the "good" part of the microbiome and *S. aureus* being the "bad" causing infections and disease. Indeed, cutaneous colonization of severe barrier-disrupted murine skin with *S. aureus* leads to expression of IL-1β, IL-6, and TNFα lasting for up to 6 days demonstrating a pivotal role for *S. aureus* in promoting skin inflammation in susceptible hosts (66). After skin wounding and subsequently initiated inflammation due to TLR3-mediated detection of damaged cells, the skin commensal *Staphylococcus epidermidis* was identified to mediate resolution of cutaneous inflammation. Binding of *Staphylococcus epidermidis* derived lipoteichoic acid to TLR2 on keratinocytes was crucial in mediating this anti-inflammatory function via a TRAF1 dependent pathway (67). Recently, it was shown that recognition of *S. epidermidis* by the innate immune system profoundly shapes adaptive immune responses (68). Colonization of the skin with *S. epidermidis* activates antigen-specific T cells secreting IL-17 and IFN-γ in an IL-1R- and MyD88-dependent manner at steady-state conditions (68). Lack of commensals lead to a failure of induction of dermal IL-17- and IFN-γ-producing αβ T cells but raised the numbers of FoxP3$^+$ regulatory T cells in the skin. To identify functional consequences of cutaneous *S. epidermidis* exposure, a model of Leishmania infection was applied showing that in the absence of sufficient immune sensing of *S. epidermidis*, Leishmania infection could not be controlled sufficiently (68). Further analysis of the cellular pathways involved in this process showed that IL-17-producing T cells induced by *S. epidermidis* belong to the CD8+ subset displaying a unique and previously unknown role for these T cells in providing immune defense mechanisms in the skin (69). Thus, colonization of the skin with the commensal *S. epidermidis* provides innate immune signals to set up a functional threshold for adaptive immunity to establish pathogen control. Adaptive immune responses induced by the microbiota are required not only to fight pathogens but also to control colonization of the skin with commensals as in the absence of adaptive immunity commensal bacteria were detected in the local lymph nodes indicating microbial invasion (70). Skin microbiota therefore seems to induce a feedback loop to control microbial colonization with commensals to maintain epithelial integrity and immune homeostasis.

Changes of the skin microbiota composition might therefore contribute to cutaneous inflammation seen in various skin diseases (71). Detailed analysis of the cutaneous microbiota of AD patients demonstrated dramatically reduced diversity of the microbiome analyzed from acute flares presenting at the antecubital and popliteal crease (1). Instead of microbiota diversity overabundance of *S. aureus* and *S. epidermidis* was detected and correlated with disease severity confirming previous observations demonstrating *S. aureus* colonization and worsening of AD (1, 72). Of note, the "hen and egg problem" is not solved. Loss of microbiome diversity leading to skin flares or skin flares orchestrating dramatically reduced diversity of the microbiome are both possible scenarios. While for humans, the latter was thought to be the more likely cascade of events, new insights were brought forward by a mouse model.

A new model to study these changes from humans in detail including possibly causal relations was established most recently: mice lacking epidermal ADAM17 (*Adam17*$^{\Delta Sox9}$, *A17*$^{\Delta KC}$) exhibit skin barrier disruption with enhanced transepidermal water loss (TEWL) and subsequently develop eczematous lesions with a lymphocytic infiltrate resembling human AD (73, 74). The skin microbiome of mice with disrupted barrier function due to epidermal

deficiency of ADAM17 (*Adam17*$^{\Delta Sox9}$) was indistinguishable from wildtype littermates in the first 2 weeks after birth but showed decreased bacterial diversity and an abundance of *Staphylococcus spp.* and *Corynebacterium spp.* compared to wildtype controls starting 4 weeks after birth (74). Administration of antibiotics in *Adam17*$^{\Delta Sox9}$ mice targeting *S. aureus* prevented the development of eczematous lesions and TEWL as well as cytokine production by CD4+ T cells and an increased number of skin-infiltrating T cells. Moreover, the microbiome showed a higher diversity – despite the use of antibiotics – and was comparable to the one of wildtype controls. In crossover experiments, antibiotic depletion of *S. aureus* in *Adam17*$^{\Delta Sox9}$ mice resulted in reduction of eczematous lesions, reduced TEWL, attenuation of skin-infiltrating T cells, and reversal of dysbiosis. By contrast, withdrawal of antibiotics in previously administered *Adam17*$^{\Delta Sox9}$ mice resulted in development of eczema, enhanced TEWL, increased skin-infiltrating T cells, and severe dysbiosis with excessive *S. aureus* colonization (74). These data clearly demonstrate that a shift of the microbiome resulting in dysbiosis with reduced microbial diversity and overrepresentation of *S. aureus* strikingly contributes to development of cutaneous inflammation and acute atopic flares in both mice with disrupted barrier and susceptible humans (1, 74). Thus enhancing the diversity of the microbiome to support colonization with putative non-pathogenic beneficial bacteria and/or targeting *S. aureus* colonization may be a therapeutic strategy in treatment of AD.

Resolution and Prevention of Atopic Skin Inflammation

While functional analysis of the skin microbiome is currently in its beginnings, much more details on the functional properties of the gut microbiota were gathered (75, 76). At steady-state conditions, IL-17A- and IFN-γ-producing T cells can be found in the gut-associated lymphoid tissue (GALT) and are required for prevention of inflammation by intestinal pathogens (77). Induction of these T cell populations has been shown to be dependent on the presence of the gut microbiota because in germ-free mice their numbers are significantly reduced (77, 78). This situation closely resembles what was found in the skin, namely turning off the local immune response by commensals to avoid inflammation by pathogens (71, 79). In contrast to the skin, FoxP3$^+$ regulatory T cells (Tregs) are abundantly present in the GALT especially lining the lamina propria and Treg induction is dependent on the intestinal microbiota (79, 80). Several distinct mechanisms for induction of these regulatory T cells by the microbiota have been elucidated. Polysaccharide A (PsA) from Gram-negative *Bacteroides fragilis* has been shown to bind to TLR2 and to exert immune modulatory capacities in murine models of intestinal inflammation (81). The regulatory effects of PsA are due to induction of FoxP3$^+$ iTregs and IL-10 secretion. Furthermore PsA is critical for maintaining immune homeostasis in the gut epithelium (81). A mixture of non-pathogenic *Clostridium spp* lacking toxins and virulence factors was identified to induce FoxP3$^+$ Tregs in a TGF-β dependent manner preventing experimentally induced intestinal inflammation (82, 83). Whether analogous approaches performed in animal models for the gut are also feasible to attenuate skin inflammation was not yet investigated.

Recently, it was shown by investigating humans that the diversity of the environmental microbiota and the prevalence of atopic diseases are interrelated and in atopic individuals a significant reduced diversity of Gammaproteobacteria at their surroundings and on their skin could be found (84). Relative abundance of Gammaproteobacteria was correlated with IL-10 secretion by PBMCs of human healthy individuals while this IL-10 secretion was lacking in atopic patients. Investigations on the level of genus revealed that the reduced frequency of Gram-negative *Acinetobacter* best correlated with diminished IL-10 production in atopic individuals (84). Moreover, it was shown that heat-inactivated *Acinetobacter Iwoffi* induces IL-10 production in DC and primary human keratinocytes *in vitro* and when applied intradermally (85). This work extends investigations that showed that *Acinetobacter Iwoffi* exposure of pregnant mice avoids allergic asthma development in their off springs (86). These findings highlighting a possibly beneficial role for Gram-negative bacteria both for prevention of atopic diseases and for maintaining skin microbiota propose to investigate new therapeutic strategies extending the regulation of the skin microbiome beyond the focus on Gram-positive bacteria such as *S. epidermidis*. We performed the first proof-of-concept RCT in humans to investigate whether signals derived from non-pathogenic Gram-negative bacteria may mediate beneficial effects for the skin with mild AD. Therefore, we initiated a randomized double-blind placebo-controlled clinical trial on AD patients using a lysate from Gram-negative *Vitreoscilla filiformis*. Groups of patients with AD received either vehicle cream or this vehicle cream supplemented with a lysate of *V. filiformis* for 29 days. A first analysis following 15 days of treatment already detected a significant change in the group treated with the *V. filiformis* supplemented cream. Following 29 days of treatment with *V. filiformis*-derived substances, a significant reduction of disease activity in patients receiving topical therapy with a cream containing *V. filiformis* was observed, moreover, intergroup comparison revealed a significant difference between groups demonstrating clinical efficacy of immune signals derived from Gram-negative *V. filiformis* (87). Consecutive work focused on underlying mechanisms mediating immune modulation induced by immune signals derived from Gram-negative *V. filiformis*. To be able to analyze this mechanism of action possibly inducing tolerogenic immune responses effective on the skin, a mouse model for AD was utilized. Topical application of a lysate of *V. filiformis*, the Gram-negative, non-pathogenic bacterium, was shown to suppress skin inflammation in Th2-dominated hypersensitivity in the AD-prone NC/Nga mice (88). To unravel the underlying mechanisms, a series of *in vitro* and *in vivo* experiments was performed. It could be shown that *V. filiformis* lysate predominantly induces IL-10-producing DC in a TLR2-and MyD88-dependent manner. Priming of naïve T cells with DC activated by *V. filiformis* lead to the induction of Tr1 cells, which are characterized by their cytokine profile with high IL-10 and low IFN-γ levels (88, 89). The induction of IL-10^{high} IFN-γ^{low} Tr1 cells by innate immune signals from *V. filiformis* was dependent on DC-derived IL-10 and TLR2-signaling in DC. *V. filiformis* induced Tr1 cells had the capacity to suppress effector T cell proliferation and function demonstrating their regulatory function (**Figure 4**). Enhanced IL-10 production, reduced IFN-γ levels and diminished T cell proliferation in skin draining lymph

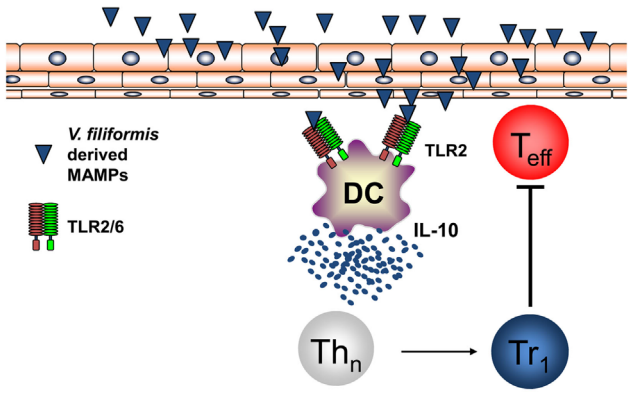

FIGURE 4 | Non-pathogenic bacterium *V. filiformis*-derived MAMPs induce tolerogenic DC and Tr1 cells. *V. filiformis*-derived MAMPs activate DC to produce IL-10 via a TLR2-dependent mechanism. DC-derived IL-10 is required to subsequently polarize naïve T helper cells into a Tr1 phenotype characterized by low IFN-γ and high IL-10 secretion. Vf-induced Tr1 cells efficiently block effector T cells (T_{eff}) demonstrating regulatory function for attenuating skin inflammation.

nodes of mice topically treated with *V. filiformis* lysate compared to controls was observed demonstrating *in vivo* functionality of this approach (88). These data demonstrate that cutaneous exposure to innate immune signals derived of non-pathogenic Gram-negative bacteria is sufficient to induce long-lasting immune tolerance by induction of IL-10, that limited cutaneous exposure is capable to induce also systemic immune modulation, and that indirect supplementation of IL-10 lacking in atopic individuals by innate immune signals from non-pathogenic Gram-negative bacteria may be a feasible therapeutic approach to stabilize both the cutaneous barrier and immune homeostasis.

Summary

T helper cells play a key role in eliciting and maintaining AD inflammation. Therefore, modulating T cell-elicited immune responses is a promising therapeutic approach. Th1- and Th1/Th17-mediated skin inflammation leading to psoriasis or cutaneous delayed-type hypersensitivity reactions could be treated successfully by immune deviation approaches targeting T helper cell polarization and inducing immune deviation (25, 26, 28). The contribution of various T helper cell subsets (Th2, Th1 Th22, maybe Th17) to AD pathophysiology precludes application of this successful principle as it could be detrimental and result in worsening of disease severity. Thus, induction of tolerogenic immune responses promises to be a feasible strategy. Innate immune recognition of specific components or bacteria derived from the gut microbiota has been shown to induce tolerance based on induction of tolerogenic DC, regulatory T cells, and anti-inflammatory cytokines. This approach could by successfully applied to dampen AD inflammation by activating DC with innate immune signals of non-pathogenic bacteria resulting in induction of tolerogenic DC, priming of regulatory Tr1 cells, and attenuation of cutaneous inflammation. After identification of diminished bacterial diversity in the skin microbiome of atopic individuals with consecutive loss of anti-inflammatory and tolerogenic IL-10, substitution of these tolerance promoting innate immune signals using microbes or microbial components is a new and promising therapeutic strategy.

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