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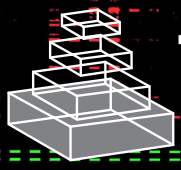
CANCER IMMUNOTHERAPY & IMMUNO-MONITORING: MECHANISM, TREATMENT, DIAGNOSIS, AND EMERGING TOOLS

Topic Editors
Chao Ma, Rong Fan and Antoni Ribas



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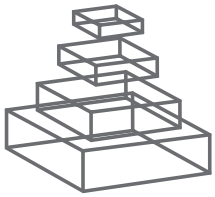

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CANCER IMMUNOTHERAPY & IMMUNO-MONITORING: MECHANISM, TREATMENT, DIAGNOSIS, AND EMERGING TOOLS

Topic Editors:
Chao Ma, California Institute of Technology, USA
Rong Fan, Yale University, USA
Antoni Ribas, University of California Los Angeles, USA

A corner of the high information-content fluorescence readout from a 5000-chamber single cell chip. Single primary human T lymphocytes were isolated into separate micro-chambers on the microfluidic chip, where the secretion of 20 functional cytokines was measured simultaneously by the barcode-shape antibody-based surface detection strips. This type of high-throughput, highly multiplexed, miniaturized technology unprecedentedly captured the profound functional heterogeneity within cellular immunity and has showed tremendous potential in uncovering the complexity underneath human biology and medicine.

In the past decade, significant progresses have taken place in the field of cancer immunotherapy. Tumor-targeting immunotherapies are being developed for most human cancers, including melanoma, prostate cancer, glioblastoma, sarcoma, lung carcinoma and hepatocellular carcinoma. The FDA has approved multiple molecular immunotherapeutics, such as Ipilimumab; cellular immunotherapies (e.g. adoptive cell transfer) are being tested in phase II/III clinical trials. Immunotherapetics has evolved into a sophisticated field: Multimodal therapeutic regimens are administrated to induce focused responses, curtail side-effects and improve therapeutic efficacy.

The lack of effective clinical assessment tools remains a major challenge. Because of the intricacy of antitumor response, it is essential to scrutinize individual tumor-targeting immune cells and their functions at the finest details – molecules. In this regard, flow cytometry analysis modernized hematology and allows characterization of surface molecular signature on individual cells. More recently, microchip technologies and new variations of cytometry have enormously expanded the spectrum, throughout and multiplexity of single cell analysis. Nowadays, tens of millions of readouts can be generated through the course of a cancer immunotherapy to monitor the abundance, phenotype and a myriad of effector functions of single immune cells. At the same time, big data analytics and data mining methodologies have been adapted to achieve sensible diagnostic interpretations. Such a marriage of technology and analytics opens the door for informative point-of-care assessment of therapeutic efficacy and ensures timely therapeutic decisions. The new generation of personalized clinical diagnostics will revolutionize healthcare in the years to come.

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Cancer immunotherapy and next-generation clinical immune assessment

***Chao Ma*[1,2]* and *Rong Fan*[3,4]**

[1] Division of Physics, Mathematics and Astronomy, California Institute of Technology, Pasadena, CA, USA
[2] David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA
[3] Department of Biomedical Engineering, Yale University, New Haven, CT, USA
[4] Yale Comprehensive Cancer Center, New Haven, CT, USA
*Correspondence: chaoma@caltech.edu

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Wolf Hervé Fridman, University Paris Descartes, France

Keywords: cancer immunotherapy, immune suppression, tumor immunity, immune assessment, single cell analysis, cytokine, functional heterogeneity, combinatory therapy, immune evasion, functional proteomics

The interplay between cancerous cells and immune cells has always been an intriguing topic in medicine and biology. Cancer cells emerge from self-cells through a series of genetic mutations. They often retain self-cells' capacity in being exempt from immune surveillance. Therefore, bringing cancer cells back under the radar of immune system has long been considered as a necessary step toward complete tumor eradication and long-term antitumor protection. Based on this rationale, a series of immunotherapies were designed and many have shown promising results. Some have gone through multiple stages of clinical trials. As a result, a successful immunotherapy is an intricate clinical procedure that affects the function of a myriad of cells. Only comprehensive studies that profile multiple aspects (e.g., cellular abundance, phenotypes, and functions) over time at the finest details can effectively monitor the convoluted immune response induced by therapy. Many recent technical developments aim to provide a solution for comprehensive clinical immune assessment.

In this book, we compiled a series of high-quality papers that summarize recent developments of immune assessment tool and methodology, as well as new biological findings in tumor immunity and cancer immunotherapy.

The book starts with a number of reviews and research articles that form an update of cancer immunotherapy. Ma et al. (1) reviewed new technologies to assess functional proteomics of single immune cells, their applications in clinical cancer immunotherapy, as well as new big-data computational methods to interpret the massive readouts. Next, a review paper by Chen et al. (2) highlighted recent advances in microfluidics tools used for functional immunophenotyping and emphasized the potential of integrated microfluidics circuitry. Klinke (3) focused on the concept of combining next-generation genome sequencing and computational power to uncover mechanism underlying tumor immunity evolution. In their opinion papers, Kwak et al. (4) and Fan et al. (5) hypothesized the importance of protein secretion profile in developing definitive correlates for cancer and immune heterogeneity.

The book goes on to the discussion of biology behind cancer immunotherapy. Monjazeb et al. (6) explored the topic of tumor induced immune suppression and proposed combinatorial therapy to induce antigen non-specific immune response and overcome immune evasion. Najjar and Finke (7) reviewed the role of myeloid derived suppressor cells (MDSC) in tumor mediated immune evasion and updated the status of pre-clinical and clinical tumor therapies designed for MDSC inhibition. Kawakami et al. (8) suggested that using combinatory therapy that targets shared immunosuppressive signaling pathway inhibitors to treat cancer. Dobrzanski (9) summarized recently discovered functions of CD4 T cell and new T cell lineages relevant to tumor immunity and tumor progression. Finally, in a research article, Milano et al. (10) showed pre-clinical evidence of nanocurcumin in improving the efficacy of dendritic cell-based immunotherapy for esophageal adenocarcinoma.

The editors thank all authors for their contributions and appreciate the valuable discussions with our reviewers. We wish that this special issue would serve as a reference book to the field and will inspire more thoughts and discussions for future investigation.

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Single cell functional proteomics for assessing immune response in cancer therapy: technology, methods, and applications

Chao Ma[1,2]*, Rong Fan[3,4]* and Meltem Elitas[3]

[1] *Division of Physics, Mathematics and Astronomy, California Institute of Technology, Pasadena, CA, USA*
[2] *David Geffen School of Medicine, University of California, Los Angeles, CA, USA*
[3] *Department of Biomedical Engineering, Yale University, New Haven, CT, USA*
[4] *Yale Comprehensive Cancer Center, New Haven, CT, USA*

Edited by:
Antoni Ribas, University of California Los Angeles, USA

Reviewed by:
Franca Ronchese, Malaghan Institute of Medical Research, New Zealand
Jianping Fu, University of Michigan, USA

****Correspondence:***
Chao Ma, Division of Physics, Mathematics and Astronomy, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125, USA
e-mail: chaoma@caltech.edu;
Rong Fan, Department of Biomedical Engineering, Yale University, 55 Prospect Street, MEC 213, New Haven, CT 06520, USA
e-mail: rong.fan@yale.edu

In the past decade, significant progresses have taken place in the field of cancer immunotherapeutics, which are being developed for most human cancers. New immunotherapeutics, such as Ipilimumab (anti-CTLA-4), have been approved for clinical treatment; cell-based immunotherapies such as adoptive cell transfer (ACT) have either passed the final stage of human studies (e.g., Sipuleucel-T) for the treatment of selected neoplastic malignancies or reached the stage of phase II/III clinical trials. Immunotherapetics has become a sophisticated field. Multimodal therapeutic regimens comprising several functional modules (up to five in the case of ACT) have been developed to provide focused therapeutic responses with improved efficacy and reduced side-effects. However, a major challenge remains: the lack of effective and clinically applicable immune assessment methods. Due to the complexity of antitumor immune responses within patients, it is difficult to provide comprehensive assessment of therapeutic efficacy and mechanism. To address this challenge, new technologies have been developed to directly profile the cellular immune functions and the functional heterogeneity. With the goal to measure the functional proteomics of single immune cells, these technologies are informative, sensitive, high-throughput, and highly multiplex. They have been used to uncover new knowledge of cellular immune functions and have been utilized for rapid, informative, and longitudinal monitoring of immune response in clinical anti-cancer treatment. In addition, new computational tools are required to integrate high-dimensional data sets generated from the comprehensive, single cell level measurements of patient's immune responses to guide accurate and definitive diagnostic decision. These single cell immune function assessment tools will likely contribute to new understanding of therapy mechanism, pre-treatment stratification of patients, and ongoing therapeutic monitoring and assessment.

Keywords: immune function, cytokine, cancer therapy, single cell method, immune assessment, antitumor immune response

The field of targeted cancer therapeutics and immunotherapy has gone through significant maturation in recent years. For example, Ipilimumab, an antibody that blocks a T-cell function-regulating surface receptor (CTLA-4), was approved by the Food & Drug Administration (FDA) for treatment of metastatic melanoma (Hodi et al., 2010); Adoptive cell transfer (ACT) therapy that utilizes T cells expressing transgenic T cell receptor (TCR) or chimeric antigen receptor (CAR) has demonstrated high objective response rate (>40%) in Phase II clinical trials (Rosenberg, 2012). The newly approved small molecule drug, vemurafenib, or PLX 4032, that targets BRAF oncogenic mutation (V600E), has been found to induce T-cell mediated antitumor response (Sosman et al., 2012; Liu et al., 2013). Through these studies and other pre-clinical investigations, it has been increasingly recognized that immune cells play an important, yet paradoxical, role in malignancy. Cytotoxic and helper T cells, natural killer cells, and antigen presenting cells can mediate tumor destruction; whereas regulatory T cells, indoleamine-2.3-dyoxigenase (IDO) positive dendritic cells, and myeloid-derived suppressor cells (MDSCs) can protect malignancy (Hunder et al., 2008; Kantoff et al., 2010). Therefore, a deep understanding of the antitumor immune response and ways to control and maintain it are crucial for designing successful cancer therapeutics.

Immune cells execute their functions primarily through the secretion of effector or signaling proteins, jointly called cytokines. Hundreds of such molecules have been found and these cytokines can mediate a myriad of functions, from direct target killing, to self-renewal, to recruitment of other immune cell types, and to promotion or inhibition of local inflammation. Further, due to the variety of the pathogens it needs to target, cellular immunity is inherently heterogeneous at the single cell level. Individual immune cells can possess differential capacities in producing these

cytokines. Therefore, a survey of immune cell function would require the development of high-throughput, highly multiplex single cell assays that can characterize the properties of single immune cells in producing multiple relevant effector cytokines, collectively called functional proteomics. An additional technical challenge is that the assays should have the capacity to relate the released proteins back to their cellular producers.

In this review, we will focus on recent progresses in the development of single cell proteomics tools, with an emphasis on those that can be used for immune diagnostics and monitoring in cancer therapeutics. These technologies are necessarily sophisticated and can generate large amounts of high-dimensional protein readouts. Therefore, advanced data modeling and analysis methods that can help interpret and visualize the readout are highly desirable. We will review some useful methods for data processing, analysis, and presentation in the second section. It is exciting that several technologies have been used to study primary human samples. Pilot studies using these technologies have provided a fresh view on the functional heterogeneity of immune cells and the dynamics of antitumor immune response. Therefore we will review some of the recent applications and propose potential roles of these technologies in cancer therapy.

SINGLE CELL PROTEOMICS TECHNOLOGIES

Mass spectrometry in combination with liquid chromatography (MS-LC) was the first tool developed for proteomics studies. It is high-throughput and has the potential to reveal the full protein spectrum. Due to the limited amount of materials retrievable from single cells, the application of MS-LC toward single cells is challenging (Choudhary and Mann, 2010; Altelaar et al., 2013). Further, MS-LC requires input of fragmented or enzyme-digested samples and thus does not allow the recovery of viable cells for downstream usage. There have been exciting developments recently; however, the application of MS-LC in a clinical setting remains to be seen (Choudhary and Mann, 2010; Altelaar et al., 2013).

Flow cytometry, invented in the 1970s, is one of the most advanced, versatile tools for studying single cells in immunology. It utilizes photon detectors to measure laser-activated fluorescence signals that are emitted from cells stained by fluorophore labeled antibodies and uses fluidics to handle the individual cells. The technology can be used to profile cell surface markers, phosphorylation during intracellular signaling and, to a limited capacity, cytokine production. With the increasing number of fluorophores available, currently 20 parameters can be measured; of them, up to 5 can be cytokines (**Table 1**; **Figure 1A**) (Perfetto et al., 2004; Betts et al., 2006). Cells can be measured at a high-throughput rate of up to 10,000/s. The potentially complicated calibration procedure to compensate the overlaps in fluorophore optical spectrum has been standardization and automated. Multiple clinical centers have established centralized flow cytometry facilities (Maecker and McCoy, 2010). A version of the flow cytometry technology, called Fluorescence Activated Cell Sorting (FACS), allows retrieving live cells with desired surface properties. Currently, as many as four cell populations can be purified in parallel. However, because of the limited multiplexity (<5), the required un-physiological blockage

Table 1 | Comparison of existing single cell technologies for profiling functional proteomics.

Technology	Reference	Minimum sample (cells)	Current multiplexity for cytokines	Readout	Throughput	Multiplexity limitation (cytokines)	Cell recovery	Single cell level
Flow cytometry (intracellular staining)	Appay et al. (2008), Betts et al. (2006), Seder et al. (2008), Darrah et al. (2007), Bendall et al. (2012)	10^5	3–5	Antibody staining based Fluorescence	10^4 cells/s	<10, intracellular space, fluorophore spectrum overlapping	Yes	Yes
Mass cytometry (intracellular staining)	Bendall et al. (2011), Newell Evan et al. (2012), Bodenmiller et al. (2012), Bendall et al. (2012)	10^5	9	Isotope	10^3 cells/s	~10, intracellular space, availability of isotopes	No	Yes
ELISpot	Moodie et al. (2010)	10^5	1–3	Enzyme, fluorescence	10^6–10^7 cells/dish	<5	No	Quasi-single cell
Single cell barcode chip	Ma et al. (2011, 2013), Wang et al. (2012), Lu et al. (2013), Ma et al. (2012a,b)	10^4	20	Fluorescence	10^3–10^4 cells/chip	100–1,000	No	Yes
Micro-engraving	Han et al. (2012), Varadarajan et al. (2011, 2012), Yamanaka et al. (2013)	10^4–10^5	3	Fluorescence	10^3–10^5 cells/chip	<5	Yes	Yes

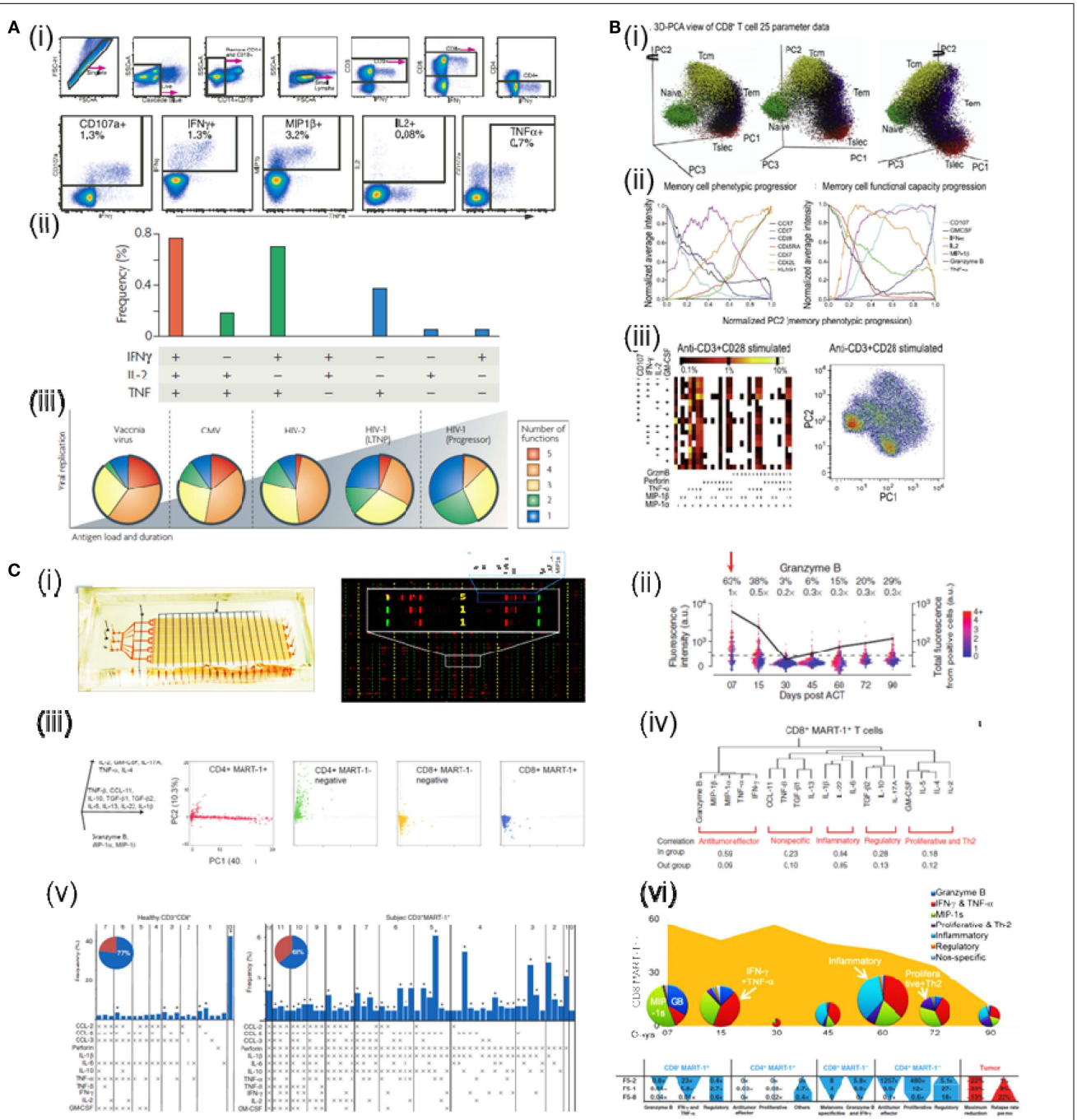

FIGURE 1 | Functional proteomics analysis by existing and emerging technologies. **(A)** Detection of 5 concurrent T-cell functions and characterization of CD8 T-cell functionality by flow cytometry. **(i)** Gating scheme for identification of multifunctional CD8 T-cell responses. **(ii)** The T-cell response is composed of multiple functional subpopulations. Each dot denotes IFN-g, IL-2, and/or TNF-a positivity. **(iii)** The functional profile of T-cells by pie charts. For simplicity, responses are grouped by number of functions. **(B)** CD8+ T cell data measured by mass cytometry. **(i)** One data set is plotted on the first three principal component axes. **(ii)** These average expression for each phenotypic (left plot) and functional (right plot) parameters were normalized and plotted as a function of normalized PC2 values. **(iii)** Left: the combinatorial diversity of 9 T cell functions were assessed in response to anti-CD3+anti-CD28. The heat of each block represents the log scale frequency of cells displaying each combination of functional capacity. Right: psuedo-colored density-dot plots of the first two principal components are shown for cells stimulated with anti-CD3+anti-CD28. **(C)** Dynamics of antitumor immune response measured by SCBC. **(i)** The design of the single cell barcode chip (left) and sample image readout of cell cytokine production (right). **(ii)** Gated and background subtracted one-dimensional scatterplots of a representative cytokines produced by single cells at different time. **(iii)** Cytokine secretion florescence intensity data analyzed by PCA. **(iv)** Hierarchical clustering of the 19 functional cytokines produced by CD8 T cells. **(v)** Functional diversity plots for antitumor CD8 T cells. **(vi)** Time-dependent changes of T cell cytokine polyfunctional strength and comparison between three patients analyzed. (Reprint permission obtained where needed.)

of protein secretion and the non-viability of cells analyzed, this technology is less optimal for measuring cytokine production.

One recent technical breakthrough along the direction of flow cytometry is mass cytometry, also known as cytometry by time-of-flight (cyTOF) (Bendall et al., 2011). The technology is based on the detection of isotopes that do not naturally exist in biologically samples. Cells are stained by isotope-labeled antibodies and are then "evaporated" into clouds of molecules in the machine; thereby the isotope labeling is detected. The application of this technology in immunology was first reported in 2011 (Bendall et al., 2011). With proper combinatorial barcoding, the technology has been showed to detect 30 surface markers and 9 cytokines simultaneously (**Table 1**; **Figure 1B**) (Bodenmiller et al., 2012). Unlike flow cytometry, whose multiplexity is limited by the overlaps in fluorophore spectrum, mass cytometry can potentially detect a huge number of markers simultaneously (Bendall et al., 2012). Currently, the technology is limited by the comparatively low-throughput rate and the low fraction of sample analyzable; however, it is expected to improve (Bendall et al., 2012). Because cells are "evaporated" during the assay, cells cannot be retrieved for downstream analysis. Thanks to many shared components and established experience available from flow cytometry, this technology grows very fast. It has been used to study the hierarchy of hematopoietic stem cell differentiation, the natural killer cell intracellular signaling and the T cell functional heterogeneity (Bendall et al., 2011; Bodenmiller et al., 2012; Newell Evan et al., 2012), as we will review later.

The enzyme-linked immunosorbent spot (ELISpot) or fluorospot assay is a widely used quasi-single cell technique (Moodie et al., 2010). In the assay, cells are cultured on a petri dish that is pre-coated with cytokine-specific antibodies. Cytokines released from individual cells are captured by surrounding antibodies. Subsequently, these captured cytokines are detected by applying secondary antibodies and fluorophore labels or through enzymatic reaction. After the assay, the number of spots on the petri dish, each relating to a cytokine-producing cell, can be enumerated. ELISpot can achieve a high sensitivity (<0.1%) and allows the detection of one to three cytokines at the same time (**Table 1**). Because single cells are not separated during the measurement, the protein level cannot be quantitated and individual cells cannot be distinguished when cells are too close together.

Recent developments in microfluidics have revolutionized the traditional ELISpot assay. These microchip-based technologies utilize arrays of highly miniaturized nano- to pico-liter volume micro-chambers to achieve ultra-sensitive protein measurement and the separation of single cells. Because single cells are separated in different micro-chambers, their protein levels can be quantitated in parallel. About 1,000–10,000s micro-chambers can be integrated into one microchip, to achieve high-throughput measurements. The amenability of these technologies to integrate with upstream cell purification and on-chip optical imaging further enhances their utility. Moreover, microchips are highly portable, low-cost, and are sample-efficient.

One version of these microchips is called the Single Cell Barcode Chip (SCBC) (Ma et al., 2011). It couples a microfluidics-generated antibody microarray substrate with a microfluidics chip containing a large array of micro-chambers. The antibody microarray serves to detect cytokines secreted and the microchip is designed to fit a full panel of antibodies in each micro-chamber. During the assay, single cells are loaded into these 100-pl size micro-chambers. Because of a 1-million fold miniaturization, the microchip can achieve ultra-sensitivity down to 100 molecules and only requires 10,000 cells as starting material. Currently, more than 20 proteins can be measured simultaneously from 5 to 10 thousand micro-chambers (**Table 1**; **Figure 1C**) (Ma et al., 2011, 2012a,b, 2013; Wang et al., 2012; Lu et al., 2013). The technology has been applied across many fields, including studying adaptive, innate immune cells, hematopoietic stem cells, and intracellular signaling in malignancy (Ma et al., 2011, 2012a,b, 2013; Wang et al., 2012). In particular, this technology has been used to study the functional heterogeneity of human T cells and clinical immune responses in an ACT immunotherapy to metastatic melanoma (Ma et al., 2011, 2013).

Another version of the microchips employs the micro-engraving technique to fabricate micro-chambers (Varadarajan et al., 2011; Yamanaka et al., 2013). In this technology, hundreds of thousands nano-liter sized micro-chambers can be integrated into one chip, wherein up to three types of cytokines can be measured by antibody on the substrate (**Table 1**). At the same time, cells can be stained by three colors. Immune cell – target cell interaction can be measured by on-chip imaging and temporal cytokine production profile can be acquired by periodically switching the antibody substrates (Varadarajan et al., 2011; Han et al., 2012). This technology also has the capacity to retrieve viable individual cells with desirable properties from the microchip, as has been showed in the case of T cell cloning (Varadarajan et al., 2012). Moreover, it has also been used to show the discordant cytokine production dynamics of human T cells (Han et al., 2012; Yamanaka et al., 2013).

The features of the technologies reviewed are summarized in **Table 1**.

ANALYSIS METHODS

The massive, high-dimensional data generated by cytometry and microchips has spurred the development of computational analysis methods.

The cytokine signals are normally measured in fluorescence intensity. To compare data acquired from different samples and from different experiments, the background level specific for each protein needs to be identified and subtracted. One logical way to characterize cells is to divide them into cytokine-producing and non-producing fractions by a gate in fluorescence level. Then, one can focus on properties of the cytokine-producing fraction by calculating their relative abundance as well as their cytokine production intensity.

For flow cytometry, commercial software, such as BD Diva and FlowJo, has been developed that can provide simple data analysis capacity. Such software can generate one-dimensional distribution plots and density-based two-dimensional plots and allows the user to manually gate out desirable cell subpopulations (see example in **Figure 1Ai**). However, manual gating is subjective and laborious, and can generate inconsistent results when a large number of proteins and samples are analyzed.

An alternative approach to detect background and determine gate is to utilize computational algorithms to fit the density distribution. Finite mixture models and their variants are commonly used (Reynolds and Rose, 1995). Some models take into account the skewness and kurtosis of the measured distribution and could generate good result in many cases (Pyne et al., 2009). In parallel, non-parametric methods have been developed to extract features of the distribution (Walther et al., 2009; Ma et al., 2013).

The single cell functional heterogeneity can be characterized after cytokine-producing and non-producing cells are identified. For example, cells can be grouped into subpopulations that produce different number of cytokines and the relative abundance of each group can be showed in a pie graph (Betts et al., 2006; Seder et al., 2008; Ma et al., 2012a,b) (**Figures 1Aii,iii**). Such a plot reflects the functional distribution. Different pie charts can be compared for statistically significant differences (Betts et al., 2006; Seder et al., 2008). A more thorough way to look at this functional heterogeneity would be to further subdivide cell population into subpopulations producing different combinations of cytokines. Then, the distribution can be showed as a bar group with an accompanying matrix denoting the function combinations (**Figures 1Aii, Bi–iii, Ci–v**) (Betts et al., 2006; Ma et al., 2011; Newell Evan et al., 2012). This type of representation is informative and has been used to show the profound functional heterogeneity existed in T cell populations actively attacking tumor, comparing to that of resting T cells (Ma et al., 2011). Furthermore, statistical indicators summarizing the functional heterogeneity can also be defined based on this information (**Figure 1Cvi**) (Seder et al., 2008; Ma et al., 2013).

Since the ultimate goal of gating is to identify biologically significant cell subpopulations based on the type and level of cytokines produced, computational methods have been developed that directly model the distribution of the multi-dimensional cytokine data. Such methods utilize different versions of clustering method, such as k-means clustering, hierarchical clustering, and their variants (**Figure 1Civ**) (Johnson and Wichern, 2007; Aghaeepour et al., 2011). The basic idea is to group the data points by certain measure of point–point distance in the high-dimensional space representing the cytokines measured. One of the challenges to utilize clustering methods is to predefine the number of clusters exist. Most of time, such information is not known beforehand, therefore additional indicators and trial-and-error iterations are necessary. The gating methods and grouping methods have provided very promising results in many cases; however, due to the often-existed complexity and irregularity of cell population, none of these methods has showed widespread successes (Zare et al., 2010; Aghaeepour et al., 2011).

High-dimensional analysis is especially susceptible to multiple data defects, an effect called curse of dimensionality (Johnson and Wichern, 2007). First, the amount of data required to allow meaningful analysis increases exponentially with the number of proteins measured. Second, spurious correlation is more likely to happen in high-dimensional data and the measure of distance used for clustering analysis is prone to be invalid. Lastly, statistical tests need to be redesigned when repetitively used for high-dimensional data, as true type I error can be much larger than expected.

To address these challenges, methods have been developed to "concentrate" the information by reducing the dimensionality. Such an approach is also biologically sound: due to the interrelating nature of gene transcription and protein expression, protein signals are normally correlated with each other. Therefore, only a small number of truly independent variables or "degrees of freedom" exist that define the biological process. In this regard, principal component analysis (PCA) and its variants are powerful resorts (Johnson and Wichern, 2007; Ma et al., 2012a,b, 2013; Newell Evan et al., 2012) (**Figures 1Bi,ii, Ciii**). When data is meaningful and the analysis is applied correctly, different components representing different aspects of biological information can are discovered. At the same time the noise is reduced. Other recent development in this direction utilized minimum spanning tree and clustering methods to characterize and display the high-dimensional data on a two-dimensional plane and provided a revealing illustration of hematopoietic stem cell differentiation (Bendall et al., 2011).

CLINICAL APPLICATIONS AND FUTURE DIRECTIONS

The application of these new technologies has greatly advanced our understanding of functional heterogeneity within immune cells. Initial studies (Ma et al., 2011; Newell Evan et al., 2012) on human T cells showed the existence of profound functional heterogeneity within a population of genetically and phenotypically similar T cells and demonstrated that the level of functional heterogeneity reflects the functional activity of T cells (Ma et al., 2011, 2012a,b, 2013). The functional heterogeneity has also been showed to be highly focused and the distribution of functional subsets is significantly different from a random distribution (Ma et al., 2011; Newell Evan et al., 2012). Thus, the functional heterogeneity contains valuable biological information, rather than random biological noise.

A new insight emerges from flow cytometry and microchip analysis is that a fraction of cells, called the polyfunctional cells, can simultaneously secrete a large number of cytokines. They also secreted each of these cytokines in large amounts (Betts et al., 2006; Darrah et al., 2007; Seder et al., 2008; Ma et al., 2011, 2013). Thus, they produced a predominant amount of cytokine in an immune response (Ma et al., 2013). One explanation of this phenomenon is that the cytokine functions are coordinated at the level of single cells and new parameters have been defined to summarize this information of polyfunctionality (**Figure 1Cvi**) (Darrah et al., 2007; Seder et al., 2008; Ma et al., 2013). These parameters have been found to correlate with the quality of T response in human and animal models (Darrah et al., 2007; Seder et al., 2008; Ma et al., 2013). For example, an index, named polyfunctional strength index (pSI), is developed to summarize the joint functional intensity from polyfunctional T cells and its distribution among cytokines (Ma et al., 2013). It is used in a recent study that monitored the temporal changes of antitumor T cells retrieved from metastatic melanoma patients participating in a transgenic TCR ACT immunotherapy. By comparing the changes in the frequency, phenotype, and polyfunctionality (summarized by pSI) of these T cells, the study showed that only the functional changes are highly distinguishable between patients and that the changes correlated with the clinical outcome (Ma et al., 2013) (**Figure 1Cvi**).

These studies demonstrated the importance to understand the functional heterogeneity of immune cells and its preliminary value in clinical diagnostics and monitoring. Because both the cellular immunity and tumor are heterogeneous at the single cell level, successful cancer therapeutic scheme is necessarily personalized. Therefore, personalized diagnostic and monitoring tools, such as the single cell functional analysis, are highly desirable and can be a integrative component in the cancer therapeutics. By understanding the functional characteristics of their immune cells, patients can be stratified pre-treatment for the best available treatment and their immune response can be monitored during the therapy so that further intervention can be applied timely. The massive information acquired are also valuable feedbacks to guide further improvements of cancer therapy.

AUTHOR CONTRIBUTION

Chao Ma conceived and composed the paper. All authors reviewed the manuscript.

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Emerging microfluidic tools for functional cellular immunophenotyping: a new potential paradigm for immune status characterization

Weiqiang Chen[1,2†], Nien-Tsu Huang[2†], Xiang Li[1,2], Zeta Tak For Yu[1,2], Katsuo Kurabayashi[2,3] and Jianping Fu[1,2,4]*

[1] *Integrated Biosystems and Biomechanics Laboratory, University of Michigan, Ann Arbor, MI, USA*
[2] *Department of Mechanical Engineering, University of Michigan, Ann Arbor, MI, USA*
[3] *Department of Electrical Engineering and Computer Science, University of Michigan, Ann Arbor, MI, USA*
[4] *Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, USA*

Edited by:
Rong Fan, Yale University, USA

Reviewed by:
Sindy K. Tang, Stanford University, USA
Rohit N. Karnik, Massachusetts Institute of Technology, USA

****Correspondence:***
Jianping Fu, Integrated Biosystems and Biomechanics Laboratory, Department of Mechanical Engineering, University of Michigan, Ann Arbor, 2350 Hayward Street, Ann Arbor, MI 48109, USA.
e-mail: jpfu@umich.edu

[†]Weiqiang Chen and Nien-Tsu Huang have contributed equally to this work.

Rapid, accurate, and quantitative characterization of immune status of patients is of utmost importance for disease diagnosis and prognosis, evaluating efficacy of immunotherapeutics and tailoring drug treatments. Immune status of patients is often dynamic and patient-specific, and such complex heterogeneity has made accurate, real-time measurements of patient immune status challenging in the clinical setting. Recent advances in microfluidics have demonstrated promising applications of the technology for immune monitoring with minimum sample requirements and rapid functional immunophenotyping capability. This review will highlight recent developments of microfluidic platforms that can perform rapid and accurate cellular functional assays on patient immune cells. We will also discuss the future potential of integrated microfluidics to perform rapid, accurate, and sensitive cellular functional assays at a single-cell resolution on different types or subpopulations of immune cells, to provide an unprecedented level of information depth on the distribution of immune cell functionalities. We envision that such microfluidic immunophenotyping tools will allow for comprehensive and systems-level immunomonitoring, unlocking the potential to transform experimental clinical immunology into an information-rich science.

Keywords: immunophenotyping, microfluidics

INTRODUCTION

The immune status of patients with infectious diseases and immune dysfunctions are dynamic and patient-specific, and such complex heterogeneity has made immunomodulatory therapies challenging in the clinic (Hotchkiss and Karl, 2003; Monneret et al., 2008). An accurate and real-time measurement of the immune status of patients is thus critical in disease diagnosis and prognosis, evaluating efficacy of immunotherapeutics, and tailoring drug treatments (Monneret et al., 2008). Functional cellular immunophenotyping, which measures the functional status of immune cells upon proliferation, cytolysis, and cytokine production, is arguably among the best methods to determine immune dysfunctions (Hotchkiss and Karl, 2003; Monneret et al., 2008; Lu et al., 2013). Immune cells in blood constitute a complex, heterogeneous mixture of multiple cell types including granulocytes, lymphocytes, and monocytes (Re and Strominger, 2004; Gordon and Taylor, 2005; Kaech and Wherry, 2007; O'Shea et al., 2008). The numbers, proportions, and cytolytic and cytokine production activities of leukocyte subsets change drastically in the presence of infections, malignancies, and autoimmune disorders (Revzin et al., 2012). As such, there is a significant need for reliable technologies that can perform rapid and accurate functional cellular immunophenotyping on patient immune cells and their subtypes to define and characterize the "immune phenotype" of patients.

Several approaches currently exist for assessment of the immune status of patients based on measuring cytokine production of immune cells. Enzyme-linked immunosorbent assay/spot (ELISA/ELISpot), for example, is a gold standard for quantifying cellular cytokine production (Cox et al., 2006; Cornell et al., 2012). ELISA/ELISpot has been commonly used for patients infected by malaria (Aidoo and Udhayakumar, 2000), HIV (Kern et al., 1999; Betts et al., 2000), and mycobacterium tuberculosis (Pathan et al., 2000) and monitoring the immune response of cancer patients undergoing immunotherapeutics (Janetzki et al., 2000; Lewis et al., 2000). However, ELISA/ELISpot usually requires numerous reagent manipulation processes that involve multiple staining, washing, blocking, and sample transfer steps, which are laborious and time-consuming. The complexity in implementing ELISA/ELISpot has been prohibitive for standardization and their utility in real-time clinical decision making. Further, ELISpot cannot quantify the amount of cytokine secretion, and it requires isolation and purification of desired subpopulations of immune cells prior to analysis, necessitating extensive sample preparation of blood specimens.

Functional cellular immunophenotyping can also be performed using intracellular cytokine staining (ICS) flow cytometry for single-cell cytokine production measurements with a high-throughput ($>10^3$ cells/s) (Seder et al., 2008). However,

ICS flow cytometry has so far only enabled detection of up to five cytokines, providing only a partial picture of the functional immune system. ICS flow cytometry also requires a large number of cells in suspension ($>1 \times 10^7$ cells in 1 mL solution) and is sample destructive, thus precluding downstream functional assays that require live cells. ICS flow cytometry has so far remained highly variable with regard to sample handling, reagents, instrument setup, and data analysis, thus standardization of ICS flow cytometry has been proved difficult if not impossible.

The limitations associated with conventional approaches to define the functional immune status of patients need to be fully addressed to realize rapid and accurate analysis of immune phenotype of patients, a key step that provides crucial information relating to staging, treatment choice, monitoring of efficacy, safety and dose adjustment of immunomodulation, as well as biological assessment of remission.

Recent advances in integrated microfluidics have made possible miniaturization and integration of biosample preparative and analytical techniques on a single chip to enable rapid, sensitive, and multiplexed high-throughput on-chip cell-based assays. Some of these microfluidic tools have been demonstrated as promising immune monitoring technologies with cell trapping and analytic functionalities and a minimum sample requirement. This review will highlight the recent development of microfluidic platforms that can perform rapid and accurate whole-blood immunoassays of plasma components as well as functional cellular immunophenotyping assays for quantitative analysis of cytokine secretion properties of patient immune cells (**Figure 1**). We will particularly discuss the future potential of integrated microfluidics to perform rapid, accurate, and sensitive cellular functional assays at the single-cell resolution on immune cell subpopulations isolated directly from patient blood, and their potential to provide an unprecedented level of information depth on the distribution of immune cell functionalities on a patient-by-patient basis.

MICROFLUIDIC WHOLE-BLOOD IMMUNOASSAYS OF PLASMA COMPONENTS

Whole-blood immunoassay is a most commonly used method to examine patient immune status, which provides useful information for diagnosis (Boomer et al., 2011; Cornell et al., 2012), prognosis (Azizia et al., 2012), and deepening the biological understanding of immune and infectious diseases (Bernard et al., 2001; Hotchkiss and Opal, 2010). Conventional whole-blood immune tests are based on proteomic identification of biomarkers in blood, relying on antibody-based heterogeneous or homogeneous immunoassays (e.g., ELISA) to capture and recognize soluble biomarkers in blood specimens. Recently, to achieve rapid on-chip immunoassays with a minimum amount of blood, several microfluidic whole-blood immunoassay devices have been developed. A notable example is the integrated blood barcode chip reported by Fan et al. (2008) that can achieve on-chip plasma separation from microliter quantities of whole-blood and rapid *in situ* multiplexed protein biomarker measurements (**Figure 2A**). The marked performance of the blood barcode chip comes from its two integrated functional components: (1) a plasma-skimming channel that separates blood plasma based on the Zweifach–Fung effect; (2) a protein detection region using a patterned DNA-encoded antibody library (DEAL) barcode immobilized on the surface of the plasma-skimming channel. Specifically, the DEAL technology involves DNA-directed immobilization of antibodies to convert a prepatterned ssDNA barcode microarray into an antibody array, thus providing a powerful means for spatial encoding. The integrated blood barcode chip and its recent improvement reported by Wang et al. (2010) is capable of detecting picomolar concentrations of cancer biomarkers and more than 10 cytokines simultaneously from cancer patient blood.

MICROFLUIDIC WHOLE-BLOOD FUNCTIONAL IMMUNOASSAYS

In addition to proteomic analysis for soluble biomarkers in blood using microfluidic immunoassays, a recent exciting trend is to

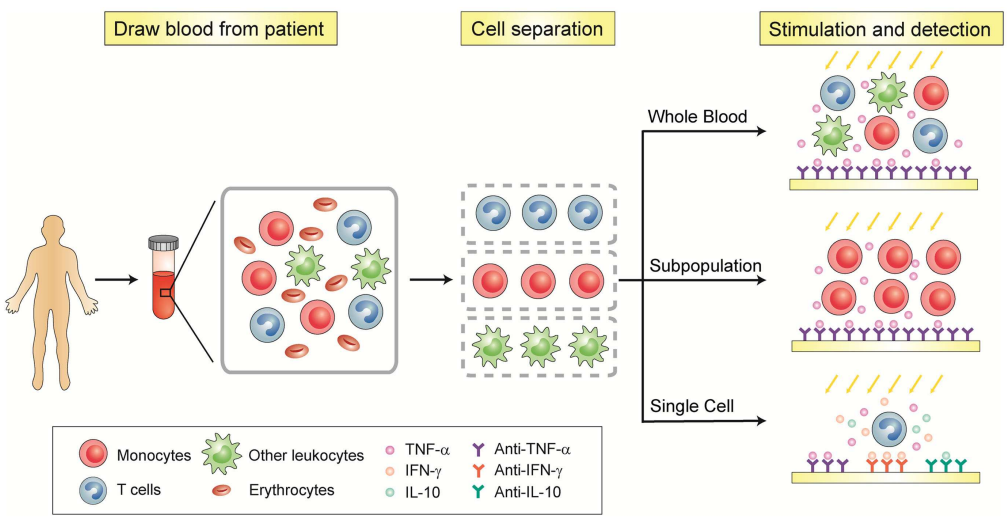

FIGURE 1 | Schematic of functional immunophenotyping of immune cells.

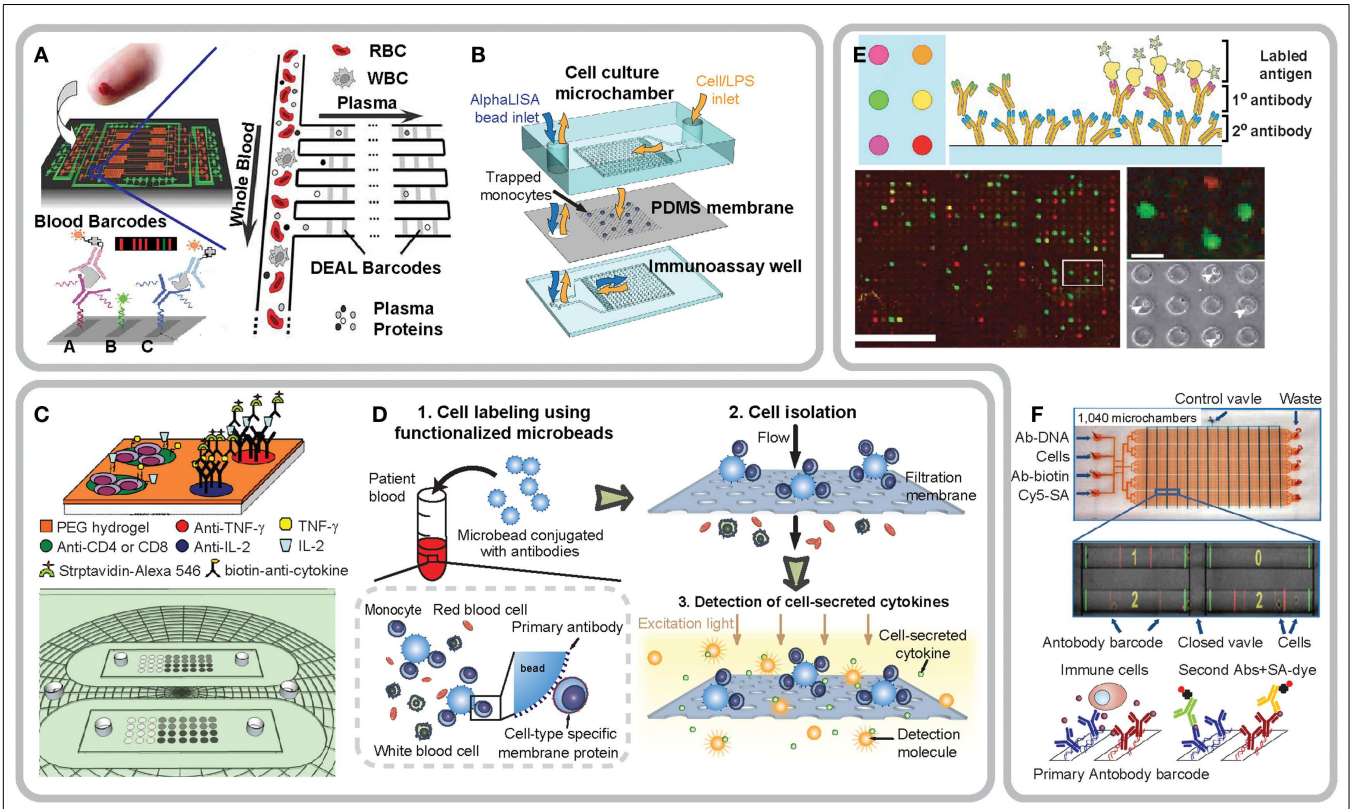

FIGURE 2 | Integrated microfluidic devices for functional immunophenotyping of immune cells in whole blood (A,B), subpopulations of immune cells (C,D) and single immune cells (E,F). **(A)** Design of the integrated blood barcode chip (IBBC). Adapted from Fan et al. (2008), Copyright ©2008, with permission from Nature Publishing Group. **(B)** Schematic of a multi-layered MIPA device consisting of a cell culture chamber, a PDMS microfiltration membrane (PMM), and an immunoassay chamber. Reproduced from Huang et al. (2012). **(C)** Conceptual design of an antibody-coated microarray for detection of cytokines secreted by CD4+ or CD8+ T-cells. Reproduced from Zhu et al. (2008) by permission of The Royal Society of Chemistry. **(D)** Schematic of isolation and immunophenotyping of subpopulations of immune cells from blood specimens by a combined use of both PMM and functionalized microbeads. Reproduced from Chen et al. (2013). **(E)** Working principle of the microengraving array for capture and immunomonitoring of single immune cells. Adapted from Love et al. (2006), Copyright ©2006, with permission from Nature Publishing Group. **(F)** Photograph and working principle of the single-cell barcode chip for polycytokine analysis of single immune cells. Adapted from Ma et al. (2011), Copyright ©2011, with permission from Nature Publishing Group.

develop microfluidics-based cellular functional immune assays, which is arguably a more direct measurement of the functional status of immune cells. To achieve this, Huang et al. (2012) have recently developed a microfluidic immunophenotyping assay (MIPA) device for rapid and efficient on-chip isolation of peripheral blood mononuclear cells (PBMCs), their stimulation and cellular cytokine secretion measurements (**Figure 2B**). A key component of the MIPA device is a surface micromachined polydimethylsiloxane (*PDMS*) microfiltration membrane (PMM) for both isolation of PBMCs from blood and allowing cytokines secreted from lipopolysaccharide (LPS)-stimulated PBMCs to diffuse rapidly into a biosensing chamber for quantitative immunosensing. The MIPA device can achieve efficient on-chip cell isolation and enrichment from blood owing to the high porosity of the PMM as compared to existing polycarbonate filters (Vona et al., 2000; Hofman et al., 2011) or parylene-based micropore membranes (Zheng et al., 2011). For quantitative immunosensing, the MIPA device utilizes a commercially available homogeneous chemiluminescence technique, the "AlphaLISA," which does not require any washing or blocking step, greatly shortening the total assay time and enhancing dynamic range for analyte detection. Owing to a miniaturized on-chip microfluidic environment, the MIPA device can achieve highly sensitive cellular immunophenotyping with 20-fold fewer cells as compared to standard whole-blood stimulation assay. The total assay time of the MIPA device using AlphaLISA is seven times faster than that of whole-blood stimulation assay using conventional ELISA.

Several microfluidics-based label-free, real-time detection techniques have also been developed recently for immunosensing. Development of real-time immunosensing techniques allows detailed examination of the temporal dynamics of cytokine secretion from immune cells, which may provide an informative and unique signature about the functional status of patient immune system (Revzin et al., 2012). The ability to assess dynamic cytokine secretion from immune cells, for example, can allow detection of the onset of the signaling process and study of intercellular communications via cytokine-mediated paracrine and autocrine signaling. Monitoring both the location and timing of cytokine

secretion events among a heterogeneous population of individual immune cells can also determine which individual cells initiate the immune response and which cells are then activated by such initial immune response. In clinical diagnosis such as tuberculosis detection, pathogen biomarkers (e.g., pathogen-specific antibodies) are not yet available. As such, cytokine production by T-cells is commonly used as a diagnostic marker for tuberculosis. If detection of dynamic response of antigen-specific T cells becomes available, it will enable early pathogen detection before pathogen biomarkers are produced or the pathogen proliferates in the host.

A noteworthy microfluidic label-free immunodetection method has been recently reported by Stern et al. (2007) based on CMOS-compatible semiconducting nanowires for real-time measurements of antibodies and early signals responsible for T-cell activation. Another label-free biosensing technique reported by Endo et al. (2008) has applied immobilized antibodies and localized surface plasmon resonance (LSPR) to continuously monitor concentration levels of cytokines secreted from mouse thymus cells. The LSPR-based biosensor provides a promising platform with attractive advantages of real-time detection of cellular responses in a simplified experimental setup with a low sample volume requirement. Overall, label-free cellular immunophenotyping permits real-time quantifications of dynamic cytokine secretion, providing the unique functional signature of immune cells such as how fast and strong immune cells secrete cytokines in response to antigen stimulations.

MICROFLUIDIC IMMUNOPHENOTYPING OF SUBPOPULATIONS OF IMMUNE CELLS

Microfluidic whole-blood immunoassays measure the overall capacity of the whole population of leukocytes in blood to produce cytokines. Thus, microfluidic whole-blood immunoassays may not be informative enough to accurately reveal the immune status of patients, as in these "bulk" assays it is difficult to pinpoint the phenotype or real identity of reactive immune cells involved. Recently, there are great efforts from different research groups to integrate cell separation techniques into microfluidic immunoassay devices and systems to achieve cellular functional analysis on subpopulations of immune cells. Zhu et al. (2008) for example, have recently developed a microarray device uniformly coated with both T-cell capture antibodies (anti-CD4 and anti-CD8) and cytokine capture antibodies (anti-IFN-γ and anti-IL-2) on top of a poly(ethylene glycol) (PEG) hydrogel layer (**Figure 2C**). To enable capturing and positioning of single CD4+ and CD8+ T-cells, the antibody-coated microarray was covered with photolithographically patterned PEG hydrogel microwells on top of the antibody containing hydrogel layer. The antibody-coated microarray can directly process red blood cell (RBC) depleted human whole-blood samples for capture of individual CD4+ and CD8+ T-cells and subsequent functional examination of IFN-γ and IL-2 secretion from single T-cells.

The antibody-based microarray platform reported by Zhu et al. has simplified the sample preparation process and also reduced the required volume of blood specimens. Although immobilized antibodies offer a heightened cell isolation purity and cytokine measurement sensitivity, it still suffers from several limitations, including the need of multiple washing and blocking steps and the difficulty to achieve real-time dynamic cytokine secretion measurement. To address these limitations, the same research group has recently applied DNA and RNA-based aptamers as an alternative to antibodies and immobilized aptamers on an array of micropatterned gold electrodes (Zhu et al., 2009; Liu et al., 2012). The aptamers have been thiolated for assembly on gold and functionalized with a methylene blue redox reporter for electrochemical signal transduction and detection with gold electrodes. Instead of using fluorescence-based biosensing methods, the authors have successfully demonstrated electrochemical measurements to access dynamic cytokine secretion from human monocytes and T-cells with a detection sensitivity of ∼ng/mL (Zhu et al., 2009; Liu et al., 2012).

In addition to antibody- and aptamer-based immunophenotyping methods for subpopulations of immune cells, Chen et al. (2013) have recently developed an integrated microfluidic device employing a combined use of the PMM and antibody-conjugated polystyrene microbeads for isolation, purification, and functional immunophenotyping of subpopulation of immune cells directly from unprocessed blood specimens (**Figure 2D**). In their method, Chen et al. have first applied functionalized microbeads conjugated with monoclonal antibodies against specific cell surface proteins to label and enlarge targeted subpopulations of immune cells in blood specimens. After labeling using microbeads, blood specimen is introduced into the microfluidic device which contains the PMM. The cell/microbead conjugates are readily trapped and isolated on the PMM, whereas other untargeted blood cells unbound to microbeads can freely pass through the PMM. Following cell isolation, the AlphaLISA is applied for quantitative measurements of cytokine secretion from LPS-stimulated immune cells captured on the PMM.

MICROFLUIDICS TO STUDY FUNCTIONAL HETEROGENEITY OF SINGLE IMMUNE CELLS

Functional and phenotypic variation among individual single cells, or single-cell functional heterogeneity, is a common feature for hematopoietic cells including immune cells. Thus, quantitative functional analysis of immune cells down to a single-cell resolution is required for a precise assessment of patient immune status. Over the last decade, significant research efforts have been directed toward applying microfluidics for manipulation and functional analysis of single immune cells. One of the most notable example entails plating and stimulating single immune cells in an array of microfabricated wells, transferring soluble molecules secreted from immune cells onto a secondary solid surface coated with capture antibodies, and labeling captured molecules with fluorescently tagged proteins prior to subsequent optical detection. For example, Love et al. (2006) have pioneered the development of engraved microarrays made in PDMS using soft lithography to monitor cytokines secreted from single immune cells (**Figure 2E**). The engraved microarray consists of 25,000 microwells (50–100 μm in diameter), each of which confines single immune cells in a nanoliter volume. After individual immune cells trapped and stimulated, the engraved microarray can be flipped against an antigen- or secondary antibody-immobilized glass slide to capture primary antibodies secreted from cells. Compared to ELISpot, the engraved microarray enables a rapid (<12 h)

and high-throughput (>10,000 individual cells) system for identification, recovery, and clonal expansion of single immune cells producing antigen-specific antibodies. More recently, studies from the same research group have demonstrated the capability of the engraved microarray for characterization of dynamic cytokine secretion from individual human T-cells after activation *ex vivo* (Han et al., 2012; Varadarajan et al., 2012).

In addition to the microengraving method, Jin et al. (2009) have recently independently developed a functional immunosensing technique called "immunospot array assay on a chip" (or ISAAC) to detect production of monoclonal antibodies by immune cells. The ISAAC method offers a rapid and high-throughput system for screening and analysis of antigen-specific antibody-secreting cells (ASCs) on a single-cell basis. Similar to the microengraving assay, the ISAAC also includes an array of microwells for trapping of single live immune cells. The top surface of ISAAC is functionalized with antibodies against immunoglobulin, and antibodies secreted by individual ASCs trapped in the wells are captured and bound to the device surface around the well. The ISAAC method is useful for detecting ASCs in response to different antigens as well as for selection of ASCs secreting high-affinity antibodies. Although both the microengraving and ISAAC methods have used a high-density array of microwells to trap and isolate single immune cells, the two methods utilize different detection techniques. Fundamentally, the microengraving method pioneered by Love et al. (2006) is based on ELISA, whereas the ISAAC is based on ELISpot (Jin et al., 2009).

Ma et al. (2011) have recently applied the single-cell barcode chip for high-content assessment of the functional heterogeneity of antigen-specific T-cells (**Figure 2F**). The single-cell barcode chip consists of 1,040 microchambers with a nanoliter volume, and each microchamber can trap single or a small number of immune cells. On the bottom surface of each microchamber, a spatially encoded antibody barcode array is pre-printed to capture cytokines secreted from immune cells trapped in the microchamber. Protein concentrations are measured with immunosandwich assays from the spatially encoded antibody barcode. A full barcode from each microchamber represents a complete panel of multiple cytokine species produced by a single immune cell (or a few cells). The single-cell barcode chip permits highly multiplexed (more than 10 proteins) on-chip detection of a few thousand proteins or less from thousands of immune cells simultaneously. The single-cell barcode chip reported by Ma et al. represents an exciting and informative microfluidic single-cell immunophenotyping tool for analyzing functional signatures of immune cells with high sensitivity, throughput and multiplicity, and a small sample size requirement.

All the microfluidic devices and systems discussed in this section provide a promising potential for high-throughput study of the functional heterogeneity of single immune cells. However, one critical issue common with these approaches is that they require off-chip isolation and purification of target cells from whole-blood prior to on-chip analysis. As such, there is still an unmet need for a highly integrated microfluidic technology platform for efficient isolation and informative systems-level cellular characterization of immune cells down to the single-cell level and using unprocessed or minimally processed blood samples.

CONCLUSION

Developing reliable, multiplexed biosensing techniques that permit simultaneous characterization of the functional status of different subpopulations of immune cells at a single-cell resolution is an exciting emerging concept. This concept holds a great promise for unraveling pathogenesis as well as for translating newly available therapeutic options into optimal personalized treatments. Continued progress in many fields ranging from fundamental immunology studies and clinical discoveries to patient managements critically hinges on the availability of such immune monitoring systems. Recent exciting developments in microfluidic technology have provided promising tools for functional cellular immunophenotyping of blood specimens. These microfluidic immunophenotyping techniques can potentially provide an unprecedented level of information depth on the distribution of immune cell functionalities. We envision that such microfluidic immunophenotyping tools will allow comprehensive and systems-level immunomonitoring in the future, thus unlocking the potential to transform experimental clinical immunology into an information-rich science.

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An evolutionary perspective on anti-tumor immunity

David J. Klinke II [1,2,3] *

[1] Department of Chemical Engineering, West Virginia University, Morgantown, WV, USA
[2] Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV, USA
[3] Department of Microbiology, Immunology, and Cell Biology, West Virginia University, Morgantown, WV, USA

Edited by:
Chao Ma, California Institute of Technology, USA

Reviewed by:
Jianping Huang, National Institutes of Health, USA
Yu Wu, Yale University, USA

****Correspondence:***
David J. Klinke II, Department of Chemical Engineering, West Virginia University, P.O. Box 6102, Morgantown, WV 26506, USA.
e-mail: david.klinke@mail.wvu.edu

The challenges associated with demonstrating a durable response using molecular-targeted therapies in cancer has sparked a renewed interest in viewing cancer from an evolutionary perspective. Evolutionary processes have three common traits: heterogeneity, dynamics, and a selective fitness landscape. Mutagens randomly alter the genome of host cells creating a population of cells that contain different somatic mutations. This genomic rearrangement perturbs cellular homeostasis through changing how cells interact with their tissue microenvironment. To counterbalance the ability of mutated cells to outcompete for limited resources, control structures are encoded within the cell and within the organ system, such as innate and adaptive immunity, to restore cellular homeostasis. These control structures shape the selective fitness landscape and determine whether a cell that harbors particular somatic mutations is retained or eliminated from a cell population. While next-generation sequencing has revealed the complexity and heterogeneity of oncogenic transformation, understanding the dynamics of oncogenesis and how cancer cells alter the selective fitness landscape remain unclear. In this technology review, we will summarize how recent advances in technology have impacted our understanding of these three attributes of cancer as an evolutionary process. In particular, we will focus on how advances in genome sequencing have enabled quantifying cellular heterogeneity, advances in computational power have enabled explicit testing of postulated intra- and intercellular control structures against the available data using simulation, and advances in proteomics have enabled identifying novel mechanisms of cellular cross-talk that cancer cells use to alter the fitness landscape.

Keywords: proteomics, Bayesian inference, next generation sequencing, simulation

INTRODUCTION

The transformation of a normal cell into a cancerous cell involves the acquisition of a series of genetic and epigenetic changes that daughter clones inherit (Hanahan and Weinberg, 2011). Next generation sequencing has reveal the breadth of genomic rearrangement that occurs in cancer (Stephens et al., 2009; Pleasance et al., 2010b; Gerlinger et al., 2012). These genetic and epigenetic changes can cause abnormal overexpression of proteins involved in cellular signaling pathways and can contribute to acquisition of these traits. Collectively, these genetic alterations rewire how cells interpret extracellular cues (Pawson and Warner, 2007; Klinke, 2010b) and subvert intracellular control mechanisms that are designed to maintain genetic integrity (Hollstein et al., 1991). It is thought that cells containing mutations in specific genes that impart an inherent proliferative advantage over cells of the host and that, over time, dominate a local cellular community. Demonstrating that a mutated gene, that is an oncogene, alters the replicative potential of a transformed cell supports this view (e.g., Muller et al., 1988; Gishizky et al., 1993). In order to inhibit the growth of malignant cells, drugs have been developed to promote cell death by targeting the oncogene in oncogene-addicted cells (Weinstein and Joe, 2008).

Demonstrating a durable clinical response in cancer using molecular-targeted therapies has been difficult. In patient groups stratified by a particular molecular biomarker, molecular-targeted therapies exhibit remarkable efficacy for a window of time in a subset of patients. For instance, overexpression of the epidermal growth factor receptor (EGFR) is observed in three-fourths of primary colorectal tumors (Hemming et al., 1992; Mayer et al., 1993) and provides support for targeting these cells using panitumumab, a monoclonal antibody against EGFR. The therapeutic window is short whereby almost all patients develop resistance within several months (Amado et al., 2008; Karapetis et al., 2008). *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutations are also a common occurrence in colorectal cancer. In a recent clinical study with panitumumab, 38% of patients that were initially negative for *KRAS* mutations developed circulating tumor cells that harbor detectable mutations in *KRAS* within 5–6 months (Diaz et al., 2012). A mathematical model was used to support the idea that resistance was due to drug-induced selection of cellular variants that harbored resistant mutations. A similar phenomena was observed in response to imatinib mesylate (Gleevec) in patients with chronic myeloid leukemia (Shah et al., 2002). While these are just two examples, the emergence of resistance to almost all molecular-targeted therapies in cancer brings a renewed interest in cancer as an evolutionary process (Merlo et al., 2006; Greaves and Maley, 2012).

Inherent in the view of cancer as an evolutionary process is that: (1) tumors consist of a heterogenous population of cells with different fitness for survival, (2) the competition among cells of a population is a dynamic process, and (3) there is a competitive landscape in the tumor microenvironment that select for variants with improved fitness. The fitness landscape includes competing for limited resources and intra- and extracellular mechanisms that are designed to maintain cellular homeostasis. While genetic sequencing technology has revealed the complexity and heterogeneity of oncogenic transformation, understanding the dynamics of oncogenesis and how cancer cells alter the selective fitness landscape remain unclear. In part, this uncertainty has been due to a scientific focus on how somatic mutations alter the inherent fitness of a cell to compete for limited resources and evade intracellular control structures (Nowak, 2006). Given the contemporary view of the degree of somatic mutations in cancer, acquiring oncogenes through random mutation also comes at a cost. Passenger mutations provide a rich source of neoantigens that can be recognized by the host immune system (Matsushita et al., 2012). Innate and adaptive immune cells comprise an extracellular control structure that is intended to restore cellular homeostasis within organ systems. Recent work suggests that malignant cells manipulate this control structure early in oncogenesis (O'Sullivan et al., 2012). In the following sections, we will describe how recent advances in technology have impacted our understanding of these three attributes of cancer as an evolutionary process. In particular, we will focus on how advances in genome sequencing have enabled improved quantification of cellular heterogeneity, how advances in computational power have enabled explicit testing of postulated intra- and extracellular control structures against the available data using simulation, and how advances in proteomics have enabled identifying novel mechanisms of cellular cross-talk that cancer cells use to alter the fitness landscape.

A TUMOR CONTAINS A HETEROGENOUS POPULATION OF MALIGNANT CELLS

Cellular heterogeneity within tumors has been recognized for several decades (Fidler and Kripke, 1977). While early efforts focused on phenotypic and morphologic heterogeneity, improved experimental tools have expanded our contemporary understanding of non-genetic and genetic sources of cellular heterogeneity within a tumor. Non-genetic sources of cellular heterogeneity have been associated with sources of cellular stress within the tumor. The metabolic requirements for cell function coupled with the diffusion of nutrients and waste products within the tumor mass stratify the tumor into different regions: an actively proliferating outer shell, a senescent inner region, and a necrotic core (Venkatasubramanian et al., 2006). The conditions within the different regions impart one component of the selective fitness landscape. For instance, malignant cells have an improved ability fulfill energetic requirements under non-ideal conditions that include hypoxia, termed the Warburg effect (Warburg, 1956; Hsu and Sabatini, 2008). In addition, emerging evidence suggests that cellular stress associated with treatment promotes reversion of an epithelial to mesenchymal-like phenotype, a phenomenon associated with resistance (Knutson et al., 2006; Higgins et al., 2007; Ebos et al., 2009; Pàez-Ribes et al., 2009). Epithelial-to-mesenchymal transition (EMT) is a biological process involved in normal development. Elements of EMT are linked in cancer with the acquisition of stem cell properties, increased invasion, and metastasis (Mani et al., 2008). The acquisition of stem cell properties is also associated with a change in oncogene dependence, such as a loss in ErbB2 expression (Shipitsin et al., 2007) and a bypass of cellular dependence on ErbB1 signaling (Barr et al., 2008). This implies that clonally derived cells at different states of differentiation will vary in therapeutic sensitivity (Voulgari and Pintzas, 2009; Sharma et al., 2010). Taken together, these studies suggest that metabolic cross-talk between cells that compete for limited resources and alterations in cell phenotype due to EMT introduce a non-genetic source of variability in how cells contained within a tumor respond to therapy.

Genetic sources of heterogeneity among malignant cells arise from the action of mutagens, such as compounds found in tobacco and UV radiation. While different mutagens have different signatures of DNA damage (Greenman et al., 2007), the random nature of DNA damage and repair implies that there are multiple ways in which tumors can originate and that many cells within a population may harbor mutations, each with a different pattern of genetic alteration. To assess the diversity of cancers that arise in a particular organ, large collaborative efforts have focused on sequencing cancer genomes (e.g., Sjoblom et al., 2006; Ding et al., 2008; McLendon et al., 2008; Pleasance et al., 2010a). In early studies, resolution was limited to coding exons associated with protein-coding genes to identify base substitutions and small insertions or deletions (Sjoblom et al., 2006; Ding et al., 2008; McLendon et al., 2008). Next generation sequencing has enabled expanded genome coverage where chromosomal rearrangement and copy number changes could also be detected (Stephens et al., 2009; Pleasance et al., 2010a,b). While many of these studies still average over the collective tumor genome, the results highlight the heterogeneity among patients with a given cancer. In focusing on a specific cancer, a recent series of papers highlight the complexity of genomic rearrangement that occurs in breast cancer (Banerji et al., 2012;Curtis et al., 2012; Ellis et al., 2012; Shah et al., 2012; Stephens et al., 2012). Collectively the results suggest that the genomes of breast cancer cells are modified extensively such that individual breast cancers carry a few consistent and functionally characterized abnormalities and tens to thousands of other alterations about which little is known. More recently, the genomic alterations in single cells have also been reported, which highlight the heterogeneity among cells of a population (Gerlinger et al., 2012; Hou et al., 2012; Xu et al., 2012).

While these sequencing efforts have focused on clinically diagnosed tumors, autopsy studies suggest that alterations in the somatic genome may be much more prevalent within an organism than has been thought previously, a stage termed "occult cancer." Nearly forty percent (39%) of women in their forties have histologic breast cancer and a similar percentage of men in their forties have histologic prostate cancer (Bissell and Hines, 2011). In support of occult cancer, these cancer sequencing studies highlight that many tumors emerge after a prolonged period of DNA damage and repair (Pleasance et al., 2010a). To illustrate the progressive change in the genome, phylogenetic trees associated with oncogenesis have been reconstructed using high resolution sequences

(Greenman et al., 2012; Nik-Zainal et al., 2012). In breast cancer, the reconstructed phylogenetic trees suggests that a majority of the time associated with oncogenesis focuses on diversifying the tumor population and selecting among nascent cancer cells. The extent of genetic rearrangement in cancer cells also highlights the frequency of mutagen-induced DNA damage and repair. For instance in lung cancer, sequencing suggests that lung epithelial cells acquire an additional mutation for every 15 cigarettes smoked, despite intracellular mechanisms designed to restore the integrity of DNA (Pleasance et al., 2010b). As the pattern of mutations is not significantly different than expected by chance, the majority of these mutations are thought not to confer a selective advantage to the cancer cell. However, these passenger mutations may provide a source of potent tumor neoantigens, as was observed in carcinogen-induced mouse models of sarcoma (Prehn and Main, 1957; Matsushita et al., 2012). In addition, these sequencing studies also suggest that metastasis may occur at different stages in different cancers. Breast cancer metastasis may occur early in oncogenesis (Kuukasjarvi et al., 1997; Torres et al., 2007; Shah et al., 2009) while prostate cancer metastasis occurs late in oncogenesis (Liu et al., 2009). Clinically, cellular heterogeneity in cancer implies that clonally homogeneous tumors may respond more favorably to treatment using a molecular-targeted therapy while a clonally heterogeneous tumor increases the likelihood that the population contains tumor cells that can survive therapy-induced changes in the fitness landscape.

THE TUMOR MICROENVIRONMENT IS A DYNAMIC SYSTEM

The second attribute of evolutionary processes is that the different cell types contained within the tumor microenvironment – stromal cells, malignant clones, and cells of the immune system – and their collective interactions create a dynamic system. This dynamic system interacts with a control structure associated with tissue homeostasis. Homeostasis is a central theme in physiology, where causal mechanisms are used to maintain the physiological state associated with life in the presence of external perturbations. These causal control mechanisms span multiple levels of organization (Klinke, 2010a) – from the cellular level, such as the intracellular mechanisms that control sodium and potassium concentrations in neurons following excitation, to the organisms level, such as organ-level mechanisms that regulate body temperature following changes in activity level. The challenge in tumor immunology is trying identify the immune-related control mechanisms that regulate the homeostatic composition of cells within an organ and how tumor cells interfere with this control structure.

To identify these control structures, one frequently creates a mental model of how one thinks a system behaves based upon prior knowledge of the system (i.e., a hypothesis); designs a controlled experiment; and acquires data to infer using statistics whether the mental model is a valid representation of the causal mechanisms that regulate system behavior. Conventionally, the mental models are "tested" against the observed data using tools of inferential statistics that were originally developed in the early 1900s (Neyman and Pearson, 1933; Fisher, 1935). Collectively, this process is called strong inference (Platt, 1964) or alternatively *in cerebello* model-based inference. There are five challenges with the conventional approach to identifying the control structure associated with tissue homeostasis and oncogenesis: (1) the interactions among cells occur locally in the tumor microenvironment, (2) robust control typically involves redundant mechanisms, (3) the control structures can be non-linear, (4) the roles that specific mechanisms play in regulating system response can change with time, and (5) many control structures are still unknown (i.e., lurking mechanisms exist). To address these challenges, we will first examine the weaknesses associated with the conventional *in cerebello* model-based inference and propose an alternative approach for inference that leverages contemporary advances in computational power.

One particular challenge in how classical tools of inferential statistics are used in practice is that one formulates the inference test in terms of two alternative hypotheses: the null hypothesis – the experimental perturbation introduces no change in the system – and an alternative hypothesis – the observed response is consistent with the proposed mechanistic hypothesis. If the data observed under control and perturbed conditions are sufficient different, the null hypothesis is rejected. Conventionally, the alternative hypothesis is then accepted. This conclusion depends on assuming that there are no other lurking mechanisms at work in the system. To highlight the problematic nature of this assumption, we consider recent controversial findings related to anti-tumor immunity. Two recent papers suggest that the adaptive immune system does not influence tumorigenesis and metastasis formation nor chemotherapy response in a spontaneous HER2-driven genetically engineered mouse model for breast cancer (Ciampricotti et al., 2011, 2012). These studies were in response to work that suggests that adaptive immunity does influence tumorigenesis (Shankaran et al., 2001; Dunn et al., 2002) and clinical response to chemotherapy (Apetoh et al., 2007; Obeid et al., 2007; Ghiringhelli et al., 2009; Mattarollo et al., 2011). de Visser and colleagues argue that transplantable models for cancer do not resemble established spontaneous tumors and use a genetically engineered mouse model (GEMM) where the mouse mammary tumor virus (MMTV) is used to induce tissue-specific expression of rat Her2 (Neu) in the mammary glands (i.e., the MMTV-NeuT model, Boggio et al., 1998). In contrast, Jacks and coworkers suggest that GEMMs of cancer may underestimate the mutational and antigenic load of most human cancers (DuPage et al., 2012).

Histological presentation of spontaneous breast cancer in the MMTV-NeuT may resemble the human equivalent (van Leeuwen and Nusse, 1995) but the molecular underpinnings of oncogenic transformation in humans may be completely different. While exome sequencing has yet to be reported, MMTV-NeuT tumors exhibit distinct and homogeneous patterns of gene expression that are unlike the human HER2+/ER-subtype (Herschkowitz et al., 2007). Oncogenes, like HER2, are a well-characterized subset of genes that upon amplification or silencing result in oncogenic transformation. While cancers commonly contain altered oncogenes, the random nature of DNA damage and repair implies that there is a mutational cost associated with malignancy. In thermodynamic terms, the conversion of one state to another state always comes at a cost, this cost is an increase in disorder

(i.e., entropy)[1]. So while the MMTV promotes the expression of the oncogene, the available data suggests that the MMTV-NeuT GEMM of breast cancer does not reproduce the degree of mutational heterogeneity observed in human breast cancers. Moreover, HER2/Neu overexpression has been suggested to downregulate major histocompatibility complex (MHC) class I expression based upon clinical data (Maruyama et al., 2010), GEMMs (MMTV-Neu; Lollini et al., 1998), and cell models (Herrmann et al., 2004).

To aid in interpreting the reported MMTV-NeuT GEMM data, we will consider a simple mathematical model for tumor growth. The fate of a malignant clone in a tissue microenvironment can be described as a dynamic system where competing cellular fates are regulated by a combination of intracellular mechanisms, such as initiation of cell proliferation or cell death, and extracellular control mechanisms, such as the role that immune cells play in eliminating microbes and foreign cells from the system. Mathematically, these causal mechanisms regulate the change in tumor size (C_T) as a function of time:

$$\frac{dC_T}{dt} = \overbrace{(k_p - k_d)}^{\text{oncogenes alter k's}} \cdot C_T - \underbrace{k_{dI} \cdot C_{\text{II}} \cdot C_T}_{\text{innate immmunity}} - \underbrace{k_{dA} \cdot C_{\text{AI}} \cdot C_T}_{\text{adaptive immmunity}}, \quad (1)$$

where k_p and k_d are the propensity for a given transformed clone to either proliferate or die through an intrinsic mechanism within a period of time, respectively. The last two non-linear terms $k_{dI} \cdot C_{\text{II}} \cdot C_T$ and $k_{dA} \cdot C_{\text{AI}} \cdot C_T$ refer to the rates of cell death elicited by innate and adaptive immunity, respectively, and C_{II} and C_{AI} are the number of innate and adaptive immune cells within a given tissue volume. These non-linear terms are the product of three quantities: the abundance of immune cells within a given tissue volume, the abundance of cancer cells within a given tissue volume, and the propensity for a tumor cell to be killed following contact with an immune cell within a given period of time. In this simple model, the terms represent different biological control mechanisms. On the surface, innate and adaptive immunity may be considered redundant. However, as illustrated in **Figure 1**, the control exerted by innate and adaptive immunity changes with time. Our prior knowledge of relevant control mechanisms (i.e., that Neu overexpression downregulates MHC class I and the lack of diversity of neoantigens decreases the likelihood for an effective cytotoxic immune cell response) can be implemented in the model in the form of a reduced value for k_{dA}. Then as the value of k_{dA} goes to zero, the presence or absence of adaptive immune cells does not alter the tumor growth trajectory. As these papers provide no information regarding the killing efficacy of cytotoxic T cell–tumor cell interaction, the data presented are insufficient to support the stated conclusions. As alluded to in this example, there are new methods for model-based inference that involve the use of mathematical models and simulation to test hypotheses.

In contrast to *in cerebello* model-based inference, *in silico* model-based inference is the statistical reasoning about our understanding of cause and effect in natural systems from experimental observation using computer simulation. Similar to a microscope that assists our natural ability to see small objects, mathematical models assist our natural intuition as they require an explicit statement of underlying assumptions and establish formal relationships between cause and effect. While mathematical modeling, *per se*, is not new to biology, there are recent advances in how our current understanding of a reactive system can be tested against the observed data. Conventionally called scientific hypothesis testing, this process aims to protect against the possibility that a discovery is based upon natural chance alone and not upon a new mechanism. The methods used for scientific hypothesis testing were developed in the early 1900s. These methods were well suited to the questions of the day, as we had very limited knowledge of biological systems and we were limited to pencil-and-paper calculations. Today, the intellectual landscape is different. High performance computing and high-throughput assays have fundamentally changed the way we study biology and motivate a contemporary approach. This contemporary approach is called *in silico* model-based inference and draws on ideas from high performance computing, statistics, and chemical kinetics. The combination of high performance computing with statistics is an active field of research that focuses primarily on data regression problems using correlative (or empirical) models (for a discussion of data regression in systems biology see Jaqaman and Danuser, 2006). Incorporating ideas drawn chemical kinetics enables *in silico* model-based inference and reshapes how these existing computational statistics tools are applied to problems of biological network inference.

In traditional chemical kinetic applications, mechanistic models of reaction networks are used for different objectives. Objectives include developing a mechanistically inspired empirical model for interpolating reaction data, developing reduced-order models of chemical kinetics to incorporate into more complicated models that account for fluid transport and reaction, and developing unbiased mechanistic models to aid in identifying key reaction steps that are at work under particular conditions. This last application is important if the resulting reaction model is going to be used to predict reactive behavior under new conditions and bears the most similarity to the challenges in biological network inference. It has also been known that mechanistically inspired empirical models have limited value in identifying novel reaction mechanisms as postulated mechanisms impose bias *a priori* (Green, 2007). This shortcoming of mechanistically inspired empirical models motivated generating mechanistic models of reaction networks using rule-based methods (Green, 2007). More recently, rule-based methods have also been embraced by the systems biology community (e.g., Faeder et al., 2009; Feret et al., 2009; Bachman and Sorger, 2011). One of the advantages of a rule-based method is that, instead of hand-crafting a reaction network using

[1]To make the thermodynamic analogy, we assume that the genome is a closed system and initially is comprised of a single genetic microstate. Mutations are introduced through a random process associated with DNA damage and repair. The acquisition of a genetic microstate that exhibits an improved fitness using this random process is also associated with the population acquiring additional microstates that exhibit neutral or negative fitness. Entropy is proportional to the number of possible genetic microstates that cells within a population can occupy. The analogy implies that adaptive immunity is an entropy detector. Cancers that exhibit a simple mutation signature may not engage adaptive anti-tumor immunity but may be more responsive to molecular-targeted therapy. Alternatively, cancers that exhibit a complicated mutation signature may not exhibit a durable response to molecular-targeted therapy and may be controlled by re-establishing adaptive anti-tumor immunity.

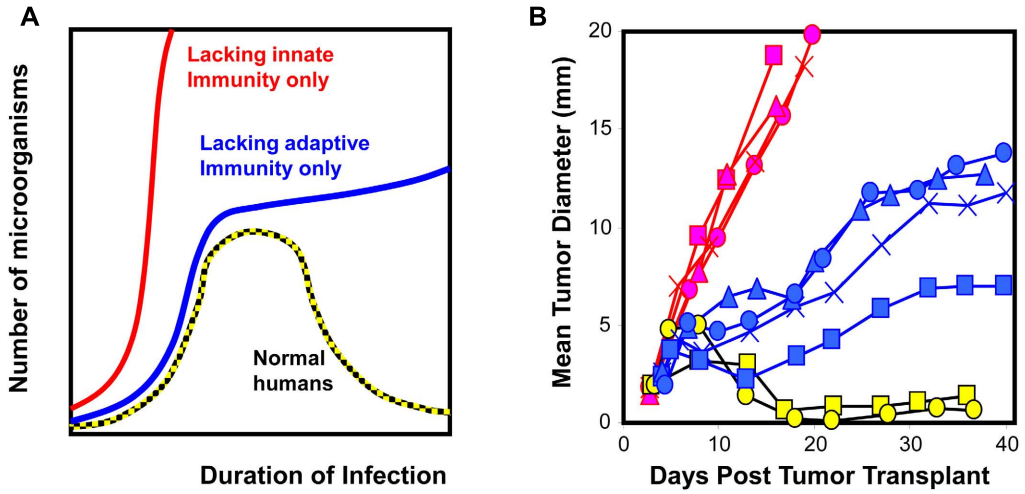

FIGURE 1 | Cellular homeostasis is a dynamic process that includes both innate and adaptive immunity. (A) The dynamics associated with immune surveillance to microorganisms in humans and mice (Murphy et al., 2007). **(B)** Similarly, clones derived from 3′ methylcholanthrene-induced sarcomas exhibit different phenotypic dynamics upon transplantation [WT clones transplanted into RAG2$^{-/-}$ hosts (red) and RAG2$^{-/-}$ clones transplanted into WT hosts (blue and yellow; O'Sullivan et al., 2012)]. Restoring homeostasis that microorganisms or tumor cells are not present in the system requires both innate and adaptive immunity. The contribution of innate versus adaptive immunity changes with time; innate immunity dominates at early time points and initiates adaptive immunity that dominates at late time points. Results for highlighted clones imply that WT clones (red) have acquired ability to evade innate immunity and their ability to evade adaptive immunity is unknown. RAG2$^{-/-}$ clones have acquired ability to evade adaptive immunity (blue) or are unable to evade innate and adaptive immunity (yellow).

a set of implicit assumptions, computer algorithms are used to generate a reaction network given a set of reactants and a set of transformations that are thought to act within the system. It is relatively easy then to change the set of transformations and see how a different set of transformations impacts the predictive power of the resulting reaction network.

The rules represent fundamental transformations, such as protein—protein interactions or elementary reactions steps, that are associated with the flow of chemical information within reaction networks. Each transformation has an associated rate constant that quantifies how quickly a transformation can occur given the presence of the reactants – a time scale. Moreover, the rate constants associated with each rule can be different. This implies that the overall flow of chemical information within reaction networks is governed by the slowest transformation. In traditional chemical kinetic applications, slow reactions are called rate-limiting steps. The rate-limiting steps correspond to sensitive levers within the reaction network that one can manipulate to achieve a desired objective – such as an improved conversion rate or selecting flow patterns within the reaction network to improve selectivity or yield of a desired product. Generally, this behavior is called the slaving principle [see comments on pg 6 of Klinke (2009, 2010a)].

In Klinke and Finley (2012), the time scales associated with the model parameters are linked to the fundamental transformations (i.e., protein–protein interactions or elementary reaction steps) that transmit chemical information within reaction networks. We show that only a subset of time scales can be uniquely identified using the observed data (i.e., exhibit two-sided bounded distributions). Transformations that are fast – such a pre-formed multi-protein complexes – and that are kinetically unimportant – such as extremely slow reactions – exhibit one-sided distributions. More importantly, this work demonstrates that the Adaptive Markov Chain Monte Carlo algorithm described in Klinke (2009) was the first to provide posterior distributions in the model parameters that are consistent with the slaving principle. Of note is that the prior statistical inference studies applied to biological network inference questions provide posterior distributions in the model parameters that have two-sided bounds all supposedly informed by data, such as a multivariate Gaussian distribution (e.g., Brown and Sethna, 2003; Brown et al., 2004; Gutenkunst et al., 2007; Vyshemirsky and Girolami, 2008; Toni et al., 2009; Toni and Stumpf, 2010; Calderhead and Girolami, 2011; Erguler and Stumpf, 2011). Given that none of the prior statistical inference studies provide "posterior" distributions that are consistent with the slaving principle, this raises the question as to whether these "posterior" distributions really reflect the data or whether they reflect an arbitrary selection of a prior or biased model formulation. For instance in Calderhead and Girolami (2011), the authors assume *a priori* that all of the postulated mechanistic steps encoded in the model are kinetically important – i.e., that there are no fast or extremely slow reactions. They also fixed *a priori* parameters that were structurally non-identifiable. Two-sided bounded distributions for all of the model parameters reported in these studies is not surprising as conventional Markov Chain Monte Carlo methods are used for regressing empirical models to data and tests of Markov chain convergence are applied to the model parameters.

As illustrated in Klinke et al. (2012), the *in silico* model-based inference approach can incorporate the best available domain knowledge, including competing hypotheses regarding topology, and search for all possible parameter combinations that provide model predictions consistent with the best available data. This

paper illustrates three possible results from *in silico* model-based inference. First, the model predictions may be consistent with the observed data and only one competing topological hypothesis is favored, which suggests that the observed data is able to discriminate among the competing topological hypotheses and that the corresponding topology is of sufficient complexity to explain the observed data. The autocrine Tumor necrosis factor (TNF)-alpha feedback mechanism illustrates this result. Second, model predictions that are unable to match the observed data suggest that the topology is missing important connections, such as paracrine feedback mechanisms that may be important *in vivo* but have no effect under conventional *in vitro* conditions (e.g., see discussion of high density results at the top of pg 4). Third, the model predictions are consistent with the observed data but are unable to discriminate among competing topological hypotheses. The discovery of differential STAT1/STAT4 activation by interleukin (IL)-12 illustrates the third type of result. According to the editor of Science Signaling, this work "serves as an example of how mathematical modeling can refine our understanding of signaling pathways." Ultimately, determining whether the topology of a reaction network can be uniquely identified from the available data is essential for identifying the right control structures at work in biological systems.

THE SELECTIVE FITNESS LANDSCAPE IN CANCER CONTAINS INTRA- AND EXTRACELLULAR CONTROL ELEMENTS

The third attribute of evolutionary processes is that local cellular environment provides a selective fitness landscape for the retention or removal of malignant variants from a population. This local fitness landscape includes competing for limited resources – such as limited oxygen or glucose or stromal support – and active intra- and extracellular control mechanisms that aim to restore cellular homeostasis. Intracellular control mechanisms include p53, a protein that helps control genomic integrity and is mutated in more than half of all cancers (Hollstein et al., 1991), and the retinoblastoma tumor suppressor gene (pRb), which encodes a protein that regulates cell cycle (Friend et al., 1986). An example of an extracellular control mechanism is the role of innate and adaptive immunity in eliminating foreign or pathogenic organisms from the cellular population. As highlighted in an influential review (Hanahan and Weinberg, 2011), decades of cancer research have revealed how intracellular control mechanisms are evaded during oncogenesis. While it is well-known that tumor load limits the efficacy of immune cells in controlling tumor growth (e.g., Maccubbin et al., 1989; Pulaski and Ostrand-Rosenberg, 1998; van Elsas et al., 1999), our understanding of how cancer cells evade extracellular control mechanisms is still emerging.

As summarized in Eq. 1, immune-mediated tumor regression is proportional to the product of three terms: the number of tumor cells recognized by the host's immune cells, the number of immune cells present in the tumor microenvironment that can elicit tumor-directed cytotoxicity, and the cellular efficiency of immune cells in eliciting tumor-directed cytotoxicity. Recent large-scale studies that aim to quantify the diversity of human cancer can also be used to identify the phenotype associated with different immune cells recruited to the tumor microenvironment. Understanding the composition and phenotype of cells contained within tumors may help inform future cancer immunotherapies (Kerkar and Restifo, 2012). As illustrated in **Figure 2**, mRNA expression results from 224 colorectal tumor and normal pairs reported as part of the Cancer Genome Atlas (TCGA) provide an overview of the immunological bias present in colorectal cancer (Muzny et al., 2012). These gene expression signatures can be used to infer the extent of natural killer (NK) cells, T cells, and tumor-associated macrophages recruitment into the tumor (see **Figure 3**) and the corresponding phenotype of immune cells within the tumor microenvironment (see **Figure 4;** Wei et al., 2009; Movahedi et al., 2010). Within this TCGA colorectal data set, three patient clusters were identified based upon a subset of genes associated with anti-tumor immunity and immunosuppressive mechanisms. Group 1 corresponds to normal tissue with a mixed Th1 and iTreg CD4+ T helper cell and M2 macrophage signatures. Groups 2 and 3 correspond to colorectal cancer samples with different immune signatures. Group 2 has a slightly lower gene signature associated with NK, T cell, and macrophage infiltrate compared to normal tissue samples while the immune cell infiltrate exhibits a preference for Th1 T helper cell and mixed M1 and M2 macrophage signatures. The gene signature associated with NK, T cell, and macrophage infiltrate is lowest in Group 3 and exhibits a mixed Th17 and Th2 T helper cell signature and a macrophage signature similar to group 2. Due to the short follow-up time associated with the colorectal study, the relationship between overall survival and these immune cell signatures is unclear. While these gene expression studies provide insight into the number and phenotype of immune cells present within the tumor microenvironment, identifying the control mechanisms that become altered during oncogenesis are difficult to identify from static snapshots of a biological state. Generally, identifying causal mechanisms at work in multi-component systems is one of the most pervasive problems in the analysis of physiological systems (Khoo, 2000).

In engineering, this problem is called a system identification problem where causal relationships between system components are inferred from a set of input (i.e., biological cue) and output (i.e., response) measurements (Khoo, 2000). In context of anti-tumor immunity, an input may be the influx of cytotoxic T lymphocytes that recognize tumor-specific antigens and an output may be tumor regression. Many approaches exist for the identification of open-loop systems, where a change in input causes a unique change in output. Reductionist methods have revealed a wealth of knowledge regarding how isolated components of physiological systems respond to biological cues. However, the different cell types contained within the tumor microenvironment constitute a closed-loop system, as implied by the observation that tumor load influences the efficacy of immune cells that enter the tumor microenvironment. A closed-loop system is defined as a multi-component system where the output (i.e., response) of one component provides the input (i.e., biological cue) to another component. Closed-loop systems are particularly challenging as it is impossible to identify the relationships among components of a system based upon overall input (e.g., T cell infiltrate) and output (e.g., tumor regression) measurements. One of the reasons for this is that changes in the internal state of the system, such as an increase in biological cues associated with tumor load, may alter

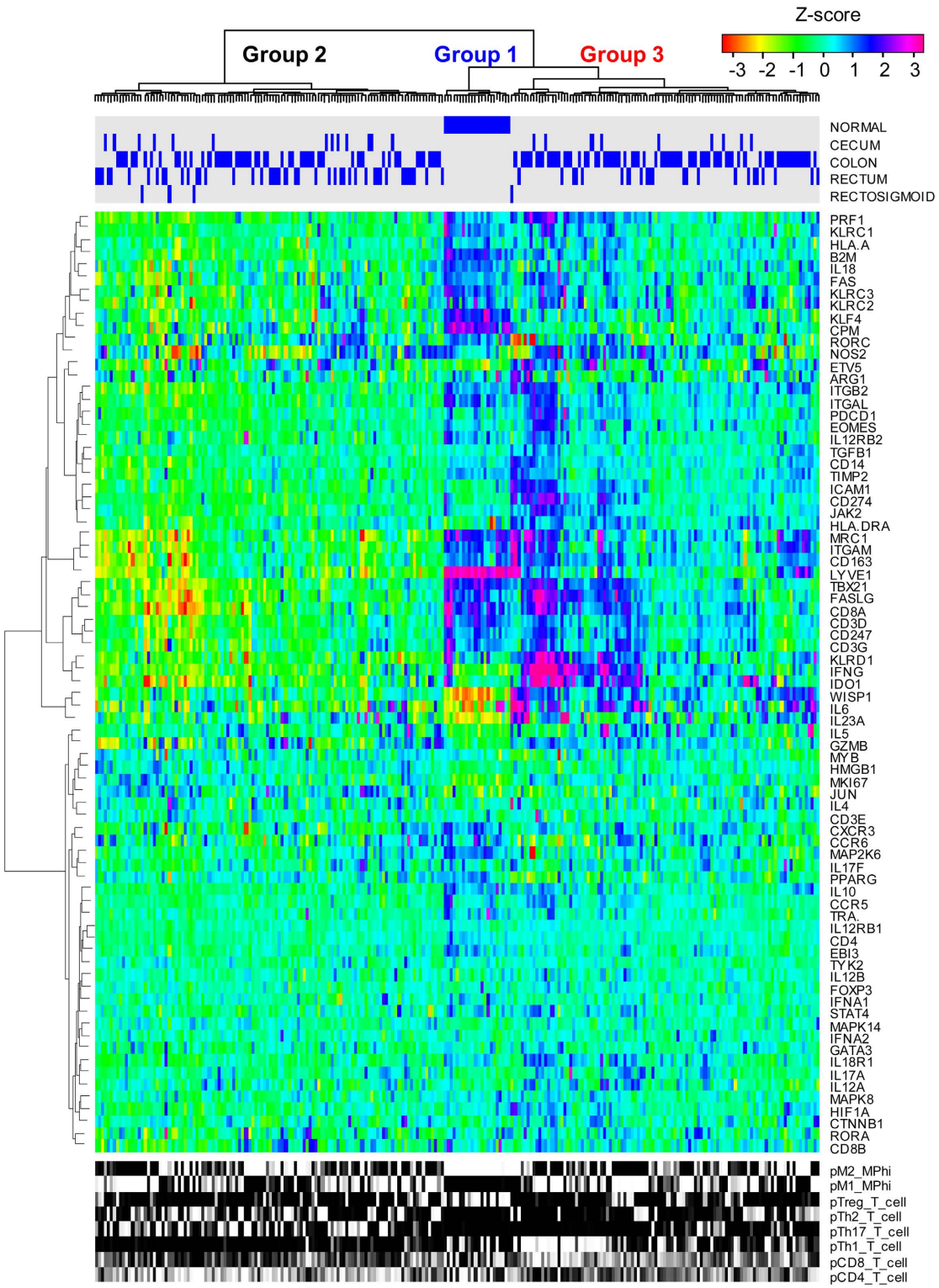

FIGURE 2 | Immune gene expression signatures in colorectal cancer clusters into three groups. mRNA expression obtained from normal colorectal and cecum, colon, rectum, and rectosigmoid adenocarcinoma tissue samples (columns) were hierarchically clustered into three groups based upon the log2 median normalized expression ratio for genes (rows) related to cell-mediated cytotoxic immunity and tumor immunosuppression. The tissue of origin is highlighted by the blue bars on top and gene expression is shown as a row-normalized heatmap. Red denotes under-expressed and violet denotes overexpressed relative to the population mean. Dendrogram indicates the degree of similarity among genes (rows) or samples (columns) using the Ward's minimum distance method in R.

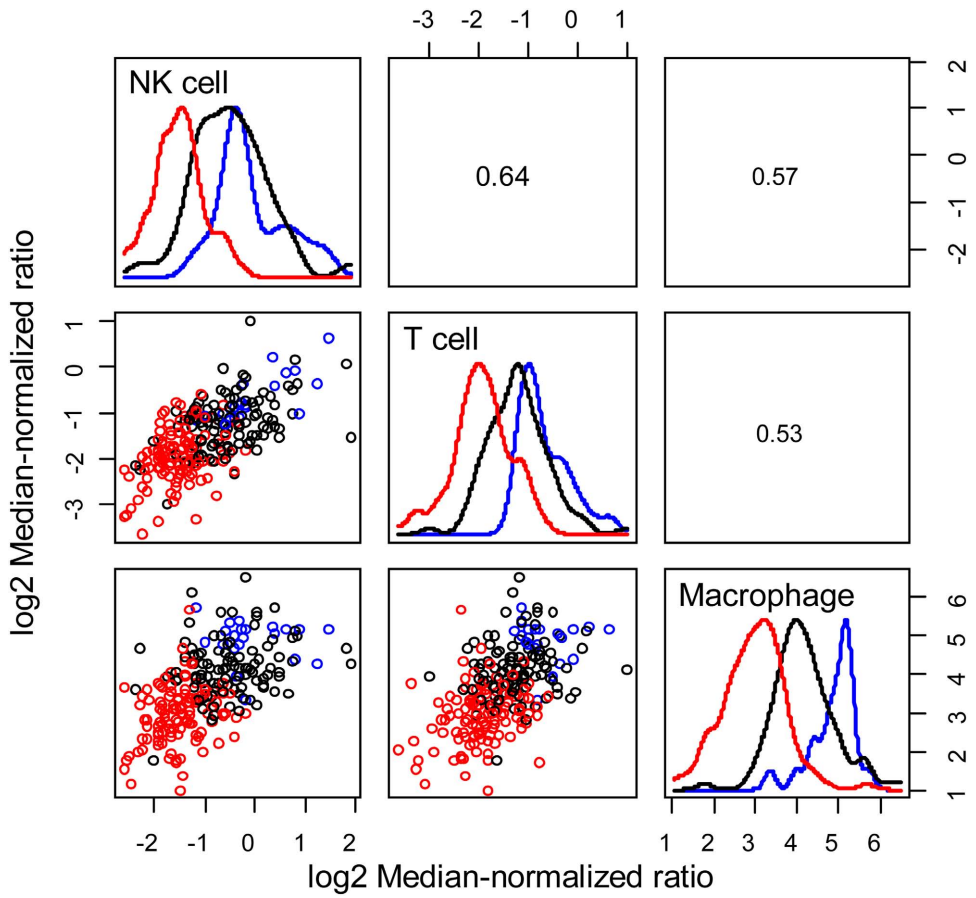

FIGURE 3 | Patient clusters exhibit different immune cell signatures. Relative immune cell infiltrate was estimated based upon the average expression of genes associated with NK cells (*KLRD1*, *KLRC1*, *KLRC2*, *KLRC3*), T cells (*CD247*, *CD3G*, *CD3D*, *CD3E*), and macrophages (*CD14*, *CPM*, *MRC1*, *HLA-DRA*, *ITGAM*). Bivariate scatter plots are shown below the diagonal, marginalized histograms stratified by the three groups are shown on the diagonal, and correlation coefficients are shown above the diagonal. Results are colored by group (Group 1: blue, Group 2: black, Group 3: red).

the response of the system to a defined input, such that there is not a direct causal relationship between overall system input and output. Historically, the causal mechanisms underlying the behavior of closed-loop systems in physiology have been identified via ingenious methods for isolating components within the integrated system, that is methods for "opening the loop." A classic example of this is the discovery of insulin by Dr. Frederick Banting and Charles Best in 1921 and it's role in connecting food intake to substrate metabolism (Roth et al., 2012). In this case, the biological cues – insulin and glucagon – that facilitate communication between components – endocrine pancreas, liver, and muscle – can be easily assayed in the blood. Observing and regulating these endocrine hormones in the blood enable one to disassemble the closed-loop system into a series of coupled open-loop systems. Each open-loop system responds in defined ways to biological cues, as depicted in the minimal model for the regulation of blood glucose (Bergman et al., 1979).

There are two key differences in applying systems identification methods to help identify the control mechanisms that regulate anti-tumor immunity compared with the control mechanisms that regulate substrate storage and metabolism. First, the biological scales are different: coordinated substrate storage and metabolism is an organ-level phenomenon while anti-tumor immunity is a cell population-level phenomenon. Second, the cross-talk among components occurs locally through secreted proteins or cell-to-cell contact. While the dynamics of cell-to-cell interactions within the tumor microenvironment can be observed using intravital live imaging (Engelhardt et al., 2012), the biochemical cues responsible for cell cross-talk are more difficult to identify *in vivo*. Moreover, samples from the peripheral blood may not be representative of the local biological cues responsible for cell cross-talk. Conventionally, immunohistochemical methods have been used to identify local biological cues present in the tissue microenvironment, a discovery process associated with experimental bias. The experimental bias stems from the fact that the method for detecting a local biological cue must be selected *a priori* and that methods for detecting this biological cue must exist (i.e., an antibody must exist). Similar to the development of rule-based modeling methods as a way to minimize bias, proteomics provide less biased methods for identifying local signaling mechanisms that contribute to homeostatic control.

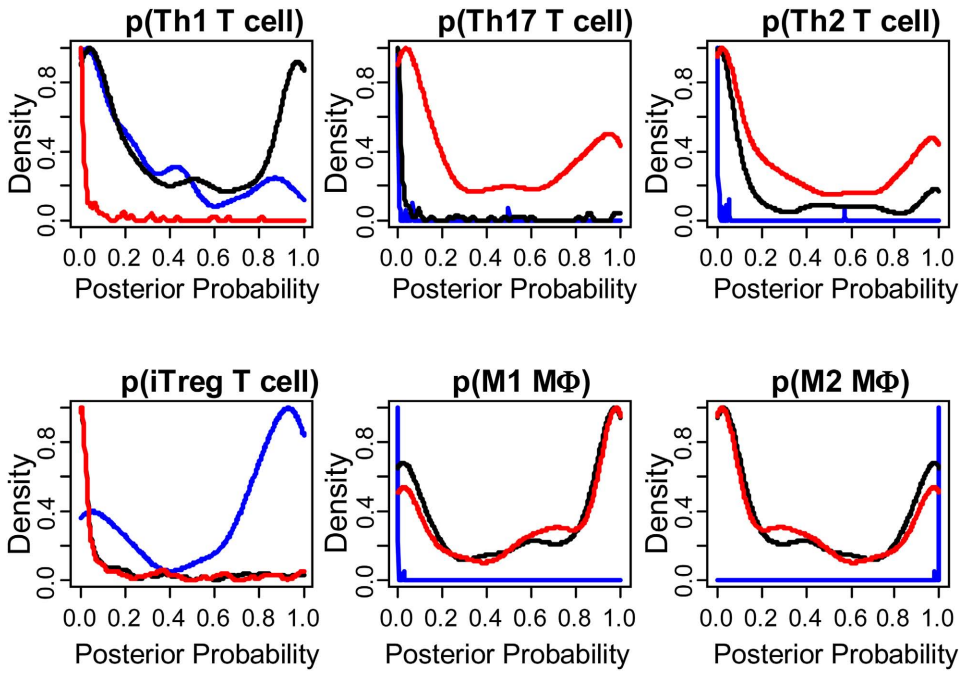

FIGURE 4 | The phenotype of immune cells within the tumor microenvironment are different among the three groups. Posterior probability distribution for T helper cell and macrophage phenotypes stratified by group, where probability was based on mutually exclusive gene expression patterns that are associated with each cell subset. T helper cell subsets were based upon gene clusters associated with Th1 (*CD4, TBX21, EOMES, FASL, IFNG, IL10*), Th17 (*CD4, RORA, RORC, IL17A, IL17F*), Th2 (*CD4, GATA3, PPARG, IL4, IL5, IL6, IL10*), and iTreg (*CD4, FOXP3, RORC, TBX21, CCR6, IRF4, MYB, TGFB1, IL10, EBI3, IL12A*) differentiation (Wei et al., 2009). Macrophage subsets were based upon gene clusters associated with M1 (*IL6, IL12B, IL23A, NOS2, IDO1*) and M2 (*TIMP2, LYVE1, ARG1, KLF4, CD163*; Movahedi et al., 2010). Results are colored by group (Group 1: blue, Group 2: black, Group 3: red). Posterior probability for each patient is also shown in the bottom row of **Figure 2** (Gray scale where 0 = white and 1 = black).

Proteomic methods have been incorporated into a variety of workflows for identifying biochemical cues that underpin cell population-level control mechanisms. Analogous to immunohistology, recent work describes imaging protein, lipid, and small molecule profiles in biological tissues using direct laser-assisted ionization followed by time-of-flight mass spectrometry (Nemes et al., 2010; Stauber et al., 2010). The distribution of lipid and small molecular profiles can be obtained at a lateral resolution of 350–35 μm (Campbell et al., 2012). However, discriminating between extracellular and intracellular localization and identifying higher molecular weight proteins is difficult given the current technology, although improvements are likely (Jungmann and Heeren, 2012). Another approach is to create minimal co-culture model systems that reproduce critical aspects of the cellular cross-talk that occurs within the tumor microenvironment. To identify mechanism of resistance to anti-cancer therapies, Golub and coworkers assayed the *in vitro* response of 45 different cancer cell to 35 anti-cancer drugs while co-cultured with one of 23 different stromal cell lines (Straussman et al., 2012). They used a reverse phase protein array to identify that stromal cells secrete hepatocyte growth factor (HGF) that confers tumor cell resistance to RAF inhibitors (e.g., vemurafenib). This mechanism for cellular cross-talk was supported by immunohistology results showing that stromal cell expression of HGF correlates with innate resistance to RAF inhibitor treatment in human melanoma. While the results highlight that local paracrine cues can influence therapeutic response, using a reverse phase protein array still assumes that the proteins responsible for the observed behavior are measured by the array. As a less biased alternative, mass spectrometry can be used to identify proteins that are secreted within the co-culture system. In Kulkarni et al. (2012), Klinke and coworkers used a 2D-gel electrophoresis MALDI-TOF/MS workflow in conjunction with a high content co-culture assay to identify that malignant melanocytes secrete exosomes and Wnt-inducible signaling protein-1 (WISP1). Exosomes are nanometer-sized endogenous membrane vesicles that are produced by a diverse range of living cells and are thought to play key roles in shaping intercellular communication, such as immunity (Théry et al., 2009). By co-culturing the malignant melanocytes with a Th1 cell line, they found that WISP1 inhibits the functional response of the Th1 cell to IL-12. From a systems identification perspective, *in silico* model-based inference was used to confirm that, in isolation, the Th1 cell line can be described as an open-loop system and that the *in vitro* co-culture model recreates a closed-loop system. *In silico* model-based inference was also used to infer that WISP1 is expressed at the periphery of B16-derived tumors *in vivo*, a similar pattern of WISP1 expression was observed in human melanoma. In addition to secreting WISP1, they also found that the B16 model for melanoma overexpresses one component of the IL-12 receptor, IL12Rβ2, that creates a local cytokine sink for IL-12. In other work, they report that STAT4 is phosphorylated irreversibly, creating a short term memory to IL-12 signaling (Klinke et al., 2012). The duration of this memory

is limited by cell proliferation. Other groups have shown that local delivery of IL-12 to the tumor microenvironment promotes tumor regression in the B16 melanoma model (Kerkar et al., 2011, 2010) and in the El4 thymoma model (Pegram et al., 2012). Collectively, these studies imply that signaling by endogenous IL-12 within the tumor microenvironment may help maintain T cell polarization when cognate tumor antigens induce T cell proliferation (Wang et al., 2007) and that manipulating this extracellular control mechanism may impart a survival advantage to the collective tumor population. In summary, these examples illustrate that coupling co-culture models with proteomics can uncover important local control mechanisms and that choosing a particular proteomics workflow involves a trade-off between selecting the degree of abstraction from reality in designing the experimental system and observing biochemical cues, given the current limits of the technology.

CONCLUSION

It has been over a decade since molecular-targeted therapies revolutionized the treatment of cancer. The clinical reality observed in intervening years has dampened the initial enthusiasm, as efficacy is limited to defined patient groups and durable response is difficult to achieve. Contemporary understanding of oncogenesis paints a more complex picture of cancer as an evolutionary process. As an evolutionary process, cancer has three hallmark characteristics: (1) that malignant cells within the tumor microenvironment are heterogeneous, (2) that interactions among cells within the tumor microenvironment comprise a dynamic system, and (3) that intra- and extracellular control mechanisms constitute a selective fitness landscape that determines the survival of cells within the tumor microenvironment. Innate and adaptive immunity function as important extracellular control mechanisms. Observed in a subset of melanoma patients, durable response to a new immunotherapy provides hope that restoring these extracellular control mechanisms can be used as an effective weapon in the battle against cancer (Hodi et al., 2010). However, increasing the subset of patients that receive clinical benefit requires an improved understanding of cancer as an evolutionary process. Here, we have reviewed some of the emerging technologies that have improved our understanding of these evolutionary hallmarks. A common theme in this review is how new technology improves our ability to limit unintended bias. At the same time, advances in computing power motivate new methods for model-based inference that leverage the rich body of knowledge accumulated over decades of oncology and immunology research. Only through an integrated approach, will we be able to deliver a true revolution in cancer treatment.

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Single-cell protein secretomic signatures as potential correlates to tumor cell lineage evolution and cell–cell interaction

Minsuk Kwak[1], Luye Mu[2], Yao Lu[1], Jonathan J. Chen[1], Kara Brower[1,3] and Rong Fan[1,4]*

[1] Department of Biomedical Engineering, Yale University, New Haven, CT, USA
[2] Department of Electrical Engineering, Yale University, New Haven, CT, USA
[3] Isoplexis Inc., New Haven, CT, USA
[4] Yale Comprehensive Cancer Center, New Haven, CT, USA

Edited by:
Chao Ma, California Institute of Technology, USA

Reviewed by:
Luis De La Cruz-Merino, Hospital Universitario Virgen Macarena, Spain
Jianping Fu, University of Michigan, USA

***Correspondence:**
Rong Fan, Department of Biomedical Engineering, Yale University, 55 Prospect Street, New Haven, CT 06511, USA.
e-mail: rong.fan@yale.edu

Secreted proteins including cytokines, chemokines, and growth factors represent important functional regulators mediating a range of cellular behavior and cell–cell paracrine/autocrine signaling, e.g., in the immunological system (Rothenberg, 2007), tumor microenvironment (Hanahan and Weinberg, 2011), or stem cell niche (Gnecchi et al., 2008). Detection of these proteins is of great value not only in basic cell biology but also for diagnosis and therapeutic monitoring of human diseases such as cancer. However, due to co-production of multiple effector proteins from a single cell, referred to as *polyfunctionality*, it is biologically informative to measure a panel of secreted proteins, or secretomic signature, at the level of single cells. Recent evidence further indicates that a genetically identical cell population can give rise to diverse phenotypic differences (Niepel et al., 2009). Non-genetic heterogeneity is also emerging as a potential barrier to accurate monitoring of cellular immunity and effective pharmacological therapies (Cohen et al., 2008; Gascoigne and Taylor, 2008), but can hardly assessed using conventional approaches that do not examine cellular phenotype at the functional level. It is known that cytokines, for example, in the immune system define the effector functions and lineage differentiation of immune cells. In this article, we hypothesize that protein secretion profile may represent a universal measure to identify the definitive correlate in the larger context of cellular functions to dissect cellular heterogeneity and evolutionary lineage relationship in human cancer.

Keywords: intra-tumor heterogeneity, protein secretion profile, single-cell analysis, immunomonitoring, tumor microenvironment

THE SECRETOMIC PROFILE OF SINGLE T CELLS DEFINES A CORRELATE TO PROTECTIVE IMMUNE RESPONSES

To establish our hypothesis and elucidate the strategies, we would like to start with the important discoveries in the field of immunology that have enhanced our understanding of protective immune responses elicited by T cells in response to infection and vaccination. T cells demonstrate diverse and important functional activities in mediating immune response that provide protection against various infections (Precopio et al., 2007; Sallusto and Lanzavecchia, 2009; Bhatia et al., 2012). Upon encountering specific pathogenic antigens that generates polarizing stimulus that induces development of specific phenotype, immune cells are activated and proliferate. After their activation, immune cells differentiated into highly heterogeneous functional lineages and attain a wide variety of effector functions (O'Garra, 1998; Darrah et al., 2007; Precopio et al., 2007; Betts et al., 2006; O'Shea et al., 2008; Seder et al., 2008; Zhu and Paul, 2010; Ma et al., 2011). Effector T cells can regulate and prime their effector mechanisms to clear the infection by producing and secreting diverse cytokines, which play important roles in orchestrating immune responses and controlling pathogenic conditions (Wong and Goeddel, 1986; Harty et al., 2000; Sandberg et al., 2001). T cells develop into highly heterogeneous subpopulations, which can be classified by their differentiation states based on surface marker phenotypes and then by diverse functional profiles (**Figure 1**), as reflected by distinct cytokine production patterns (Sandberg et al., 2001; Appay et al., 2008; Seder et al., 2008; Ma et al., 2011; Han et al., 2012).

The critical issues in developing effective vaccines have been comprehensive characterization of these complex T cell responses (O'Garra, 1998; Darrah et al., 2007; Seder et al., 2008). It is important to identify the correlation of both quality and magnitude of T cell immunity with the protective responses generated following infection or vaccination (De Rosa et al., 2004). Due to increasing phenotypic and functional heterogeneity of effector T cells and the plasticity of T cell differentiation, there have not been clearly defined correlates of immune protection against specific pathogens. Correlate of immune protection is a measurable predictor of an individual's immunity to a pathogen following infection or vaccination (Zhu and Paul, 2010). Defining correlates of protective T cell immunity has been particularly challenging for immunologists because the degrees of protection does not clearly match with any known T cell phenotypes (O'Shea et al., 2008).

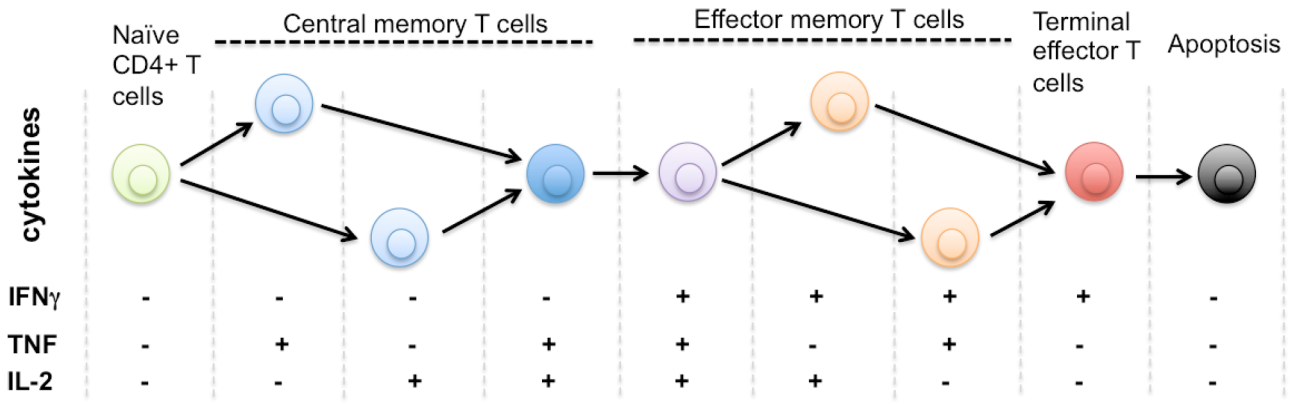

FIGURE 1 | Protein secretion profile defines phenotype and stage of CD4$^+$ T cell differentiation. It is known that human naïve CD4$^+$ T cells differentiate through multiple stages including central memory and effector memory T cells to become terminally differentiated effector T cells that are monofunctional (IFNg$^+$), less immunoprotective, and approaching the commitment to apoptosis. The triple positive polyfunctional T cells were found more potent and durable to generate effective immune protection over a prolonged time. Due to the limitation of single-cell cytokine profiling technology, it remains unclear how many effector functions are associated with single T cells. The general trend is polyfunctionality positively correlates to the effectiveness of T cell responses against infection or tissue dysfunction. There is also a diverse range of functional phenotypes defined by distinct cytokine profiles during the process of T cell activation and differentiation. The entire repertoire of heterogeneous T cells collectively defines the quality and protection of T cell-mediated immunity.

Quantification and characterization of these complex and heterogeneous T cell responses have become critical to understand disease pathogenesis and develop preventive or therapeutic vaccines that elicit potent, durable, and specific immune responses (Precopio et al., 2007; Betts et al., 2006; Zhu and Paul, 2010).

The functional profiles of T_H1 cells (type I helper T cells), one of major functional subsets differentiated from naïve CD4 T cells, demonstrated marked heterogeneity (O'Garra, 1998; Sandberg et al., 2001). Functional analysis of effector T cells using multiparameter flow cytometry could delineate a number of distinct functional subsets that produce and release different combinations of cytokines within immune response elicited by bacterial infection (De Rosa et al., 2001; Perfetto et al., 2004). The study by De Rosa et al. (2004) measured and characterized the secretion profiles of five cytokines at the single-cell levels using multiparameter flow cytometry, and discovered that the activated T cells express diverse cytokine profiles. Specific subsets with the ability to produce and secrete multiple cytokines simultaneously conferred more effective and durable protection and other effector functions than the subsets that secreted single cytokines (O'Garra, 1998; Darrah et al., 2007; Appay et al., 2008; Betts et al., 2006; Seder et al., 2008; Han et al., 2012). Frequency of polyfunctional T cells that secreted three distinct cytokines simultaneously and the quality of cytokine secretion best correlated to the degree of protection (De Rosa et al., 2001; Campbell and Polyak, 2007; Polyak, 2011; Marusyk et al., 2012). The induction and maintenance of polyfunctional CD8$^+$ T lymphocytes that produce 5+ cytokines contributes to effective anti-viral immune protection (O'Garra, 1998). The immune responses elicited by vaccination that generated optimal protection and resulted in a low level of pathogenic antigens are dominated by multifunctional T cells (Precopio et al., 2007; Zhu and Paul, 2010). Recently a microchip technology allows for simultaneous measurement of up to 12 cytokines to functionally profile antigen-specific CTL (cytotoxic T lymphocytes), which are the main effectors targeting intracellular pathogens (Wong and Goeddel, 1986; Seder et al., 2008; Attig et al., 2009). This device has enabled the detection and characterization of polyfunctional heterogeneity within a phenotypically homogeneous T cell population at single-cell levels. Haining (2012) and Han et al. (2012) used serial microengraving method to design an array of nanowells in which single T cells are isolated and stimulated to cytokine secretion, and characterized the dynamic evolution of cytokine secretion by individual T cells. Recent single immune cell studies also suggest that the ability of effector immune cells to secrete multiple cytokines simultaneously, named polyfunctionality, correlates with protective immune responses (Darrah et al., 2007; Precopio et al., 2007; Betts et al., 2006; O'Shea et al., 2008; Seder et al., 2008; Zhu and Paul, 2010; Ma et al., 2011).

CELLULAR HETEROGENEITY IN HUMAN CANCER

Almost all solid and metastatic tumors display startling phenotypic and morphologic heterogeneity between and within tumors as well as among different cancer-afflicted individuals (Campbell and Polyak, 2007; Polyak, 2011). Tumor is comprised of highly heterogeneous subpopulation of cells that frequently exhibit substantial variability in virtually all discernible phenotypic features, especially the traits associated with tumorigenesis such as self-renewal capacity, proliferative, invasive, and metastatic potential (Heppner and Miller, 1983; Heppner, 1984; Marusyk and Polyak, 2009; Denysenko et al., 2010; Polyak, 2011; Marusyk et al., 2012). Tumors are not rigid and terminally differentiated cell mixtures, but dynamic organisms which continuously change their properties to adapt to hostile surroundings (Gatenby and Gillies, 2008).

The basic mechanisms by which tumor heterogeneity is evolved and regulated have not been clearly understood and the subject of much discussion (Tu et al., 2002; Michor and Polyak, 2010). Recently, there have been two ideas proposed to elucidate the

establishment of tumor heterogeneity (Hanahan and Weinberg, 2011). First, the concept of cancer stem cells (CSCs) postulates that only a small population of cells, or "cancer stem cells," are responsible for growth, maintenance, and progression of tumors (Reya et al., 2001; Bjerkvig et al., 2005; Ichim and Wells, 2006; Marusyk and Polyak, 2009; Michor and Polyak, 2010). Second, there is the clonal evolution model. The model states that tumor progression is driven as cancer cells over time accumulate highly diverse combinations of genetic and epigenetic alterations (Maley et al., 2006; Marusyk and Polyak, 2009; Sottoriva et al., 2010; Polyak, 2011; Ding et al., 2012). To design an effective and robust personalized therapy that prevents tumor relapse, it is essential to understand the causes and mechanisms of tumor heterogeneity.

Tumor heterogeneity also significantly complicates and impedes investigation and clinical diagnostics of cancer. Because tumor subpopulations exhibit substantial variability in sensitivities to various therapeutic interventions such as chemotherapy, radiation therapy, and immunotherapy, designing effective cancer therapies has posed a major challenge (Håkansson and Tropé, 1974; Hill et al., 1979; Olsson and Ebbesen, 1979; Heppner and Miller, 1983; Schilsky, 1987). One of the major reasons for failure of current cancer therapies is relapse or tumor recurrence after initial remission. Although most cancer cells initially respond to treatment that attempt to selectively kill dividing tumor cells, cancer therapy often fails because there is a small population of cells that re-establish the tumor (Marusyk et al., 2012). Those cells often exhibit potent tumor-initiating capabilities, have intrinsic resistance to treatment, or acquire the mutations that reduce efficacy of treatments (Roche-Lestienne et al., 2003; Mullighan et al., 2008; Ding et al., 2012; Marusyk et al., 2012). In order to stratify patients and predict the therapeutic response, it is required to identify the correlates that can define tumor cell heterogeneity, differentiation stage, lineage relationship, and interactions within a complex microenvironment in the clinical settings.

SECRETOMIC PROFILES OF SINGLE TUMOR CELLS AS A DEFINITE CORRELATE OF TUMOR HETEROGENEITY AND EVOLUTION

In this article, we would like to introduce a new strategy that may help to assess the extent of tumor heterogeneity, elucidate the fundamental mechanisms of how tumor heterogeneity influence tumor progression and therapeutic responses, and provide valuable insights for designing effective personalized cancer treatments. We hypothesize that a single-cell proteomic secretion profile may be identified as a definite correlate to tumor heterogeneity and evolution. A major challenge in investigating tumor heterogeneity and developing effective diagnostic and therapeutic tools has been the lack of adequate strategies to comprehensively characterize intra-tumor heterogeneity. To fully characterize genetic and phenotypic heterogeneity exhibited within a tumor, the new technologies with the ability to analyze almost every aspect of phenotype at the single-cell level must be developed (Bhatia et al., 2012). Analyzing secretion profiles of soluble mediators such as cytokines and growth factors at single-cell levels is particularly interesting because secretomic profiles of effector T cells can be used to characterize the magnitude and quality of T cell responses and predict a degree of immune protection (Betts et al., 2006; Darrah et al., 2007; Precopio et al., 2007; Seder et al., 2008). Like diverse mixtures of cells constituting tumors, effector T cells exhibit substantial functional and phenotypic heterogeneity, so the similar strategy will be employed to define the extent of tumor heterogeneity and predict tumorigenic potential and drug-resistance. Our preliminary result also suggests that the protein secretion profile evolves as tumor stem cells differentiate.

EMERGING MICROCHIP TECHNOLOGIES TO ANALYZE SINGLE-CELL PROTEIN SECRETION PROFILES

Defining molecular signatures that indicate the status of human disease or the protective immune response following interventions like vaccines has become one of the central goals in molecular medicine. Characterizing protein secretomic signatures at the single-cell resolution would improve studies of the roles of cellular heterogeneity in pathogenesis, responses to drugs, and cell differentiation (Tay et al., 2010; Agasti et al., 2012). Several new technologies that enabled quantitative single-cell proteomic analysis and characterization of functional and phenotypic heterogeneity shown by diverse cell types have recently been introduced (Fan et al., 2008; Han et al., 2011, 2012; Ma et al., 2011). Many analytical tools have been developed using a wide range of materials and techniques to achieve more efficient isolation of single cells, and multiplexed detection and characterization of secreted proteins (Chin et al., 2004; Rettig and Folch, 2005; Love et al., 2006; Zhu et al., 2009; Han et al., 2011). Recent efforts have reported the development of a novel integrated microfluidic barcode chip platform that enables the rapid, high-content, and multiplexed detection and quantitative assessment of various biomarkers of single cells (Fan et al., 2008; Ma et al., 2011). The integrated blood barcode chip (IBBC) enabled the multiplexed and rapid measurement and quantification of a panel of plasma proteins, including the low abundance cytokines, chemokines implicated in tumor–immune interaction, from a finger prick of human blood (Fan et al., 2008). By integrating microfluidic hydrodynamic principles, the platform enables rapid and effective on-chip blood separation. It employed DNA-encoded antibody library (DEAL) technique, which involves DNA-directed immobilization of antibodies, to create antibody barcode array for *in situ* measurement of plasma proteins (Fan et al., 2008). The single-cell barcode chip (SCBC) has been developed to enable comprehensive characterization of the functional and phenotypic heterogeneity of single immune cells (Ma et al., 2011). The SCBC module consists of a microfluidic system comprised of two polydimethylsiloxane (PDMS) layers and the microscopic slide coated with antibodies (high-density antibody barcode array). The platform has demonstrated multiplexed measurement of a large number of proteins at a single-cell level, and on-chip, rapid, and high-content assessment of protein secretion patterns (Ma et al., 2011). Its capability was validated by detecting multiple cytokine secretions from single macrophages and then polyfunctional profiling of tumor antigen-specific cytotoxic T cells from patients being treated by adoptive T cell transfer therapy (Ma et al., 2011). Varadarajan et al. (2012) reported the design of integrated single-cell analysis to detect and recover antigen-specific $CD8^+$ T cells based on their cytokine secretion profiles. Han et al. (2011) introduced an approach based on

microengraving that permits quantitative measurements of the rates of cytokine secretion from single immune cells (Olsson and Ebbesen, 1979; Seder et al., 2008). The design minimizes the total number of cells to be interrogated by using a nanowell-array that could retrieve and characterize single $CD8^+$ T cells (Love et al., 2006; Han et al., 2011; Varadarajan et al., 2012).

To determine and characterize the protein secretomic profiles of single tumor cells, we have developed and optimized a novel single-cell analysis microchip. This technology will allow for rapid, high-content (more than 1000 single cells), and highly multiplexed measurement of single-cell protein secretion (>14 proteins). The module will be comprised of two major components: ultra-high-density antibody barcode chip and microfluidic single capture platform. We have successfully fabricated a PDMS chip consisting of a sub-nanoliter cell capture microchamber array (unpublished data). The PDMS-based microwell array can rapidly and efficiently capture more than 1000 single cells in a single chip, and the captured cells can be cultured and monitored inside the microchambers that provide physiologically relevant microenvironment. We also aim to employ spectral and spatial multiplexing to significantly increase the number of functional proteins (up to 45 proteins) and single cells (up to 4000 cells) to be analyzed.

To make our platform a more versatile research tool and effective for clinical applications, the high-content and fully automated imaging scheme to image and analyze an entire chip need to be developed. We are in a process of creating novel imaging algorithms with the capacity for detection, counting, and characterization of captured single cells in a rapid and fully automated manner. In order to comprehensively characterize the diverse cellular components, especially highly heterogeneous immune cell compartments, of tumor microenvironment, we are in a process of developing four-color fluorescence imaging to identify phenotypic surface markers of captured single cells for rapid identification of their diverse phenotypes in conjunction of single-cell protein secretion profiling. Integration of these two approaches in a single microchip might provide an effective strategy to define a correlation between distinct cell phenotypes and cytokine secretion, which may lead to improved understanding of the roles of highly heterogeneous cellular components in the tumor microenvironment in promoting tumor development.

PROTEIN SECRETOMIC PROFILING AS A TOOL TO STUDY THE CYTOKINE NETWORKS MEDIATING COMPLEX TUMOR–MICROENVIRONMENT INTERACTION

Although tumor growth is typically initiated when a single cell acquires genetic abnormalities that confer its proliferative advantages and drive the malignant transformation, tumors do not develop alone, nor are they mere collections of malignant cells with unrestricted proliferation rate (Weiner, 2008; Marusyk et al., 2012; Wu et al., 2012a,b). The decades of research have led to the view that tumor cells actively interact with the tumor microenvironment composed of heterogeneous cell types, and their interplay significantly promotes tumor growth, progression, and metastasis, also drives co-evolution with tumor microenvironment (Mocellin et al., 2001; Dranoff, 2004; Weiner, 2008; Marusyk et al., 2012; Wu et al., 2012a,b). The interplay between these cells comprising the tumor microenvironment are orchestrated by the complex autocrine and paracrine signaling networks, which are mediated by the sets of small soluble proteins such as cytokines, growth factors, and chemokines (Irish et al., 2006; Huang et al., 2007; Raman et al., 2007; Ma et al., 2011). Cytokines are secreted or membrane-bound protein mediators that are involved in diverse biological functions (Dranoff, 2004; Elsawa et al., 2011). When produced in the malignant microenvironment, cytokines and tumor cells form a comprehensive network that have profound influences on tumor growth and progression by modulating the tumor microenvironment (Dranoff, 2004; Sheu et al., 2008; Elsawa et al., 2011). The cytokines such as the tumor-necrosis factor (TNF) are produced by immune cells, and can improve the efficacy of the T cell priming and induce adaptive anti-tumor immunity (Zou, 2005). On the other hand, certain cytokines have been associated with poor patient outcomes, and reported to promote tumor growth and inhibit anti-tumor immune response (Wojtowicz-Praga, 1997; Mocellin et al., 2001; Raman et al., 2007). For example, imbalanced production of interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), or macrophage colony-stimulating factor (M-CSF) inhibit adaptive anti-tumor immunity by suppressing dendritic cell maturation and activating regulatory T cells (T_{reg}) to aid tumor cells in evading immune-surveillance (Zou, 2005). Transforming growth factor beta (TGF-β), which is abundantly expressed in many pathological conditions, heavily influence tumor growth and maintenance as the cytokine plays important roles in forming tumor microenvironment, and facilitating angiogenesis (Wojtowicz-Praga, 1997; Zou, 2005; Bierie and Moses, 2006; Sheu et al., 2008).

Targeting and manipulating the cytokine balance have shown the therapeutic efficacy in previous trials (Wojtowicz-Praga, 1997; Zou, 2005; Bierie and Moses, 2006; Sheu et al., 2008; Weidle et al., 2010; Dinarello, 2011). The elucidation of the composition and function of cytokine networks in the tumor microenvironment may identify the targets for potent cancer therapy (Dranoff, 2004; Weiner, 2008). But, a systems-level study, which not just investigates the roles of individual factors, but comprehensively assesses complex signaling networks and recapitulates the dynamics of tumor microenvironment, has yet to be realized (Wu et al., 2012a,b). Despite the importance of characterizing the composition and function of cytokines during tumor development, there have been only a few studies to characterize the complex interplay among different cell types and cytokines within the microenvironment (Egeblad et al., 2008; Shi et al., 2012; Wang et al., 2012; Wu et al., 2012a,b). Shi et al. (2012) developed the SCBC for quantitative and multiplexed assay of intracellular signaling proteins in single tumor cells. The platform can provide a systematic approach to analyze the nature of perturbed signaling transduction networks in the tumor. Wang et al. (2012) utilized the single-cell microchip to assess how cell signaling pathways associated with tumorigenesis are influenced by cell–cell interaction at single-cell levels. To study the tumor microenvironment *in vivo*, Egeblad et al. (2008) developed a multicolor imaging technique to analyze the dynamics and interactions of multiple stromal cell types within the tumor microenvironment via direct observation. Most recently, Yu and his colleagues performed *in silico* stochastic study of glioblastoma

multiforme (GMB) microenvironment (Wu et al., 2012a,b). Their model reconstructed the complex cell-to-cell communications in the tumor microenvironment to assess the effects of cytokine-mediated signaling pathways in GMB development. Their model comprises 5 cell types, 15 protein mediators, and 69 signaling pathways, reflecting highly heterogeneous tumor microenvironment (Wu et al., 2012b). This study provides insights into the dynamics of diverse cell populations comprising the tumor microenvironment and the roles of cytokine signaling in the evolution of tumor microenvironment. The cytokine network analysis also identified several key molecules and pathways that play an important role in tumor development and consequently new therapeutic strategies can be designed to target cytokines such as IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF), in tumor microenvironment to treat human cancer (Weiner, 2008; Marusyk et al., 2012).

We speculate that the analysis of single tumor cell secretion profiles from a novel clinical microchip will lead to a more complete model that predicts the dynamics of tumor evolution and aids in developing more effective personalized medicine. Each individual tumor cells display unique protein secretion profiles as they secrete unique combinations of cytokines at differing kinetics to regulate widely diverse functions during tumor progression. Significant research efforts have been made recently to develop single-cell proteomics technologies and powerful clinical tools to examine the heterogeneity of tumor microenvironment and complex cytokine-mediated signaling networks, and enable personalized therapy that targets the tumor microenvironment (Irish et al., 2006; Xu et al., 2006; Huang et al., 2007; Ma et al., 2011). Our recently developed single-cell analysis microchip will be employed to experimentally measure the magnitude, quality, and dynamics of cytokine secretion by the cells comprising tumor microenvironment. The single-cell cytokine secretion profile of the tumor will, for the first time, allow reconstruction of a systems-level and large-scale intercellular cytokine signaling network at a single-cell resolution. We also propose to develop new multi-color fluorescence imaging technologies that identify single-cell phenotypic markers and enable rapid molecular phenotyping. By integrating the imaging technologies with single-cell proteomics microchip, we expect to directly assess the behavior of the cells in tumor microenvironment and study how tumor cell cytokine secretion correlates to their phenotypic characteristics and interaction with other cells at the single-cell level. We anticipate that this approach will not only improve cancer diagnosis and stratification but also represents an informative tool to monitor the response of patients, in particular, the one treated by immunotherapy such as cytokine therapeutics, antibody therapy (anti-CTLA4 and anti-PD1), or adoptive T cell therapy that augment the function of anti-tumor immune response in tumor microenvironment to cue cancer (Weiner, 2008).

IDENTIFICATION OF CANCER STEM CELLS AND LINEAGE DIFFERENTIATION – UNDERSTANDING TUMOR EVOLUTION AND HETEROGENEITY

The CSCs perspective suggests that a small subset of cells with stem cell properties including indefinite proliferative potential is responsible for driving tumor initiation and progression (Reya et al., 2001; Michor and Polyak, 2010). It is one of two major mechanisms that have been proposed to elucidate the origins of tumor heterogeneity. CSCs possess the high self-renewal capacity and unique ability to differentiate, which gives rise to highly heterogeneous cell types that constitute the majority of the tumors, and generates intra-tumor heterogeneity (Hwang-Verslues et al., 2009; Marusyk and Polyak, 2009; Michor and Polyak, 2010). The study by Vermeulen et al. (2008) observed that CSCs from human colon cancer possess multi-lineage differentiation capacity. Recent studies have observed that stem cells are usually preferential targets for mutations that accumulate to cause neoplastic transformation (Bonnet and Dick, 1997; Miyamoto et al., 2000; Betts et al., 2006). CSCs might explain why majority of conventional therapies fail due to tumor relapse after initial remission. It has been suggested that more aggressive cancers that are more likely to relapse contain more CSCs (Al-Hajj et al., 2004; Singh et al., 2004; Bao et al., 2006; Zhou et al., 2009). Many CSCs are relatively more resistant to chemotherapy due to their anti-apoptotic pathways and resistance to oxidative or DNA damage (Reya et al., 2001; Li et al., 2006; Diehn et al., 2009).

However, the definitive cellular or molecular biomarkers that identify tumor-initiating cells have not yet determined. The study by Hwang-Verslues et al. (2009) identified different subpopulations of cells displaying distinct tumorigenic abilities within the breast cancer cell line. The discoveries suggest that there are multiple lineages of CSCs, which can subsequently be differentiated into more diverse cells. The CSC perspective views the tumors as hierarchical organization composed of multiple lineages of differentiated cells with distinct phenotypes. The analysis of single-cell secretomic profiles shows that while every single cell exhibits distinct secretomic profiles, there are groups of single cells with comparable secretomic profiles. Based on single-cell secretomic profiles, the entire tumor cell population may be compartmentalized into multiple clusters, each of which is a group of cells that have similar or related cytokine secretion patterns. The multiple groups of single cells classified based on the secretion profile may represent distinct lineages originated from the differentiation and evolution of CSCs. Our study has shown marked change of protein secretion profiles from human brain tumor cells undergoing differentiation to mature tumor cells, suggesting the possibility of using secretomic signatures to define tumor cell differentiation and heterogeneity (**Figure 2**).

The mechanisms by which CSCs acquire their tumorigenic and metastatic abilities to promote tumor growth, metastasis, and resistance to therapy have not been fully understood. As normal stem cells are influenced by their "niche," CSCs are regulated by, and in turn regulate, the extrinsic signals generated within the tumor microenvironment (Karnoub et al., 2007; Weiner, 2008; Korkaya et al., 2011). Heterogeneous cell types that constitute the tumor microenvironment secrete the pro-inflammatory cytokines such as IL-6 or IL-8 that increase tumorigenic potential and promote therapeutic-resistance (Scheller et al., 2006; Levina et al., 2008; Liu et al., 2011). In turn, tumorigenic cells also produce and secrete various factors to enhance their survival and proliferation. Recent studies have found that the capabilities of CSCs to sustain tumor growth and promote resistance to various therapies are associated with their high ability to produce soluble

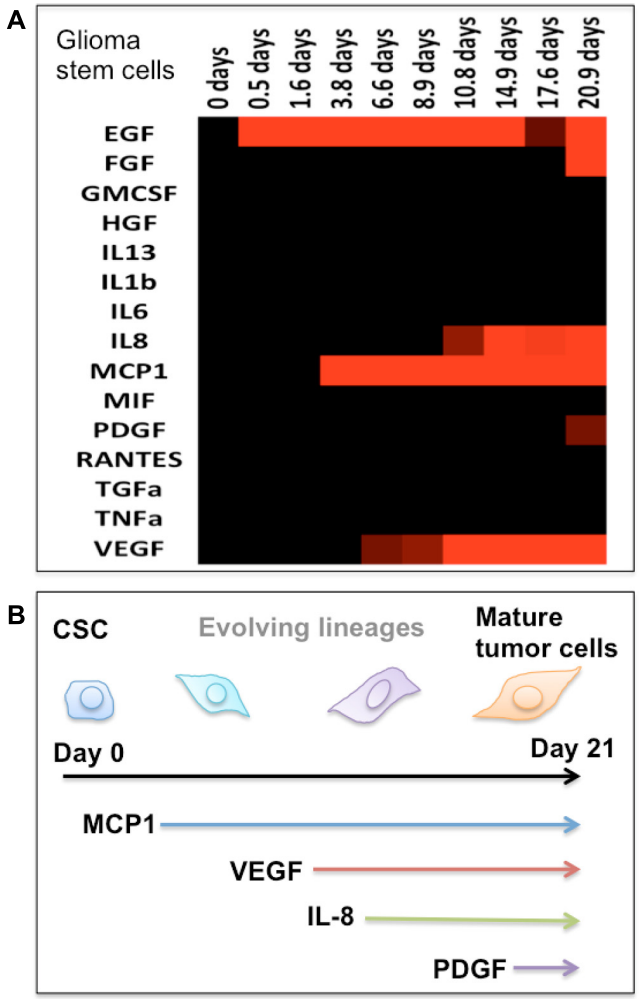

FIGURE 2 | Protein secretion profile correlates to the stage of cancer stem cell differentiation. (A) Heat map showing the protein secretion profiles measured at different times during the differentiation of human glioma stem cells *in vitro*. **(B)** Schematic depiction of the stage of glioma stem cell differentiation as identified by emergence of cytokine secretion. In our protein assay panel, glioma stem cells appear to be relatively "quiescent." During the differentiation process, these cells begin to produce a number of functional proteins and gradually become the phenotype of mature tumor cells.

mediating factors such as cytokines and growth factors (Todaro et al., 2007; Weiner, 2008; Iliopoulos et al., 2009; Tang et al., 2012). The study discovered that the levels of numerous cytokines, growth factors, and chemokines were two- to threefolds higher in isolated CSC-derived tumors than parental tumor cells (Levina et al., 2008; Tang et al., 2012). The production of IL-4 by colon CSCs contributes to higher therapeutic-resistance as IL-4 promotes the expression of anti-apoptotic genes and upregulates resistance to apoptosis of CSCs (Todaro et al., 2007; Iliopoulos et al., 2009). These studies suggest that the greater ability to produce multiple cytokines has been correlated to tumorigenic and metastatic potential. From the single-cell secretion profiles, we can identify the groups of tumor cells characterized by the significant secretion of multiple, specific cytokines. These groups may elicit greater tumorigenic potentials and promote the evolution of more aggressive and invasive cancer phenotypes. Because our single-cell analysis microchip allows comprehensive characterization of phenotypes of captured single cells, including their surface phenotypes, motility, and viability, we hope to determine the correlation between specific cytokine secretion profiles of individual cells and their tumorigenic potentials and differentiation stages. The cytokine secretion profiles of single tumor cells can be used to characterize a tumor hierarchy and serve as biomarkers for tumor-initiating cells or different lineages with varying tumorigenicity and treatment-resistance.

OUTLOOK, CLINICAL APPLICATION, AND UTILITY

The effective targeting of cancerous cells with greater tumorigenic potential and intrinsic drug-resistance can prevent cancer relapse or persistent growth, and when combined with conventional therapy that kills the rapidly dividing cells, it can potentially cure cancer (Vermeulen et al., 2008; Zhou et al., 2009; Chen et al., 2010; Michor and Polyak, 2010). Our single cell-based cytokine secretion analysis would provide framework and new insight for designing effective therapeutic strategies by dissecting hierarchical organization of tumor microenvironment in hope to identify the specific cell subsets with higher tumorigenic and metastatic potential, and resistance to treatment. Single-cell secretomic profiling could become a new means for quantitative characterization of the extent of tumor heterogeneity, with which oncologists can diagnose the stage of cancer and likelihood of development of metastatic cancer for individual patients, leading to personalized medicine and treatments. Because distinct cytokine secretion patterns are associated with distinct differentiation lineages, secretomic profiling may aid in understanding of CSC differentiation and tumor evolution.

One of the major challenges in designing effective personalized cancer therapeutics and early diagnosis has been the lack of adequate technologies to comprehensively characterize inter- and intra-tumor heterogeneity in the clinical settings. Single-cell analysis of cytokine profiles are possible correlates to evaluate whether there is a high degree of intra-tumor heterogeneity of cancer phenotypes, and provide valuable insights into the origins of tumor heterogeneity, the mechanisms of the complex signaling networks that mediate the characteristics of individual tumor cells, and the extent of tumor differentiation and evolution, that has the potential to enable the development of more effective personalized medicines for human cancers.

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Erratum: Single-cell protein secretomic signatures as potential correlates to tumor cell lineage evolution and cell–cell interaction

Minsuk Kwak[1], Luye Mu[2], Yao Lu[1], Jonathan J. Chen[1], Yu Wu[1], Kara Brower[1,3] and Rong Fan[1,4]*

[1] *Department of Biomedical Engineering, Yale University, New Haven, CT, USA*
[2] *Department of Electrical Engineering, Yale University, New Haven, CT, USA*
[3] *Isoplexis Inc., New Haven, CT, USA*
[4] *Yale Comprehensive Cancer Center, New Haven, CT, USA*
**Correspondence: rong.fan@yale.edu*

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We noticed an error that the name of a key contributor, Dr. Yu Wu, was left out from the author list. The corrected author list and the affiliations are the following.

Minsuk Kwak[1], Luye Mu[2], Yao Lu[1], Jonathan J. Chen[1], Yu Wu[1], Kara Brower[1,3] and Rong Fan[1,4*]

[1] Department of Biomedical Engineering, Yale University, New Haven, CT, USA
[2] Department of Electrical Engineering, Yale University, New Haven, CT, USA
[3] Isoplexis Inc., New Haven, CT, USA
[4] Yale Comprehensive Cancer Center, New Haven, CT, USA

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Immunoediting and antigen loss: overcoming the Achilles heel of immunotherapy with antigen non-specific therapies

Arta Monir Monjazeb[1], Anthony E. Zamora[2], Steven K. Grossenbacher[2], Annie Mirsoian[2], Gail D. Sckisel[2] and William J. Murphy[2,3]*

[1] Department of Radiation Oncology, Comprehensive Cancer Center, University of California at Davis Medical Center, Sacramento, CA, USA
[2] Department of Dermatology, University of California at Davis Medical Center, Sacramento, CA, USA
[3] Division of Hematology Oncology, Department of Internal Medicine, Comprehensive Cancer Center, University of California at Davis Medical Center, Sacramento, CA, USA

Edited by:
Antoni Ribas, University of California Los Angeles, USA

Reviewed by:
Sandra Demaria, New York University School of Medicine, USA
Howard L. Kaufman, Rush University Medical Center, USA

Correspondence:
William J. Murphy, Department of Dermatology, Institute for Regenerative Cures, University of California at Davis, 2921 Stockton Blvd., Room 1614, Sacramento, CA 95817, USA
e-mail: wmjmurphy@ucdavis.edu

Cancer immunotherapy has emerged as a mainstream therapy option in the battle against cancer. Pre-clinical data demonstrates the ability of immunotherapy to harness the immune system to fight disseminated malignancy. Clinical translation has failed to recapitulate the promising results of pre-clinical studies although there have been some successes. In this review we explore some of the short-comings of cancer immunotherapy that have limited successful clinical translation. We will give special consideration to what we consider the most formidable hurdle to successful cancer immunotherapy: tumor-induced immune suppression and immune escape. We will discuss the need for antigen-specific immune responses for successful immunotherapy but also consider the need for antigen specificity as an Achilles heel of immunotherapy given tumor heterogeneity, immune editing, and antigen loss. Finally, we will discuss how combinatorial strategies may overcome some of the pitfalls of antigen specificity and highlight recent studies from our lab which suggest that the induction of antigen non-specific immune responses may also produce robust anti-tumor effects and bypass the need for antigen specificity.

Keywords: cancer immunotherapy, radiotherapy, immune tolerance, bystander T-cells, immune suppression, immune surveillance

INTRODUCTION

The allure of cancer immunotherapy as a potential magic bullet against cancer has intrigued researchers for over a century. The rationale underlying anti-cancer immunotherapy stems from the concept of immune surveillance first attributed to Erlich and colleagues over a century ago (1). This concept, founded in the idea that there is no evolutionary purpose to the tissue rejection immune response, states that tissue rejection is actually a manifestation of an immune surveillance mechanism that guards against spontaneously arising tumors. If such a mechanism does exist then it stands to reason that it can be re-invigorated and harnessed to battle malignancy in cancer patients. This idea, in its simplest form, is particularly attractive given that the immune system should be able to identify and specifically eradicate malignant cells based on the expression of abnormal antigens not expressed or present in normal tissues (2).

Interest in this concept has waxed and waned over the past century and during this time the major advances in cancer therapy were focused on cytotoxic therapies and surgical excision. Despite the continual advancement of the field, the inability to eradicate malignancy once it has disseminated remains the greatest challenge in cancer therapy. Over the past decade there has been a renaissance in cancer immunotherapy with a renewed belief by many that harnessing the immune system may be a viable strategy for successfully treating metastatic disease. This renaissance has produced a seemingly exponentially increasing number of pre-clinical and clinical studies which are serving to translate this concept into the mainstream arsenal of anti-cancer therapeutics. Numerous strategies are being explored including augmentation of antigen-presenting cells (APC) and immune effector cells, immunologic stimulants such as cytokines and pathogen associated molecular pattern (PAMP) receptor agonists, adoptive transfer of transgenic immune cells, antibodies and molecules such as anti-CTLA-4 antibody or transforming growth factor (TGF)-beta antisense aimed at reversing suppressive mechanisms, and numerous vaccines comprised of DNA, peptides, or autologous tumor cells [reviewed in Ref. (3,4)]. Two such therapies, sipuleucel-T, a pulsed dendritic cell vaccine (5), and ipilimumab, an anti-CTLA-4 antibody (6), have been amongst the first to be approved by the FDA for mainstream use (although some immunotherapies such as Bacilli Calmette–Guerin have been used clinically for decades without the fanfare of the aforementioned therapies). These two therapies, which provide overall survival benefits in castration-resistant prostate cancer and melanoma respectively, have generated enthusiasm and played a central role in re-introducing immunotherapy into the mainstream. Unfortunately, the benefit imparted by these and other immune therapies remains modest. Sipuleucel-T provides no statistical benefit in freedom from progression and improves median overall survival by about 16 weeks (5). Similarly, ipilimumab also provides a survival benefit of roughly 16 weeks (6). Thus, although these therapies may validate the concept of cancer immunotherapy and are an important first step, they fall short of the theorized potential of eradicating metastatic disease. Unfortunately, to date, clinical studies of cancer immunotherapy have

failed to manifest the pre-clinical and theoretical promise of this approach. In this manuscript we will review some of the hurdles of cancer immunotherapy including the need to overcome tumor-induced immune suppression and immune escape. We will discuss the importance of inducing antigen-specific immune responses for successful immunotherapy but also consider the need for antigen specificity as a major potential pitfall of immunotherapy given tumor heterogeneity, immune editing, and antigen loss. Finally, we will discuss how combinatorial strategies may overcome some of the pitfalls of antigen specificity and highlight recent studies from our lab which suggest that the induction of antigen non-specific immune responses may also produce robust anti-tumor effects and bypass the need for antigen specificity.

SHORT-COMINGS OF CANCER IMMUNOTHERAPY

A recent publication summarizing results from the Society for Immunotherapy of Cancer (SITC) immunotherapy summit identifies nine critical hurdles in cancer immunotherapy (7). Of these nine critical hurdles, eight are related to the development of therapeutics and one is inherent to the therapies or diseases themselves. This one: the "complexity of cancer, tumor heterogeneity, and immune escape" encompasses a huge and diverse range of biological issues. Below we will consider some of these critical hurdles as well as other potentially critical obstacles.

One obstacle recently described by Lesterhuis and colleagues is that the timing and dosing for many immunotherapy regimens is often empirically derived and further refinement of these technical aspects may improve outcomes (8). In many ways this obstacle is directly attributable to the "limited funds available to translate science into patients." The timing and dosing of many immunotherapy regimens tested in the clinic are extrapolated from pre-clinical regimens or from phase I trials assessing therapy tolerability and safety. Although the common thought with cytotoxic therapies is that the more that can be delivered, the greater the anti-tumor effect, this rationale may not hold true when trying to alter the delicate balance of the immune system for therapeutic gain. Issues such as exhaustion of effector cells and induction of suppressive networks must be considered.

Another critical hurdle is the "limitation of current animal models to predict the efficacy of cancer immunotherapy strategies in humans." A recent publication has suggested, based on discordant gene expression profiles after traumatic or inflammatory insults, that mice provide poor models of human inflammatory diseases (9). It should be noted that a single mouse strain was used to draw such broad conclusions. Although this article has recently generated attention in the lay media, its conclusions regarding the short-comings of mouse models have long been recognized by most researchers. Despite these short-comings, mouse models remain a staple of pre-clinical studies due to the complex mechanistic studies which can be performed, low cost, and short generation times amongst numerous other advantages. These authors, while acknowledging the many limitations of mouse models, do not endorse abandoning a model which has over many decades proven its utility in improving the understanding and treatment of human disease. Care must be taken, however, to make our pre-clinical models as robust and accurate as possible and to properly validate pre-clinical findings prior to clinical translation. Most mouse cancer studies are performed in young mice, however human cancers most commonly occurs in the aged and the use of aged mice would be more appropriate for cancer studies. This is particularly relevant for immunotherapy studies given the significant changes in immune functioning with age. In a series of studies examining immunotherapy in young versus aged mice we have demonstrated a significant impact of age on efficacy and toxicity (submitted). We suggest use of aged mice should be considered as part of the pre-clinical development of any cancer study. We also suggest that companion animals with spontaneous tumors provide an excellent platform for validating pre-clinical studies prior to human translation.

Two other critical hurdles which we will consider together are "lack of definitive biomarkers for assessment of clinical efficacy of cancer immunotherapies" and that "conventional clinical response criteria do not take into consideration differences between response patterns to cytotoxic agents and immunotherapies." Currently there is no reliable measure of treatment effects other than survival and imaging responses which makes it difficult to identify treatments that may have a small but important effect which needs to be further explored. The lack of validated assays that can measure immune response across trials make it difficult to determine how strategies should be altered to improve efficacy. These issues need to be explored at the pre-clinical level but also as correlative studies in clinical trials. Unfortunately, the capabilities of human immune monitoring fall short of the sophisticated assays used in pre-clinical models and further refinement and development are required (10). In many human trials immune monitoring correlatives consist of a simple characterization of various markers in the peripheral blood. Mouse (11) and human (12) studies demonstrate that the immune response observed systemically may not be representative of what is occurring in the suppressed tumor microenvironment and draining lymph nodes. Obviously, the ethical issues with justifying repeated biopsy of tumor or draining lymph nodes make this a dilemma, which is not easily resolved.

An issue not identified by the SITC summit is that clinical cancer trials of new agents are typically undertaken in patients with widely metastatic disease who, due to the large burden of disease, the immunosuppressive activities of the tumor itself, or the immunosuppressive effects of prior therapies, are unable to respond to even a very effective immunotherapy. Ohashi and colleagues have shown that anti-tumor vaccination is most effective after surgical de-bulking of the primary tumor (13) demonstrating that an effective immunotherapy alone may be unable to induce a clinically significant response if tumor doubling time is short or tumor burden is high. Not surprisingly, some of the greatest successes of immunotherapy have been produced in pre-invasive or very early stage cancers where there is a limited volume of disease and patients have received minimal prior therapy. For example, intravesicular *Bacille Calmette–Guerin* is a standard of care in the management of non-muscle invasive bladder cancer demonstrating superiority to chemotherapy in this setting (14) and an HPV peptide vaccine demonstrated a 50% complete response rate in women with pre-invasive vulvar neoplasia (15).

Another limitation of immunotherapy is the potential toxicity associated with many treatments. As we iatrogenically upset the delicate balance of the immune system we introduce the potential

for severe adverse effects. Some therapies can produce systemic inflammation and cytokine storm with disastrous effects. The systemic administration interleukin (IL)-2 has demonstrated activity against metastatic renal cell carcinoma and melanoma capable of producing durable responses in patients with metastatic disease, but toxicities can be so extreme that it limits its regular use and treatments are often provided in intensive care units. In order to limit toxicity and mortality, such treatments are generally only undertaken at centers with expertise in IL-2 therapy but access to such centers is limited. Therapy can induce a severe vascular leak syndrome that emulates sepsis and is characterized by hypotension, vasodilation, pulmonary edema, neutrophil dysfunction, and, without intervention, culminates in end-organ failure and death (16, 17). In addition to a systemic inflammation and cytokine storm, another concern with immunotherapy is the induction of immune responses which inappropriately target self or through bystander effects damage self tissues. This autoimmunity is seen in certain instances in patients treated with ipilimumab, where therapy disrupts the immune suppressive mechanism network that prevents anti-cancer immune responses but also usefully prevents inappropriate immune responses. Disruption of the latter can produce autoimmune colitis, dermatitis, hepatitis, endocrinopathy, and other adverse effects (18). A report by Morgan et al. (19) illustrates the potentially disastrous effects of disrupting immune balance. T-Cells modified with chimeric antigen receptors (CARs) to HER-2/neu were transferred to a patient with refractory metastatic colorectal adenocarcinoma. Unfortunately, this patient suffered fatal pulmonary failure as the transfused T-cells unexpectedly recognized low levels of the HER-2/neu antigen present on lung epithelial cells. The adverse effects observed within this recent trial highlights the critical need to assess the shortcomings of our pre-clinical models as a means to better foreshadow toxicity responses within the clinic.

Of the hurdles identified by SITC the "complexity of cancer, tumor heterogeneity, and immune escape" is the only one that addresses the nature of the disease itself. Under the umbrella of this one category fall a broad number of biological issues that are the subject of intense scientific investigation and will ultimately, more so than any of the other hurdles listed above, dictate the utility of anti-cancer immunotherapy. The "complexity of cancer" and "tumor heterogeneity" have been recognized for decades in mouse models. Fidler et al. demonstrated great variability in the metastatic potential of clones from a parent culture of murine melanoma (20). More recently, a genetic analysis of human renal cell cancers likewise demonstrated similar variability (21). Taking multiple spatially distinct biopsies from a single tumor the authors were able to demonstrate significant genetic changes within a given tumor providing evidence of the heterogeneity of even a single tumor. This topic and these studies will be considered in further detail later in this review. "Immune escape" can refer to a broad spectrum of mechanisms whereby an anti-tumor immune response is evaded or subverted. Two widely investigated phenomena that must be considered under this topic are immunoediting/antigen loss and tumor-induced immune suppression (**Figure 1**). Immunoediting is discussed in a separate section below and we will discuss the concept of tumor-induced immune suppression here (22, 23). The hostile nature of the tumor microenvironment and numerous mechanisms underlying this are well documented. The immune system is in a delicate balance of fluxes of activation and suppression that allow for appropriate responses but guard against potentially harmful responses that are inappropriate either in scale or target. A spectrum of suppressive cells, such as immature dendritic cells, regulatory T (T_{reg})-cells, myeloid-derived suppressor cells, and tumor-associated macrophages, are actively recruited to or generated within the tumor microenvironment (**Figure 1**). Likewise, a mélange of suppressive cytokines and enzymes, secreted by the tumor itself or resulting from the chronic inflammation associated with many tumors, contributes to the recruitment of the suppressive cells listed above and to direct suppression of effector cells. Cytokines such as TGF-beta, IL-10, and prostaglandin (PG)-E_2 with documented immune suppressive effects may be highly expressed (24). Enzymes such as indolamine-2,3-dioxygenase (IDO) and arginase, which catabolize tryptophan and arginine respectively, can create a microenvironment in which immune effectors cannot activate or proliferate and suppressive cells thrive (25, 26). They function to both deplete the aforementioned amino acids essential for effector cell activity but also produce catabolites which can be independently suppressive and can alter the phenotype of immune cells from activating to suppressive (25, 26).

IMPORTANCE OF ANTIGEN-SPECIFIC RESPONSES

The potential of cancer immunotherapy lies in the ability of the immune system to specifically distinguish and target non-self from self. Drawing on Erlichs earlier hypothesis, in the 1960s McFarlane Burnet and Lewis Thomas formally proposed the concept of immune surveillance as the true evolutionary purpose of the allograft tissue rejection response (27–29). They hypothesized that given the frequency of somatic mutations and that a proportion of these mutations will give rise to cells with malignant potential, then there must exist an evolutionary mechanism, likely immunological in nature, to deal with these potentially dangerous cells. This implies that tumors, although derived from host tissues must have some unique property whereby they can be distinguished from self. This concept of tumor antigens was confirmed in experimental animal models by Old and Boyse in the 1960s (2) who demonstrated the existence of tumor-specific antigens in murine leukemias and mammary tumors. These findings were later validated in human melanomas with the discovery of tumor-infiltrating lymphocytes able to recognize tumor antigens and lyse malignant cells (30). These findings have since been corroborated in various malignancies (31). The concept of tumor antigens has since evolved from tumor-specific antigens to tumor-associated antigens. These include inappropriately or over-expressed tissue antigens (i.e., Her-2/neu), viral oncogenes (i.e., v-*src*), idiotypic antigens (i.e., B-cell receptor), oncofetal antigens (i.e., CEA), fusion proteins (i.e., BCR-Abl), and post-translationally modified glycoproteins (i.e., MUC-1).

To date, the focal point of cancer immunotherapy research has been T-cell biology and by default tumor antigen-specific immune responses. A number of these therapies are being tested for clinical efficacy. As mentioned above, sipuleucel-T (Provenge), a pulsed dendritic cell vaccine, uses the prostatic acid phosphatase antigen, although the precise mechanism of its clinical benefit remains uncertain. Another strategy being tested is the use of

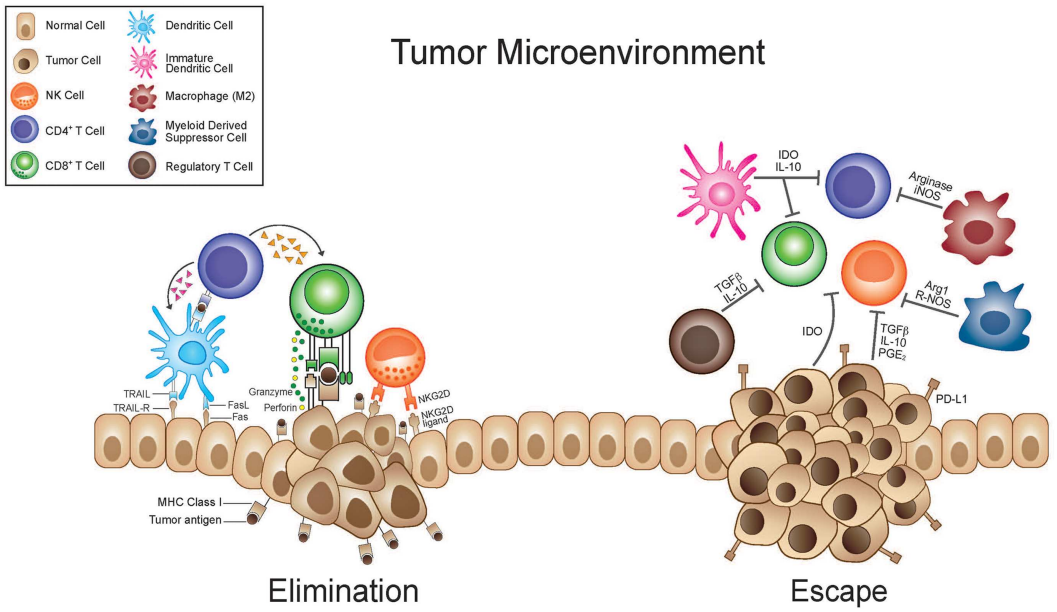

FIGURE 1 | Immunosuppressive tumor microenvironment and antigen loss mediate tumor escape. During the elimination phase immune effector cells such as CTL's and NK cells with the help of dendritic and CD4+ T-cells are able to recognize and eliminate tumor cells. This killing relies on stress ligands such as NKG2D and recognition of TAA's in the TCR-MHC complex. As a result of tumor heterogeneity, tumor cells which are less immunogenic or have up-regulated immunosuppressive factors are selected for. These cells are able to subvert the immune response and escape immune surveillance. Tumor cells can secrete cytokines that recruit suppressive cells such as regulatory T (T_{reg}) cells, immature myeloid cells [including immature dendritic cells (iDC) and myeloid-derived suppressor cells (MDSC)], and M2 macrophages. iDC can cause T-cell anergy due to lack of co-stimulatory molecules. M2 macrophages and MDSC inhibit T-cell responses through a variety of mechanisms, including nutrient sequestration via arginase, reactive oxygen species (ROS) generation, nitric oxide (NO), as well as interference with trafficking into the tumor site. Immunosuppressive cytokines and the up-regulation of immunosuppressive enzymes [like indolamine-2,3-dioxygenase (IDO) and arginase] that catabolize essential nutrients required for effector cell activation and also produce immunosuppressive catabolites, contribute to a microenvironment where immune responses are difficult to instigate and sustain. Furthermore tumor cells will down-regulate MHC molecules, loose expression of antigenic molecules, and up-regulate inhibitory molecules such as PD-L1.

CARs that engineer T-cells with receptors specific for target specific tumor antigens. Two clinical trials have demonstrated the potential of this approach. Infusion of T-cells with CARs targeting the CD19 B-cell antigen in chronic lymphoid leukemia (32) or the NY-ESO-1 antigen in synovial cell sarcoma and melanoma (33) has demonstrated the ability to induce tumor regression. It has been implicated that even therapies that are not necessarily billed as antigen-specific ultimately rely on the generation of antigen-specific T-cell responses for clinical effect. For example, clinical studies of CTLA-4 blockade with ipilimumab, which is aimed at reversing immune suppression, correlate clinical effect with the generation of T-cells specific to the NY-ESO-1 (34) and Melan-A (35) antigens. Similarly, Fong and colleagues demonstrate that clinical response of prostate cancer patients to ipilimumab is also correlated with the robustness of antigen-specific antibody and T-cell responses (36).

Given the existence of tumor antigens and their central importance in anti-cancer immunotherapy an approach being explored is the use of antigen-specific cancer vaccines. Unfortunately, these vaccinations, although successful in generating an antigen-specific immune response, have failed to produce meaningful clinical responses. For example, as announced by Therion Corporation by press release in 2006, a phase III trial of the viral PANVAC™ vaccine in pancreatic cancer patients elicited immune responses to the CEA antigen in about 70% of patients but without a survival benefit (http://www.prnewswire.com/news-releases/therion-reports-results-of-phase-3-panvac-vf-trial-and-announces-plans-for-company-sale-56997582.html). Moreover, Canvaxin™ – a melanoma vaccine – was able to induce antigen-specific responses to the glycoprotein tumor-associated antigen TA-90 (37), but Phase III trials were terminated early due to an observed survival detriment.

IMMUNOEDITING AND IMMUNE ESCAPE SUBVERT ANTIGEN-SPECIFIC T-CELL RESPONSES

One potential shortcoming of cancer immunotherapy not detailed above is the need for antigen-specific immune responses. Despite the findings chronicled above demonstrating the promise and importance of antigen-specific immune responses in cancer immunotherapy, many problems exist with this approach. One issue is the lack of clear target antigens for many tumors. Cancer arises from "self" tissues and thus the majority of antigens expressed have gained central tolerance. Few tumor-specific or tumor-associated antigens which can uniquely target malignant cells exist and those which do tend to be poorly immunogenic. This is in direct contrast to microbes that express a vast array of proteins, lipids, and carbohydrates which are foreign and strongly immunogenic. Nonetheless, some tumor antigens do exist and as demonstrated above immunity against them can be generated.

The studies above also outline that even when a target antigen is identified and a response is generated, it may fail to translate into clinical benefit.

Although the reason for these findings is likely multi-factorial one of the most plausible explanations is that of immunoediting and antigen loss (**Figure 1**). As described by Schreiber and associates, this concept takes the principles of evolution and natural selection and applies them on a microscopic scale. It suggests that during carcinogenesis, tumors which become clinically relevant – under selective pressure by the host immune system – must have sub-populations which can survive immune pressure and are thereby selected for as the tumor evolves strategies to evade the host immune response (38). They describe three processes: the first is elimination during which active immune surveillance finds and eradicates the majority of tumor cells (or all of the tumor cells when it is successful). As a tumor grows and invades surrounding tissues the release of inflammatory cytokines recruit components of the innate immune system which will in turn, via cytokines and in the draining lymph nodes, recruit an adaptive immune response. The second is dynamic equilibrium during which time the rapidly dividing and mutating tumor is being eliminated by the immune system that is simultaneously placing an evolutionary pressure on the tumor and selecting out variants which by virtue of poor immunogenicity or other mechanisms are able to survive the immune attack. In the third phase, escape, tumor subclones which are poorly recognized or eliminated by the immune system are able to grow unchecked and become clinically observable disease. Experimental evidence from this same group demonstrates that tumors generated under selective immune pressure are less immunogenic (39). They find that chemically induced sarcomas from wild-type or RAG $2^{-/-}$ grow equally well when transplanted into naïve RAG $2^{-/-}$ mice but when transplanted into immunocompetent naïve wild-type mice less than half of the tumors generated in RAG $2^{-/-}$ grow as compared to 100% of those generated in wild-type mice. If tumors can become less immunogenic during carcinogenesis due to selective pressure from the immune system then it stands to reason that once established they can also evolve less immunogenic phenotypes if exposed to a new selective pressure from the immune system, as would occur with immunotherapy. Clinically, this concept is confirmed by the loss of the MART-1 antigen in melanoma patients after adoptive transfer of MART-1 specific T-cells (40, 41). In addition to antigen loss, there is evidence that tumor cells also down regulate the ability to present antigen, either by down-regulating major histocompatibility complex (MHC) or antigen processing capabilities (42, 43). Also supporting this hypothesis, it has been demonstrated that the patients who respond to antigen-specific therapies, such as a MUC-1 peptide pulsed dendritic cell vaccine, are those who have epitope spreading where an immune response is generated against tumor antigens not targeted by the antigen-specific therapy (44). This may be one mechanism whereby antigen-specific therapies can overcome this shortcoming and it may be that only those patients who are able to overcome the outgrowth of tumor subclones which poorly express MUC-1 are able to produce a clinically meaningful response, although this is not conclusively demonstrated.

In this sense, tumor heterogeneity is a major obstacle in that the tumor subclones which do not express a given antigen or have some other trait which makes them poorly immunogenic will be selected for after immunotherapy. Recent genetic studies demonstrate the complexity of somatic mutations within a single melanoma (45) and the heterogeneity of spatially distinct biopsies from renal cell cancers (21) leading the authors of the latter to conclude that "intratumor heterogeneity, associated with heterogeneous protein function, may foster tumor adaptation and therapeutic failure through Darwinian selection." As mentioned above, part of this process may entail selection of cells which are poorly immunogenic by virtue of low expression of tumor antigens or dysfunctional antigen-presenting machinery and part of this process may entail selection of cells which induce, by any number of mechanisms, an immunosuppressive tumor microenvironment (**Figure 1**). Antigen-specific effector T-cells appear to be particularly prone to both of these mechanisms since they rely on antigen recognition and are also more sensitive to direct suppression in the microenvironment, given up-regulation of molecules like PD-1, CTLA-4, Fas, and Lag-3 on antigen-specific activated T-cells (**Figure 2**). We propose that this may be an Achilles heel of many current immunotherapy approaches that limits both the magnitude and frequency of responses.

ANTIGEN NON-SPECIFIC IMMUNOTHERAPY APPROACHES

There are some therapies that tend to be less susceptible to the short-comings of the antigen-specific therapies listed above. These types of therapies can include cytokines such as IL-2, immunostimulatory agents such as bacterial DNA, agonists and antagonists of key immunoregulatory molecules such as CD40 or PD-1, inhibitors of key enzymes such as cyclo-oxygenase or IDO, and vaccine strategies capable of encompassing the broad array and heterogeneity of tumor antigens such as syngeneic whole cell vaccines and *in situ* vaccines. As mentioned above in the discussion of ipilimumab, these therapies also rely, at least in part, on the generation of antigen-specific responses but we classify them for the purpose of discussion as antigen non-specific when they are not specifically targeted to one or a few antigens. They tend to be multi-modal and can have direct anti-tumor effects, target the suppressive tumor microenvironment, and activate innate immunity and adaptive immunity for both antigen-specific and antigen-non-specific killing (i.e., non-MHC-restricted killing by natural killer cells, macrophages, and T-cells) with most functioning through parallel mechanisms. For example, inhibition of cyclo-oxygenase can have direct cytotoxic effects on tumor cells by depriving them of necessary growth signals or inducing the intracellular accumulation of arachidonic acid (46, 47) and can also reverse immune suppression by blocking production of PGE_2 (48, 49). Similarly, CpG oligodeoxynucleotides, which are recognized as bacterial DNA products and signal through toll-like receptor-9 (50, 51), can function to activate B-cells, dendritic cells, natural killer cells, macrophages, and lymphocytes but can also inhibit immunosuppressive myeloid-derived suppressor cells (52). Numerous approaches have been developed to target the immunosuppressive tumor microenvironment. Regulatory T (T_{reg})-cells are a well-studied component of tumor-induced suppression and can inhibit the function of effector T-cells and APC (53). The CD25 molecule (high affinity IL-2 receptor) expressed on T_{reg} cells is targeted by the antibody daclizumab (Zenapax) and by the IL-2 diphtheria toxin fusion protein denileukin diftitox (Ontak).

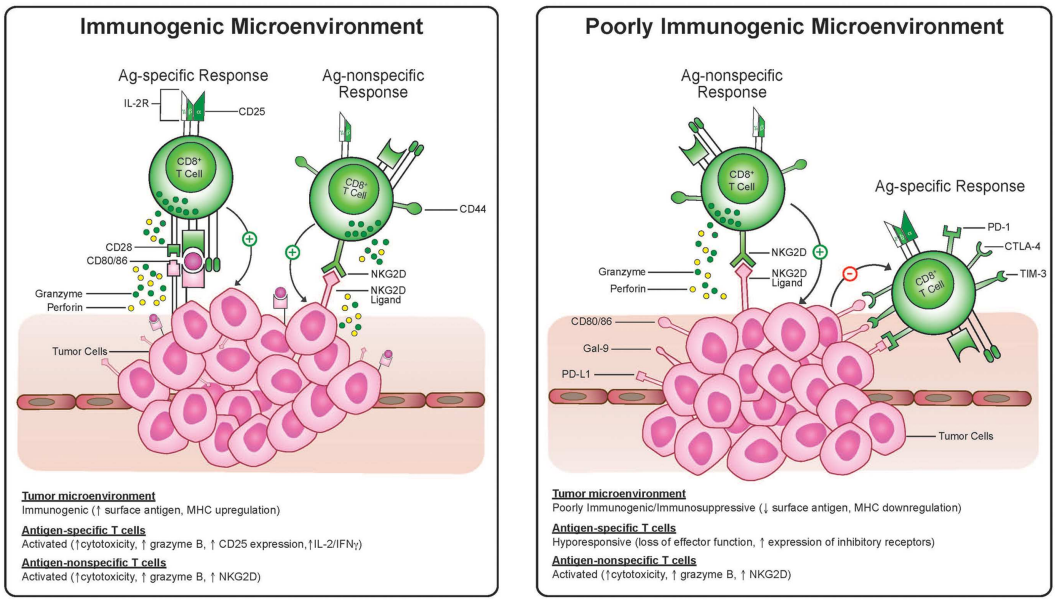

FIGURE 2 | Antigen-specific cytotoxic T-lymphocytes and antigen-non-specific bystander T-cell killing in an immunogenic and suppressive tumor microenvironment. T-cells activated via TCR engagement up-regulate markers, including CD25, CTLA-4, and PD-1. Antigen-non-specific activated cytotoxic T-cells have a $CD25^{-}$ and $NKG2D^{+}$ phenotype. In the immunosuppressive environment antigen-non-specific activated T-cells may be resistant to suppressive signaling via PD-1 or CTLA-4 and may recognize targets expressing NKG2D ligands even when antigen is lost and MHC is down-regulated whereas antigen-specific T-cells may become anergic.

Activated T-cells up-regulate expression of the inhibitory CTLA-4 molecule and it competes with the co-stimulatory molecule CD28 for binding of B7, acting as a feedback inhibitory mechanism. This feedback inhibitory mechanism is taken advantage of by tumors that use it to inactivate effector T-cells in the tumor microenvironment. Blockade of CTLA-4 with ipilimumab has demonstrated promising clinical results with regression of melanoma in some patients and a benefit in median overall survival of 2.1 and 3.6 months in two clinical trials (6, 54). These findings validate the central role of CTLA-4 in maintaining tumor immune tolerance and suppressing tumor reactive T-cells.

Another strategy being tested in the clinical trials is inhibition of the immunosuppressive enzyme IDO. IDO is an inducible tryptophan-catabolizing enzyme which can function to induce tolerance to alloantigens as demonstrated by its prevention of T-cell-mediated fetal rejection in mice (26, 55). A growing body of evidence suggests that, akin to many other immunoregulatory mechanisms, IDO is high-jacked by tumors to induce tolerance and may even act as a master switch coordinating the different aspects of the suppressive tumor microenvironment. IDO has been demonstrated to be inappropriately expressed by tumors and can coordinate the induction of Tregs and inhibition of natural killer cells and effector T-cells and can be up-regulated to counteract the effects of cancer immunotherapy (56, 57). The potential of inhibiting IDO as a means to reverse tumor-induced immune suppression and promote an anti-tumor immune response has been demonstrated repeatedly in pre-clinical studies and both competitive inhibitors and small molecule inhibitors of this enzyme are being tested in clinical trials.

Massive expansion, activation, and non-MHC-restricted killing by NK cells, macrophages, and memory T-cells can be induced by intense immune-stimulatory therapies such as CD40 agonists, IL-2, GM-CSF, IL-12, and CpG. The advantage of these approaches is that they provoke a broad immune response involving many cell types and are not dependent on a specific antigen. As mentioned above, CpGs can activate APC such as dendritic cells through toll receptor-9 signaling (50, 58) causing increased antigen presentation, co-stimulation, and pro-inflammatory cytokine secretion which can, in turn, trigger innate and adaptive cell-mediated immunity (51).

Immunotherapy with potent cytokines such as IL-2 or IL-12 can provoke tumor rejection at least partially through a non-MHC-restricted mechanism that includes NK and T-cells (59–62). These cells likely identify and lyse target malignant cells by recognition of NKG2D ligands as opposed to specific antigens. Moreover, it has been demonstrated that IL-2 can cause conversion of NK and T-cells to lymphokine-activated killers which also recognize and kill target cells through an antigen independent mechanism (63). A recent report demonstrates the ability of a CD40 agonist to induce tumor regression of pancreatic adenocarcinoma in both a pre-clinical trial and a human clinical trial (64). The authors conclude that the anti-tumor effects are mediated by the cytotoxic effects of macrophages without a dependence on antigen-specific T-cells. These examples and the innumerable other such studies demonstrate the potential of this type of therapy to induce anti-tumor immunity. The advantage, at least in theory, is that they are not dependent on a specific cell type or antigen but instead create an environment where an anti-tumor immune response is

supported and can efficiently recognize and eradicate malignant cells thus potentially rendering these therapies more resistant to the immune evasive mechanisms of tumors described above.

ANTIGEN NON-SPECIFIC T-CELL RESPONSES

Classically, T-cells require two signals via the T-cell receptor (TCR) and a co-stimulatory signal for activation and proliferation, and the need for a third signal (cytokine secretion) has been described for cytotoxic effector function of CD8+ T-cells. These requirements for activation can, however, be bypassed in a phenomenon termed "bystander activation." In the setting of very high cytokine stimulation, as occurs locally during viral and bacterial infections, memory T-cells can become activated and proliferate without the need for antigen-specific TCR engagement (65, 66). The exact function of these bystander activated T-cells is uncertain but in light of the up-regulation of cell surface NKG2D it has been suggested that they play a role in viral clearance (67, 68). Recently studies from our lab have demonstrated that bystander activated T-cells also occur after highly stimulatory systemic immunotherapy regimens (62) in a process that is similar to what is observed after infections such as influenza. These highly stimulatory immunotherapy regimens such as CD40 agonist and IL-2, CpG and IL-15, or IL-2 and IL-12 induce marked expansion of $CD8^+$ T-cell compartment that primarily consists of $CD44^{high}$ memory $CD8^+$ T-cells (62). In murine models, the bystander memory $CD8^+$ T-cells induced by these therapies proliferate and exhibit effector functions without the need for TCR engagement and produce significant anti-tumor effects which are dependent on IFN-g, IL-12, and Fas ligand expression but independent of $CD4^+$ T-cells, NK cells, and perforin (69, 70). After immunotherapy (62) these antigen-non-specific activated memory $CD8^+$ T-cells (AN-CTL) have a surface marker phenotype that is different than that of antigen-specifically activated T-cells through TCR engagement (**Figure 2**). In contrast to traditionally activated naïve or memory $CD8^+$ T-cells, these AN-CTLs do not up-regulate surface expression of CD25 and PD-1 but do express the natural killer cell activating receptor, NKG2D giving them a unique $NKG2D^+CD25^-CD8^+$ phenotype. The anti-tumor effects of these cells *in vivo* appears to be, in addition to IFN-g, IL-12, and Fas ligand, dependent on NKG2D as *in vivo* blockade of NKG2D significantly reduces the anti-tumor efficacy (62). These AN-CTLs express granzyme B and are post-therapy the only T-cells with cytolytic activity.

In TCR transgenic OT-1 mice, in which greater than 95% of the T-cells have TCRs specific for ovalbumin (OVA), vaccination with OVA produced OT-1 $CD8^+$ T-cells which were able to lyse OVA-expressing EG7 tumor cells but not the OVA-negative EL4 parental cell line. Conversely, after highly stimulatory systemic immunotherapy with CD40 agonist and IL-2, bystander activated antigen non-specific OT-1 $CD8^+$ T-cells are able to lyse both the OVA-expressing and OVA-negative targets *ex vivo* demonstrating their ability to kill without TCR engagement (62). Mirroring these results, *in vivo* therapy in OT-1 mice led to significant anti-tumor effects against OVA-negative 3LL tumors. We observed expansion of $CD8^+$ T-cells expressing the unique phenotype of up-regulation of NKG2D and Granzyme B without up-regulation of CD25 or PD-1. Importantly, it appears a similar mechanism may exist in humans. Unlike cells activated by TCR engagement, *in vitro* IL-2-treated human memory $CD8^+$ T-cells do not up-regulate PD-1 and CD25 expressing a similar bystander phenotype to that seen in mice. Furthermore, in melanoma patients, treatment with the topical toll-like receptor 7 agonist, imiquimod, produces infiltration of $CD8^+CD25^-$ T-cells compared to placebo-treated tumors (62). As a whole, these studies demonstrate that after highly stimulatory immunotherapy a pool of memory $CD8^+$ T-cells expands and has effector function which is both independent of antigen-specific TCR engagement and plays a critical part in the anti-tumor efficacy of these therapies.

The anti-tumor effects of these AN-CTL have several advantages over traditional anti-tumor cytotoxic T-cells. Given that these cells are both activated and recognize their targets in an antigen non-specific manner they are less sensitive to the mitigating effects of immunoediting, MHC down-regulation, or antigen loss. As substantiated in the above studies using OT-1 mice bearing non-OVA-expressing tumors, these cells can exhibit anti-tumor effects even when a tumor antigen recognized by their TCR is lacking. Additionally, these cells may be less prone to immune suppression as the lack of PD-1 surface expression implies that they are impervious to suppression by PD-1 ligand expression on tumor cells. Furthermore, since these cells are derived from the memory compartment, they have presumably been through multiple rounds of selection (central and peripheral tolerance) and have shown the ability to recognize foreign antigens thereby deeming them "safer" to become activated in a non-specific fashion without causing autoimmunity. Clinically, another advantage of being derived from the memory T-cell compartment, is that memory T cells increase with age. Since most malignancies occur in an aged population with limited thymic output and a limited naïve T-cell compartment, these memory cells provide an attractive pool for immunotherapy. The disadvantage of this approach is the need for a cytokine rich environment; because they are lacking in CD25, the high affinity IL-2 receptor, these cells rely on copious amounts of cytokine to maintain their activated state which, as discussed earlier, has the potential to become extremely toxic.

OVERCOMING THE ACHILLES HEEL OF IMMUNOTHERAPY WITH COMBINATORIAL STRATEGIES

It is likely that any successful immunotherapy strategy will need to rely to some extent on adaptive immunity, as any sustained response will need to rely on the development of immunological memory. This is demonstrated by the studies cited above of antigen-specific responses being correlated with outcomes in patients treated with ipilimumab. To date, the success of antigen-specific and non-specific strategies used alone have been unremarkable. Thus to overcome this Achilles heel of antigen-specific responses, yet still induce sustainable and clinically meaningful responses, will likely require the employment of combinatorial strategies using antigen-specific and non-specific approaches. This idea of targeting numerous mechanisms simultaneously to prevent the evolution of subclones which can circumvent the therapy has been highly successful in the management of HIV infection. For example, antigen-specific CD8 T-cells require TCR engagement whereas AN-CTLs recognize their targets via NKG2D ligands, thus the mechanisms of killing may be complimentary and a tumor would have to evolve strategies to overcome both of these mechanisms for immune evasion. Combinatorial strategies may also avoid some of the pitfalls of antigen-specific therapies and provide

superior outcomes. As we have previously reviewed, the use antigen non-specific therapies can induce innate immunity and AN-CTLs that can "de-bulk tumors, increase antigen release, sway the tumor microenvironment from suppressive to permissive, and induce a milieu of pro-inflammatory cytokines" all of which serve to create an environment where antigen-specific therapies can be more effective (71). The idea that combining an antigen-specific therapy may be improved by combining with an antigen non-specific therapy has been demonstrated in a melanoma vaccine trial (72). Vaccinated patients produced a measurable antigen-specific T-cell response to the vaccine antigens but these vaccine primed responses were significantly increased following CTLA-4 blockade with ipilimumab. A similar question is whether these combinatorial strategies can not only increase the robustness of the response to the targeted antigens but induce a response against new tumor antigens. In a clinical trial using nodal injection of CpG molecules in melanoma patients, a response was generated against melanoma associated antigens in 50% of the patients suggesting that this approach could increase the antigens targeted after an antigen-specific therapy (73). Clinically, there is some data that these types of combination therapies may be an effective treatment strategy as the combination of immunomodulatory cytokines such as GM-CSF or IL-12 with vaccines has shown efficacy in preliminary trials (74, 75).

CONCLUSION

Over the last decade cancer immunotherapy has evolved from a marginal idea to a reality in cancer therapy. Staggering breakthroughs are occurring with regularity and promising novel therapies that have the potential to change the paradigm of cancer treatment are on the horizon. Despite this promise and optimism the clinical efficacy of cancer immunotherapy has been modest to date. We have outlined above a number of potential obstacles to improving the effectiveness of immunotherapy. Chief amongst these is the need to overcome the tumors ability to evade an effective immune response. We suggest that combination immunotherapy regimens, working by many mechanisms simultaneously, may help address this obstacle. One approach which may be particularly useful in this regard is the induction of AN-CTLs which can bypass the need for antigen specificity and thereby help overcome the Achilles heel of cancer immunotherapy. Understanding how best to combine immunotherapy strategies is an area of active investigation which will further solidify immunotherapy in the arsenal of cancer therapeutics.

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Clinical perspectives on targeting of myeloid derived suppressor cells in the treatment of cancer

Yana G. Najjar[1]* and James H. Finke[2]

[1] *Department of Internal Medicine, The Cleveland Clinic Foundation, Cleveland, OH, USA*
[2] *Department of Immunology, The Cleveland Clinic Foundation, Cleveland, OH, USA*

Edited by:
Chao Ma, California Institute of Technology, USA

Reviewed by:
Julian Pardo, Fundación Agencia Aragonesa para la Investigación y el Desarrollo, Spain
Amedeo Amedei, University of Florence, Italy

****Correspondence:***
Yana G. Najjar, Internal Medicine Residency Program, The Cleveland Clinic Foundation, 9500 Euclid Avenue, NA10, Cleveland, OH 44195, USA.
e-mail: najjary@ccf.org

Tumors escape immune recognition by several mechanisms, and induction of myeloid derived suppressor cells (MDSC) is thought to play a major role in tumor mediated immune evasion. MDSC arise from myeloid progenitor cells that do not differentiate into mature dendritic cells, granulocytes, or macrophages, and are characterized by the ability to suppress T cell and natural killer cell function. They are increased in patients with cancer including renal cell carcinoma (RCC), and their levels have been shown to correlate with prognosis and overall survival. Multiple methods of inhibiting MDSCs are currently under investigation. These can broadly be categorized into methods that (a) promote differentiation of MDSC into mature, non-suppressive cells (all trans retinoic acid, vitamin D), (b) decrease MDSC levels (sunitinib, gemcitabine, 5-FU, CDDO-Me), or (c) functionally inhibit MDSC (PDE-5 inhibitors, cyclooxygenase 2 inhibitors). Recently, several pre-clinical tumor models of combination therapy involving sunitinib plus vaccines and/or adoptive therapy have shown promise in MDSC inhibition and improved outcomes in the tumor bearing host. Current clinical trials are underway in RCC patients to assess not only the impact on clinical outcome, but how this combination can enhance anti-tumor immunity and reduce immune suppression. Decreasing immune suppression by MDSC in the cancer host may improve outcomes and prolong survival in this patient population.

Keywords: MDSC, targeted therapy, combination therapy, cancer, immune evasion

INTRODUCTION

While several cancer treatments have been shown to illicit antigen specific immune responses, this has not correlated well with a clinical response and tumor regression. Multiple pre-clinical models have demonstrated regression of bulky tumors with immunotherapy, but the clinical response rates of several so called immunogenic tumors, including melanoma and renal cell carcinoma (RCC), remain quite low. It is widely accepted that the tumor microenvironment is immunosuppressive, both inhibiting activated immune cells and activating cells with a suppressive phenotype. Multiple cell types contribute to tumor mediated immune suppression, including regulatory T cells (T_{reg}), type 2 NKT cells, tumor associated macrophages (TAMs), and myeloid derived suppressor cells (MDSCs). MDSCs are a heterogeneous cell population characterized by the ability to suppress T cell and natural killer (NK) cell function (Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg, 2010). They arise from myeloid progenitor cells that do not differentiate into mature dendritic cells, granulocytes, or macrophages. MDSCs have been shown to be significantly increased in cancer patients of all stages relative to healthy volunteers, with a significant correlation between circulating MDSC, metastatic burden, and clinical cancer stage (Diaz-Montero et al., 2009), and therefore offer an exciting new target in cancer therapy. The goal of this review is to summarize the rationale of therapeutic targeting of MDSC numbers and/or function in patients with cancer. This includes a discussion of MDSC subpopulations, particularly those in human cancer patients, along with a very brief description of the mechanisms used by MDSC to suppress T cell function, as this topic has been extensively reviewed by others (Gabrilovich et al., 2012). Included is a discussion of the various approaches used to reduce the number or function of MDSC, along with a summary of pre-clinical studies that have examined the impact of combining immunotherapy with approaches to reduce MDSC as a means to promote anti-tumor T cell immunity and decrease tumor progression.

A HETEROGENEOUS POPULATION OF MDSCs IS INDUCED BY TUMOR MEDIATED INFLAMMATION

Two main subsets are described in mouse tumor models, granulocytic, and monocytic. Granulocytic (G) MDSC are polymorphonuclear-like and account for 70–80% of the MDSC population (Movahedi et al., 2008; Youn et al., 2008), whereas monocytic (M) MDSCs are mononuclear and account for 20–30% of MDSCs (Youn et al., 2008). Identification of MDSC subsets in humans is more complex, with multiple populations defined in solid tumors, but are broadly defined as myeloid cells expressing CD33, CD11b, and low/negative HLA-DR. In general granulocytic and monocytic subsets represent major components of human MDSC and there may be subpopulations of each based on the markers used to define them. Additionally, MDSC with the phenotype of CD33+HLA-DR−/low that are linage negative (CD15−, CD14−) have also been well documented

in cancer patients (Gabrilovich et al., 2012). The granulocytic subset expresses CD15 and/or CD66 and are typically negative for CD14 (Serafini et al., 2006a; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg, 2010; Gabrilovich et al., 2012). For some types of human cancers such as RCC, granulocytic-MDSC with immunosuppressive activity is the prevalent population in the blood, although M-MDSC, linage negative (CD15−CD14−), and other subsets are also present (Zea et al., 2005; Kusmartsev et al., 2008; van Cruijsen et al., 2008; Rodriguez et al., 2009; Ko et al., 2010; Walter et al., 2012). Similar findings have been reported in glioma and bladder cancer patients (Raychaudhuri et al., 2011; Sippel et al., 2011). While a recent study in murine tumor models demonstrates that G-MDSC are functionally distinct from neutrophils and represent immature neutrophils with suppressive activity (Youn et al., 2012), the relationship between G-MDSC and neutrophils is less clear in human cancer patients. Cells with the phenotype of activated neutrophils have been shown to co-purify with peripheral blood mononuclear cells (PBMC) and MDSC during ficoll density centrifugation (Schmielau and Finn, 2001; Zea et al., 2005; Ko et al., 2009; Rodriguez et al., 2009) and are immunosuppressive, unlike neutrophils from healthy donors. Additionally, when neutrophils from healthy donors are activated they display prolonged survival, have reduced density, and are immunosuppressive, similar to MDSC (Schmielau and Finn, 2001; Rodriguez et al., 2009; Sippel et al., 2011). Moreover, immature neutrophils (CD66b+CD16−) also co-purify with PBMC (Brandau et al., 2011), although the suppressive activity of these cells is not well defined. It seems likely that activated neutrophils and immature granulocytes (G-MDSC) contribute to immune suppression in different types of human cancers, although the specific suppressive and angiogenic activity of these two cell types requires further study. The monocytic MDSC population is also present in many different tumor types and is typically CD14+HLA-DR−/low. In patients with melanoma, multiple myeloma, prostate, and hepatocellular carcinoma, the immuosuppressive M-MDSC is a prominent population (Filipazzi et al., 2007, 2012; Hoechst et al., 2008; Mandruzzato et al., 2009; Poschke et al., 2010; Vuk-Pavlovic et al., 2010) and is thought to suppress via the production of arginase, iNOS, and suppressive cytokines.

Myeloid derived suppressor cells are induced by chronic inflammation, and several tumor-secreted factors have been implicated in MDSC induction. Prostaglandin E2 induces differentiation of c-kit+ hematopoietic stem cells into MDSCs, contributing to T cell immune suppression (Rodriguez et al., 2005; Sinha et al., 2007b). Interleukin (IL)-6, IL-1β, GM-CSF, and G-CSF, which are found in the microenvironment of many tumors, have been shown to significantly increase MDSC accumulation and T cell suppression (Song et al., 2005; Bunt et al., 2006; Sinha et al., 2008). Furthermore, IL-1β induced inflammation aids MDSC and macrophage cross-talk, thus increasing MDSC mediated innate immune suppression (Bunt et al., 2006). In addition, proteins S100A8/A9, both pro-inflammatory, induce MDSC accumulation (Sinha et al., 2008). An autocrine positive feedback loop is created by MDSC secreting pro-inflammatory factors, including IL-6 and S100A8/A9, thus further sustaining themselves in the tumor microenvironment (Sinha et al., 2008; Ostrand-Rosenberg, 2010).

MDSC ARE INCREASED IN PERIPHERAL BLOOD AND TUMOR PARENCHYMA OF THE TUMOR HOST, AND LEVELS HAVE BEEN SHOWN TO CORRELATE WITH CLINICAL OUTCOME

Several studies have shown increased MDSC levels in patients with different histologic tumors (Hoechst et al., 2008; Movahedi et al., 2008; Gabrilovich and Nagaraj, 2009). In a study of 106 patients with newly diagnosed stage I-IV solid tumors, circulating MDSC percentages were measured (Lin^{-}/Low, HLA-DR^{-}, $CD33^{+}CD11b^{+}$) prior to the start of treatment. Circulating MDSC levels were found to correlate both with clinical stage ($p < 0.0001$) and metastatic burden ($p < 0.01$). Interestingly, patients with radiographic evidence of disease progression had increased levels of circulating MDSC, whereas patients who responded to treatment had decreased MDSC (Diaz-Montero et al., 2009). A recent study identified six MDSC phenotypes using single multicolor staining: increased percentages of MDSC2–MDSC6 phenotypes were noted in patients with RCC compared to healthy donor controls ($p < 0.01$). Furthermore, a retrospective analysis found MDSC4 (monocytic; $p < 0.001$) and MDSC5 (granulocytic; $p = 0.016$) were significantly negatively associated with overall survival (Walter et al., 2012). Recently, increased circulating promyelocyte-like bone marrow derived $CD11b^{+}$/$CD16^{-}$MDSC levels correlated with reduced survival in breast cancer ($p = 0.048$) and colorectal cancer patients ($p = 0.025$) (Solito et al., 2011). Additionally, increased levels of HLA-DR $Lin1^{low/-}$ $CD33^{+}$ $CD11b^{+}$ MDSC in pancreatic, esophageal, and gastric cancer was an independent prognostic factor for survival ($p < 0.001$) (Gabitass et al., 2011).

The presence of MDSC in hematological malignancies is not as well established, but they have been described in patients with multiple myeloma, Hodgkin's lymphoma (HL), and non-Hodgkin's lymphoma (NHL) (Montero et al., 2012). In the latter two, MDSC levels were found to correlate with clinical stage, and in NHL also correlated with faster rates of disease progression and more aggressive NHL histology ($p = 0.01$) (Motzer et al., 2002; Montero et al., 2012). Collectively, these early clinical findings suggest that accumulation of MDSC levels in cancer patients contributes to tumor progression, thereby providing a target for improving immunotherapy.

MDSCs USE VARIOUS MECHANISMS TO SUPPRESS EFFECTIVE ANTI-TUMOR IMMUNITY

The regulatory function of MDSC in dampening anti-tumor immunity has been extensively shown in both *in vitro* and *in vivo* studies (**Figure 1**). MDSC inhibit both antigen specific and non-specific T cell activation in murine MDSC co-cultures with peptide activated T cells and murine and human MDSC co-cultures with anti-CD3 activated T cells (Gabrilovich et al., 2001; Sinha et al., 2005). Both $CD4^{+}$ and $CD8^{+}$ T cells are suppressed, and while suppression requires cell to cell contact, this can occur by an MHC restricted or unrestricted mechanism (Nagaraj et al., 2007). Granulocytic and monocytic MDSC inhibit T cells by depletion of L-arginine within the tumor microenvironment, thus arresting T cells in G0–G1 (Rodriguez et al., 2005; Ostrand-Rosenberg, 2010). Similarly, MDSC inhibit T cell activation by sequestering cystine. This disables T cells from obtaining cysteine, which is essential for antigen activation, proliferation, and differentiation

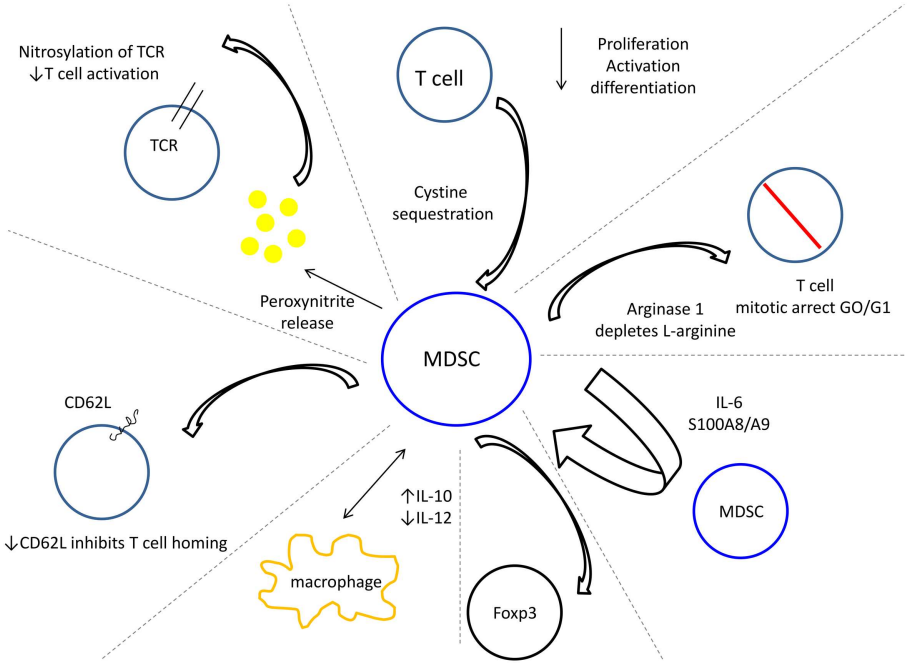

FIGURE 1 | Myeloid derived suppressor cells use multiple mechanisms to dampen anti-tumor immunity.

(Ostrand-Rosenberg, 2010; Srivastava et al., 2010). Reduced $CD4^+$ and $CD8^+$ T cell homing to lymph nodes is effected by MDSC, resulting in a down-regulation of L-selectin, which normally drives leukocyte extravasation to areas of inflammation (Hanson et al., 2009). MDSC have also been shown to impair innate immunity by their cross-talk with macrophages, which increases MDSC production of IL-10 and decreases macrophage production of IL-12, converting anti-tumor M1 cells into M2 cells that enhance tumor progression (Sinha et al., 2007a). In a murine B-cell lymphoma model, MDSC were identified as tolerogenic antigen presenting cells (APC) capable of antigen uptake and presentation to tumor-specific T_{regs} by an arginase dependant mechanism (Serafini et al., 2008). Interestingly, *in vitro* and *in vivo* inhibition of MDSC function reduced T_{reg} proliferation and tumor-induced tolerance in antigen specific T cells (Serafini et al., 2008).

DIFFERENT STRATEGIES TO DOWN-REGULATE MDSC NUMBER AND FUNCTION

Given the phenotypic and functional heterogeneity of MDSCs, therapeutic approaches that are sufficient to inhibit MDSCs across a wide spectrum of cancer patients would be a significant addition to the anti-cancer armamentarium, and several mechanisms are currently undergoing investigation (**Table 1**).

PROMOTING DIFFERENTIATION OF MDSC INTO MATURE, NON-SUPPRESSIVE CELLS (ATRA, VIT D3)

Promoting differentiation of suppressive MDSC into mature, non-suppressive cells has been studied in pre-clinical and clinical cancer models, the rationale being that conversion of MDSC may enhance anti-tumor immune responses. Increased production of reactive oxygen species (ROS) is a functional characteristic of MDSC, and all trans retinoic acid (ATRA), a derivative of vitamin A, has been shown to induce MDSC differentiation by a glutathione synthase dependant mechanism (Nefedova et al., 2007). While ATRA induced differentiation of MDSC into myeloid dendritic cells *in vitro* (Gabrilovich et al., 2001), administration *in vivo* increased MDSC differentiation and enhanced $CD4^+$ and $CD8^+$ T cell antigen specific immune responses, but did not decrease tumor burden (Gabrilovich and Nagaraj, 2009). More promising results were obtained by combining ATRA with antigen specific peptide vaccines. In two different tumor models, treatment with ATRA and peptide vaccines significantly prolonged the anti-tumor treatment effect, making this molecule a promising candidate as an adjunct to cancer immunotherapy (Gabrilovich et al., 2001). The effect of ATRA on MDSC in cancer patients was recently elucidated: 18 patients with metastatic renal cell carcinoma (mRCC) who were shown to have elevated MDSC levels were treated with ATRA. This significantly reduced the number of MDSC in patients with a high plasma concentration of ATRA (>150 ng/mL), but not in patients with lower ATRA concentrations (<135 ng/mL) (Mirza et al., 2006). Interestingly, the effect of ATRA was abrogated in patients who also received subcutaneous IL-2 (Mirza et al., 2006).

In a phase IB study, treatment with oral Vit D3 in patients with HNSCC was shown to reduce the number of immune suppressive $CD34^+$ cells (CD11b+CD33+CD14−HLA-DR−), increase HLA-DR expression, and increase plasma IL-12 and IFN-gamma levels *in vitro*, which would favor an anti-tumor Th1 immune response (Lathers et al., 2004; Ugel et al., 2009).

DECREASING MDSC LEVELS (SUNITINIB, GEMCITABINE, 5-FU)

Sunitinib is an oral receptor tyrosine kinase inhibitor that targets signaling by PDGFRs, VEGFRs, and c-kit, and was approved by

Table 1 | Summary of mechanisms of anti-MDSC agents and key study findings.

Mechanism of action	Agent	Study finding	Reference
Promoting MDSC differentiation	ATRA	Induced MDSC differentiation into myeloid DC in mice/humans. Improved T cell response	Gabrilovich et al. (2001)
		High plasma concentrations of ATRA correlated with reduced MDSC levels	Mirza et al. (2006)
	Vit D3	HNSCC pts treated with oral VitD3 had decreased MDSC	Lathers et al. (2004), Ugel et al. (2009)
Decreasing MDSC levels	Sunitinib	Treatment in RCC pts decreased MDSC (monocytic and linage negative subsets) and increased myeloid DC in patients experiencing tumor regression	van Cruijsen et al. (2008)
		Treatment in RCC pts decreased MDSC and T_{reg} levels, improved T cell function (IFNY production)	Finke et al. (2008), Ko et al. (2009)
		In mouse model (MCA 26) sunitinib reduced MDSC levels in tumor and also T_{regs}	Ozao-Choy et al. (2009)
		Synergized with immunotherapy to reduce tumor size	
		Reduced PDL-1 expression	
		In B16 Ova mouse model sunitinib reduced MDSC and T_{eg} in tumor	Bose et al. (2010)
		Reduced levels of immunosuppressive co-stimulatory molecules and chemokines involved in MDSC and T_{reg} trafficking	
		Synergy with vaccine to boost T cell anti-tumor response	
		Sunitinib reduced the viability of granulocytic-MDSC in tumor bearing mice and reduced the proliferation of monocytic MDSC	Ko et al. (2010)
	Axitinib	Reduced MDSC, T_{reg}, and enhanced T cell response in tumor bearing mice	Bose et al. (2010)
	Gemcitabine	Decreased splenic MDSC, improved CD8 and NK cell anti-tumor activity in 5 murine lung cancer models Reduces number *ex vivo* and then they show apoptosis of splenocytes *in vivo*	Suzuki et al. (2005)
		Early treatment in a murine mammary carcinoma model decreased MDSC, which correlated with tumor growth inhibition	Le et al. (2009)
	5-FU	Treatment decreased splenic and intra tumor MDSC, did not affect T, B, NK, or dendritic cells. 5-FU triggers MDSC apoptosis	Vincent et al. (2010)
	Docetaxel	Reduced MDSC in spleen; increased CTL response; and polarized MDSC to M1 phenotype	Kodumudi et al. (2010)
Inhibiting MDSC function	CDDO-Me	In a murine model, decreased MDSC inhibitory function and decreased tumor growth. In RCC patients, completely abrogated MDSC inhibitory function *in vitro*	Nagaraj et al. (2010)
		In mice, did not affect number of MDSC in spleen, but eliminated suppressive activity of MDSC on $CD8^+$ T cells *in vitro*	
Inhibiting MDSC function	PDE-5 inhibitor	In mice, treatment down regulated ARG1 and NOS2, abrogated suppressive pathways. In isolated cells from cancer patients, restored T cell proliferation	Serafini et al. (2006b)
		In melanoma patients, treatment decreased MDSC levels and weakened suppressive function	Umansky and Sevko (2012)
		Sildafenil increased survival of tumor bearing mice by a $CD8^+$ T cell dependant mechanism. Decreased MDSC number and immunosuppressive function	Meyer et al. (2011)
	COX-2 inhibitor	In a murine glioma model, treatment inhibited PGE-2 production and delayed glioma development. MDSC were decreased in bone marrow and within the tumor, CCL2 chemokine was decreased also	Fujita et al. (2011)
		In ovarian cancer pts, decreased MDSC levels in ascites correlated with CXCL12 and PGE-2 inhibition	Obermajer et al. (2011)
	Nitro aspirin	Increased the number and function of tumor Ag-specific T lymphocytes *in vitro* and *in vivo* by decreasing ARG and NOS activity in $CD11^+$ B lymphocytes	De Santo et al. (2005)

the FDA for the treatment of advanced RCC in 2007, following a phase III trial that demonstrated improved overall and progression free survival (Motzer et al., 2009). It is currently front line therapy for patients with metastatic RCC. In patients with advanced RCC, after 4 weeks of sunitinib treatment, a generalized decrease in myeloid frequencies was observed (van Cruijsen et al., 2008). Increased levels of myeloid DC subsets were noted relative to other myeloid subsets in patients experiencing tumor regression, and high levels of CD1c/BDCA-1(+) MDSC were predictive of tumor regression and improved progression free survival (van Cruijsen et al., 2008), suggesting that sunitinib may play an immunomodulatory role in the tumor bearing host. In RCC patients, one cycle of treatment with sunitinib significantly increased the percentage of IFN-gamma producing T cells, reduced IL-4 production, and diminished type 2 bias (Finke et al., 2008). This augmented T cell response was associated with decreased MDSC levels, including a reduction in the dominant population, granulocytic-MDSC (Ko et al., 2009). The increase in type-1 response may be partly related to modulation of T_{reg} cells: mRCC patients were found to have a significantly higher number of T_{reg} than healthy controls, and while an inverse correlation between the increase in type-1 and a decrease in the percentage of T_{reg} was noted, the reduction in T_{reg} after treatment did not reach statistical significance (Finke et al., 2008). Additional studies in a mouse tumor model (4T1) indicate that sunitinib treatment may function by reducing the expansion of monocytic MDSC while inducing apoptosis in the granulocytic-MDSC subset (Ko et al., 2010). In an advanced tumor murine model, sunitinib treatment decreased both MDSC and T_{reg} levels, in addition to reducing suppressive function of MDSCs and improving tumor-specific T cell function (Ozao-Choy et al., 2009). Treatment with sunitinib also resulted in reduced expression of IL-10, transforming growth factor-beta, and Foxp3, but increased expression of IFN-gamma, skewing the immune response toward a Th1 phenotype, and increased cytotoxic T lymphocyte (CTL) responses in isolated tumor infiltrating lymphocytes (TILs). Perhaps most importantly, the expression of negative co-stimulatory molecules was widely dampened: CTLA4 and PD-1 were decreased in $CD4^+$ and $CD8^+$ T cells, and PDL-1 expression on MDSC and plasmacytoid dendritic cells was also significantly decreased by sunitinib treatment (Ozao-Choy et al., 2009).

STAT3 plays a central role in MDSC function, promoting tumor invasion, and angiogenesis. There is some evidence that sunitinib may act through a STAT3 associated mechanism. In a murine kidney cancer model (RENCA), sunitinib inhibited STAT3 activity in tumor associated MDSCs, and was found to reduce the expression of several STAT3 regulated pro-angiogenic genes (Kujawski et al., 2008; Xin et al., 2009).

While some chemotherapeutic agents, such as doxorubicin and cyclophosphamide, have been shown to increase MDSC levels in peripheral blood (Suzuki et al., 2005), gemcitabine, a cytidine nucleoside analog, has been shown to decrease splenic MDSC in murine models of five advanced lung cancer cell lines (Suzuki et al., 2005). Interestingly, no significant reduction was noted in $CD4^+$ T cells, $CD8^+$ T cells or B cells, and an increase in the anti-tumor activity of $CD8^+$ T cells and activated NK cells was noted, making this a promising MDSC targeting agent. Furthermore, at specific time points after treatment, gemcitabine was shown to selectively induce MDSC apoptosis (Suzuki et al., 2005). In a more recent study, BALB/c mice inoculated with 4T1 mammary carcinoma were treated with repeated gemcitabine starting within 1 week after inoculation, or treated once after 20–25 days (Le et al., 2009). Early treatment with gemcitabine significantly decreased the proportion of MDSC in the spleen, and this correlated with a decrease in tumor growth (Le et al., 2009). While a single dose of gemcitabine in mice with large tumors did inhibit MDSC accumulation, this did not affect tumor burden. This study also suggests selective inhibition of MDSC, as gemcitabine treatment of tumor bearing mice restored $CD8^+$ T cell immune function (Le et al., 2009).

5-FU, a pyrimidine analog, is another chemotherapeutic agent that has shown selective anti-MDSC activity. Treatment of tumor bearing mice with 5-FU led to a major decrease in splenic MDSC and MDSC within the tumor parenchyma, with no significant effect on T cells, B cells, NK cells, or dendritic cells (Vincent et al., 2010). Compared to gemcitabine, 5-FU showed more efficacy in MDSC depletion and induction of MDSC apoptotic cell death, both *in vitro* and *in vivo* (Vincent et al., 2010). Furthermore, 5-FU mediated elimination of MDSC increased IFN-gamma production by tumor-specific $CD8^+$ T cells infiltrating the tumor, promoting T cell-dependent anti-tumor responses *in vivo* (Vincent et al., 2010).

FUNCTIONAL INHIBITION OF MDSC (PDE-5 INHIBITORS, COX-2 INHIBITORS, CDDO-Me)

PDE-5 inhibitors are currently widely used for the treatment of erectile dysfunction and pulmonary hypertension. Recently, multiple studies have elucidated their potential as anti-MDSC agents in cancer treatment. *In vitro*, PDE-5 inhibitors have been shown to have pro-apoptotic activity on chronic lymphocytic leukemia and colon carcinoma (Ugel et al., 2009). Experiments in immune deficient mice have clearly shown that this drugs' anti-tumor effects are immune mediated. In multiple murine tumor models, several PDE-5 inhibitors were shown to synergize with adoptive cell therapy, delaying tumor growth (Serafini et al., 2006b). Furthermore, mice treated with PDE-5 inhibitor had increased $CD8^+$ T cell intra tumor infiltration, and these lymphocytes up-regulated CD69 and CD25 (markers of activation) and secreted IL-2 (Serafini et al., 2006b). Most importantly, MDSC suppressive pathways were dampened: ARG1 and NOS2 were down regulated, in addition to IL-4-Rα expression (Serafini et al., 2006b). This strategy was also shown to be effective in cancer patients: in PBMC isolated from patients with head and neck cancer or multiple myeloma, PDE-5 inhibitors restored T cell proliferation (Serafini et al., 2006b).

More recently, studies have assessed the role of PDE-5 in melanoma. MDSC were found to be increased in melanoma lesions, and their accumulation was associated with a strong TCR ζ-chain down-regulation in T cells (Umansky and Sevko, 2012). Treatment with PDE-5 inhibitor resulted in decreased MDSC levels and partial restoration of ζ-chain expression in T cells, resulting in attenuated immunosuppressive function and significantly increased survival of tumor bearing mice, by a $CD8^+$ T cell dependant mechanism (Meyer et al., 2011; Umansky and Sevko, 2012). These studies suggest that PDE-5 may be of benefit if used in conjunction with melanoma targeted immunotherapies.

The enzyme cyclooxygenase 2 (COX-2) plays a role in the production of PGE-2, which induces expansion of MDSC (Sinha et al., 2007b). In a murine glioma model, treatment with COX-2 inhibitors inhibited systemic PGE-2 production and delayed glioma development (Fujita et al., 2011). CCL2, an MDSC-attracting chemokine, was reduced in the tumor microenvironment, and MDSC were decreased both in the bone marrow and the tumor microenvironment (Fujita et al., 2011). Furthermore, increased levels of CTLs were noted in the tumor microenvironment (Fujita et al., 2011). These results were not observed in glioma-bearing COX-2 and CCL2 deficient mice (Fujita et al., 2011).

In a recent study, it was shown that PGE-2 attracts MDSC into the ascites microenvironment of ovarian cancer patients by inducing expression of functional CXCR4 in cancer-associated MDSCs, and plays a role in the production of its ligand CXCL12, thus ensuring MDSC migration (Obermajer et al., 2011). Frequencies of MDSCs closely correlated with CXCL12 and PGE-2 levels in ascitic fluid, and inhibition of COX-2 or PGE-2 receptors in MDSCs suppressed CXCR4 expression, and thus MDSC responsiveness to CXCL12 or ovarian cancer ascites (Obermajer et al., 2011). These studies provide a rationale for targeting COX-2 in cancer therapy.

CDDO-Me belongs to a class of relatively new compounds called synthetic triterpenoids, and has been shown to be a potent activator of the transcription factor NFR2, which up-regulates several antioxidant genes (Nagaraj et al., 2010). *In vitro*, CDDO-Me completely abrogated MDSC immunosuppressive activity from tumor bearing mice (Nagaraj et al., 2010), which is not surprising given that up-regulation of ROS is an essential function of MDSC. Treatment of mice with this agent did not decrease the proportion of splenic MDSC, but did eliminate MDSC suppressive activity, and decreased tumor growth (Nagaraj et al., 2010). Furthermore, CDDO-Me completely abrogated the inhibitory effect of MDSC *in vitro* in samples isolated from RCC patients (Nagaraj et al., 2010).

COMBINATION THERAPY: TARGETING MDSC AS AN ADJUVANT TO VACCINES AND IMMUNOTHERAPY

Current studies are focused on strategies that combine approaches to reduce MDSCs as an adjuvant to different forms of immunotherapy. As previously discussed, gemcitabine has been shown to reduce splenic MDSC levels in tumor bearing mice (Suzuki et al., 2005). In this same study, combining gemcitabine with IFN-beta markedly enhanced anti-tumor efficacy (Suzuki et al., 2005). In a HER-2/neu tumor model, treatment with gemcitabine, HER-2/neu vaccine and anti-glucocorticoid tumor necrosis factor receptor related protein (GITR) mAbs showed potent therapeutic anti-tumor immunity, in addition to protection against pre-existing tumors (Ko et al., 2007). Given that Her-2/neu is a self antigen with poor immunogenicity, this study suggests that when given with antigen specific immunotherapy, gemcitabine combinational therapy may be more effective than either treatment alone (**Table 2**).

Several studies have shown that tumor-directed radiation therapy increases the effectiveness of several forms of immunotherapy (Kao et al., 2011). While the exact mechanism has yet to be elucidated, this may be due to increased uptake of tumor antigen by APCs within the irradiated field. In a recent mouse glioma model, addition of sunitinib to low-dose radiotherapy only modestly improved survival (D'Amico et al., 2012). Combining sunitinib with high dose radiation therapy resulted in fatal toxicities, though each treatment was well tolerated alone, thus limiting the feasibility of this combination (D'Amico et al., 2012). Unfortunately, success with the combination of sunitinib and radiation has been on a case by case basis, with no clinical series to date assessing the potential synergy of this combination (Dallas et al., 2012; Venton et al., 2012).

In patients with RCC, mutation the VHL tumor suppressor gene results in overproduction of vascular endothelial growth factor (VEGF). Athymic nude mice that were inoculated with human RCC cells were found to have VEGF receptor 1 (VEGFR1)/CD11b myeloid cells in the peripheral blood (Kusmartsev et al., 2008). Treatment with Avastin (humanized anti-VEGF-1 mAb) resulted in significantly reduced numbers of circulating VEGFR1+ MDSC, suggesting that elimination of VEGFR1+ cells may restore immunocompetence (Kusmartsev et al., 2008). However, treatment of metastatic RCC patients with bevacizumab either alone or combined with interleukin-2 did not reduce MDSC levels in the peripheral blood (Rodriguez et al., 2009). The difference in MDSC modulation between these two studies may be related to the timing of antibody administration, since RCC patients had advanced disease while mice were treated with antibody during early stages of tumor development.

Recent animal models have suggested that inhibiting MDSC and thus reversing immune suppression with sunitinib, a tyrosine kinase inhibitor, may be an effective adjunctive treatment to immune-based cancer therapies (Ozao-Choy et al., 2009; Bose et al., 2010; Kujawski et al., 2010). However, in a phase III trial, combining the TroVAax (MVA-5T4) vaccine with either sunitinib, IL-2, or IFN-α in RCC patients did not enhance survival relative to sunitinib alone (or IL-2 or IFN-α alone) (Amato et al., 2010). Interestingly, the lack of synergy between vaccine and sunitinib in this trial may be related to the sequence of vaccine and sunitinib administration. In an MC38-CEA murine tumor model, treatment with sunitinib followed by vaccine was most effective compared to the reverse order, suggesting that in some tumor models the sequencing of sunitinib and vaccine is important (Farsaci et al., 2012). Further studies are needed to assess the role of combining sunitinib with immunotherapy in t he clinical setting. Indeed, two company supported clinical trials are underway to test the efficacy and immune modulating activity of combining sunitinib with vaccines in metastatic RCC patients (Argos Therapeutics and Immatics Biotechnologies).

CONCLUSION

Immune evasion is a hallmark of cancer, and MDSC play a central role in tumor mediated immunosuppression. MDSC are increased in the tumor bearing host, and MDSC levels have been shown to correlate with disease stage and survival. Multiple studies show that targeting MDSC leads to an improvement in anti-tumor immunity, specifically recovery of $CD8^+$ T cell anti-tumor activity, resulting in tumor suppression, and multiple modes of targeting MDSC are in clinical development. For example, administration of ATRA to patients with metastatic RCC

Table 2 | Summary of combination therapies targeting MDSC and key study findings.

Agents	Study finding	Reference
ATRA + antigen specific peptide vaccine	In two different murine tumor models, significantly prolonged the anti-tumor treatment effect	Gabrilovich et al. (2001)
Gemcitabine + IFN-b	Significantly increased anti-tumor activity in a murine tumor model	Suzuki et al. (2005)
Gemcitabine + HER-2/neu vaccine + anti-GITR mAb	Potent therapeutic anti-tumor immunity in a murine tumor model	Ko et al. (2007)
Sunitinib + low-dose radiotherapy	Modestly improved survival in a mouse glioma model Sunitinib with high dose radiation resulted in fatal toxicities	D'Amico et al. (2012)
Sunitinib + DC based vaccine	Combination Rx had superior anti-tumor effect than either Rx alone in a murine melanoma model and enhanced anti-tumor T cell response and reduced MDSC/T_{reg}	Bose et al. (2010)
Sunitinib + adoptive T cell therapy	In murine melanoma and RCC models Inhibited Stat3 in DC and T cells Reduced conversion of T cells to T_{regs} Increased $CD8^+$ T cell infiltration and activation at the tumor site Inhibited primary tumor growth	Kujawski et al. (2010)
Sunitinib + IL-12 + 4-1BB activation	Significantly improved long-term survival rate of large tumor bearing mice in liver and lung tumor models, promoted T cell response and reduced MDSC levels	Ozao-Choy et al. (2009)
Sunitinib + CEA vaccine	In a murine colon cancer model: continuous sunitinib followed by vaccine increased tumor infiltration of Ag-specific T lymphocytes Reduced tumor volumes Increased survival Decreased T_{reg} Decreased MDSC	Farsaci et al. (2012)
Bevacizumab ± IL-2	Did not reduce MDSC levels in the peripheral blood	Rodriguez et al. (2009)
Phase III trial, TroVAax (MVA-5T4) vaccine + sunitinib, IL-2, or IFN-a	In RCC pts did not enhance survival relative to sunitinib, IL-2, or IFN-a alone	Amato et al. (2010)

increased MDSC differentiation and enhanced $CD4^+$ and $CD8^+$ T cell antigen specific immune responses (Gabrilovich and Nagaraj, 2009). In another study, treatment with oral Vit D3 in patients with HNSCC reduced the number of immune suppressive $CD34^+$ cells and skewed immune system toward an anti-tumor Th1 immune response (Lathers et al., 2004; Ugel et al., 2009). However, while multiple studies have shown effective antigen specific immunity, this has not correlated with improved survival: reduction in immune suppression by MDSC may improve outcomes using cancer vaccines and other forms of immunotherapy.

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Improvement of cancer immunotherapy by combining molecular targeted therapy

Yutaka Kawakami*, Tomonori Yaguchi, Hidetoshi Sumimoto, Chie Kudo-Saito, Tomoko Iwata-Kajihara, Shoko Nakamura, Takahiro Tsujikawa, Jeong Hoon Park, Boryana K. Popivanova, Junichiro Miyazaki and Naoshi Kawamura

Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan

Edited by:
Antoni Ribas, University of California Los Angeles, USA

Reviewed by:
Howard L. Kaufman, Rush University Medical Center, USA
Graham R. Leggatt, University of Queensland, Australia

****Correspondence:***
Yutaka Kawakami, Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan.
e-mail: yutakawa@z5.keio.jp

In human cancer cells, a constitutive activation of MAPK, STAT3, β-catenin, and various other signaling pathways triggers multiple immunosuppressive cascades. These cascades result in the production of immunosuppressive molecules (e.g., TGF-β, IL-10, IL-6, VEGF, and CCL2) and induction of immunosuppressive immune cells (e.g., regulatory T cells, tolerogenic dendritic cells, and myeloid-derived suppressor cells). Consequently, immunosuppressive conditions are formed in tumor-associated microenvironments, including the tumor and sentinel lymph nodes. Some of these cancer-derived cytokines and chemokines impair immune cells and render them immunosuppressive via the activation of signaling molecules, such as STAT3, in the immune cells. Thus, administration of signal inhibitors may inhibit the multiple immunosuppressive cascades by acting simultaneously on both cancer and immune cells at the key regulatory points in the cancer-immune network. Since common signaling pathways are involved in manifestation of several hallmarks of cancer, including cancer cell proliferation/survival, invasion/metastasis, and immunosuppression, targeting these shared signaling pathways in combination with immunotherapy may be a promising strategy for cancer treatment.

Keywords: immunotherapy, immunosuppression, MAPK, STAT3, β-catenin

INTRODUCTION

By the time cancer cells are detected clinically, they have already evaded the immune-defense system (Robert et al., 2011). During their long development process, such cancer cells have lost highly immunogenic tumor antigens and acquired immunoresistant and immunosuppressive properties through various mechanisms (Yaguchi et al., 2011). Consequently, elimination of cancer cells by immunological strategies may not be easy. However, it has been revealed that the tumor antigens expressed by cancer cells are qualitatively or quantitatively different form the normal counterpart, and that cancer cells can be eliminated by T cells using various immune-interventions in some patients. We have previously identified human tumor antigens recognized by T cells (Kawakami et al., 1994a,b), and attempted to develop various antigen-specific immunotherapies (Rosenberg et al., 1998). For instance, the administration of gp100 melanoma antigen peptide vaccine along with IL-2 resulted in 16% objective response with 9% CR in the recent multicenter randomized trial (Schwartzentruber et al., 2011). Furthermore, adoptive immunotherapy using cultured melanoma-specific T cells following lymphomyeloablative treatment, which depletes various immunosuppressive cells and induces homeostatic proliferation of administered T cells, resulted in more than 70% objective response with about 20% durable CR in advanced melanoma patients with multiple metastases (Rosenberg et al., 2011). These observations indicate that active immunization may be further improved by various immune-interventions.

DEVELOPMENT OF EFFECTIVE IMMUNOTHERAPY BY COMPREHENSIVE REGULATION OF ANTI-TUMOR IMMUNE NETWORK

Analysis of mouse tumor models and human clinical trials using the identified tumor antigens revealed that following key points need to be addressed in order to regulate the anti-tumor immune network and develop effective immunotherapy (**Figure 1**) (Kawakami et al., 2004). (1) *Identification of appropriate tumor antigens for immunotherapy*: the ideal antigens should have tumor-specific expression and they should be involved in cancer cell proliferation/survival. They must also be expressed in cancer initiating cells. We have identified human glioma antigen SOX6, which is expressed in glioma stem-like cells. SOX6 is involved in cancer proliferation and is recognized by T cells (Ueda et al., 2004, 2010). Sox6-DNA vaccination was able to inhibit growth of murine glioma in a therapeutic setting (Ueda et al., 2008). (2) *Development of in situ tumor destruction methods to induce immunogenic cancer cell death:* break down of tumor releases endogenous tumor antigens and subsequently induces anti-tumor immune response (*Immunogenic cancer cell death).* This may be achieved possibly by using chemotherapy, molecular targeted drugs, anti-tumor antibody, irradiation, cryoablation, radiofrequency ablation, or oncolytic viruses. (3) *Development of methods to enhance dendritic cell (DC) functions:* the methods include augmentation of antigen uptake, cross presentation, and T cell stimulation by using adjuvants, cytokines, or agonistic antibodies. We have previously developed several protocols for combined immunotherapy of *in situ* tumor destruction and

subsequent DC activation. An example of this is the use of oncolytic HSV, which is capable of both direct tumor destruction and DC stimulation. Intratumoral administration of HSV not only inhibited the treated tumor but also suppressed untreated tumors at remote sites via induction of systemic anti-tumor T cells (Toda et al., 2002). Another protocol involves a combination of tumor cryoablation and subsequent intratumoral administration of DCs pretreated with TLR2-stimulating BCG-CWS (Mycobacterium bovis Bacillus Calmette-Guérin cell wall skeleton). This protocol induced T cell responses to multiple endogenous tumor antigens and suppressed growth of untreated remote tumors as well (Udagawa et al., 2006). (4) *Development of methods to activate and expand anti-tumor T cells in vivo:* this may be achieved possibly by immunization with tumor antigens, administration of cytokines, or agonistic antibodies against co-stimulatory molecules on T cells, or transfer of cultured anti-tumor T cells. We are currently attempting to use tumor-specific T cells cultured *in vitro* to treat patients with melanoma. (5) *Development of methods to reverse immunosuppression*: Various immunomodulating reagents are being studied to evaluate their efficacy in recovering immunosuppressive condition in cancer patients. These reagents include antibodies (e.g., anti-CTLA-4, anti-PD-1/PD-L1), chemotherapy, and molecular targeted drugs.

In this article, we will focus on the combined use of molecular targeted drugs with immunotherapy, that could possibly reverse immunosuppression and augment anti-tumor T cell responses.

MECHANISMS OF IMMUNOSUPPRESSION IN CANCER PATIENTS

Cancer cells, more specifically oncogene activation and subsequent signal activation in cancer cells, trigger multiple immunosuppressive cascades. These immunosuppressive cascades involve various immunosuppressive molecules such as TGF-β, IL-10, IL-6, VEGF, PD-L1, COX2, and IDO/TDO as well as immunosuppressive cells such as tolerogenic DCs, myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs). Ultimately, cancer cells generate immunosuppressive microenvironments in tumor and sentinel lymph nodes (Yaguchi et al., 2011). For example, an over production of TGF-β in tumor microenvironment resulted in accumulation of MDSCs, M2 macrophages and Tregs, and impairment of DC functions in tumor tissues and sentinel lymph nodes. We have shown that TGF-β-induced-Snail not only induces metastasis-causing epithelial-to-mesenchymal transition (EMT) of cancer cells but also enhances production of immunosuppressive cytokines and chemokines, including TGF-β, IL-10, CCL2, and TSP-1 (Kudo-Saito et al., 2009), which further promotes metastasis. These cytokines impair DC function, induce Tregs, and finally inhibit induction of anti-tumor T cells. CCL2 produced by cancer cells recruits MDSCs into tumor and CCL22 produced by M2 macrophages recruits $CCR4^+$ Tregs and Th2 cells into tumor and sentinel lymph nodes (Kudo-Saito et al., 2009, 2013; Tsujikawa et al., 2013). Therefore, TGF-β production in tumor microenvironment by either cancer cells or infiltrated immune cells triggers multiple immunosuppressive cascades involving various immunosuppressive cytokines, chemokines, and immune cells. It has been reported that inhibition of TGF-β signaling by injection of plasmid DNA containing TGF-β type II receptor cDNA near the tumor sites enhanced tumor antigen-specific T cells accompanied by decrease of Tregs through blockade of TGF-β signaling (Fujita et al., 2009). Therefore, blockade of the TGF-β dependent immunosuppressive cascade at either upstream signaling for TGF-β production, TGF-β itself, or its downstream events such as Treg induction may restore immunocompetence of cancer patients.

SIGNAL INHIBITORS MAY AUGMENT ANTI-TUMOR IMMUNE RESPONSES

To effectively reverse immunosuppressive condition in cancer patients, which molecules or cells should be targeted in the immunosuppressive cascades? Where should they be blocked, upstream, or downstream? Blockade of downstream immunosuppressive molecules, such as CTLA-4 and PD-1/PD-L1, was recently shown to be effective in augmenting anti-tumor immune responses in clinical trials (Hodi et al., 2010; Topalian et al., 2012). Targeting downstream immunosuppressive molecules (e.g., TGF-β, IL-10, IL-6, VEGF, CTLA-4, PD-1, PD-L1, IDO/TDO, Cox2) and cells (e.g., MDSCs and Treg) with antibodies or small molecule inhibitors may have specific and efficient inhibitory activity against immunosuppressive cascades. However, inhibition of one molecule or one cell type may not be sufficient to reverse caner immunosuppression in patients.

In order to reverse immunosuppression in tumor-bearing hosts, we have evaluated signal inhibition at upstream molecules, such as BRAF-MAPK, STAT3, and Wnt/β-catenin (Sumimoto et al., 2006; Iwata-Kajihara et al., 2011; Yaguchi et al., 2012) (**Figure 2**). Targeting a constitutively activated signaling in cancer cells will not only inhibit multiple downstream immunosuppressive events simultaneously but also suppress multiple intrinsic malignant features of cancer cells, such as proliferation, survival, and invasion. The destruction of cancer cells may result in release of various endogenous tumor antigens and contribute to induction of anti-tumor immune response, and subsequent decrease of tumor burden decreases total immunosuppressive activity. In developing molecular targeted therapy, the idea of personalized treatment strategy is crucial. This is because the contribution of target signaling molecules in immunosuppression may be different even among patients with same type of cancer. Another factor to consider is that signal inhibitors sometimes have direct effects on immune cells, including activation of immune cells (e.g., DC) and inhibition of various immunosuppressive cells (e.g., Treg, MDSC) (Iwata-Kajihara et al., 2011; Oosterhoff et al., 2012). A combination of both upstream and downstream blockade is also an attractive strategy. For instance, administration of signal inhibitors (e.g., BRAF inhibitor) and blockade of antibodies against major immuosuppressive molecules (e.g., TGF-β, PD-1/PD-L1, CTLA-4) may be effective. However, it should be noted that such upstream blockade may affect various normal cells and cause adverse effects, including suppression of anti-tumor immune response. Therefore, a careful evaluation of total *in vivo* activity of these signal inhibitors is needed in both animal tumor models and clinical trials.

MAPK SIGNALING INHIBITORS

A common mutation of BRAF (V600E), a molecule in MAPK signal pathway, was identified by systematic DNA sequencing of

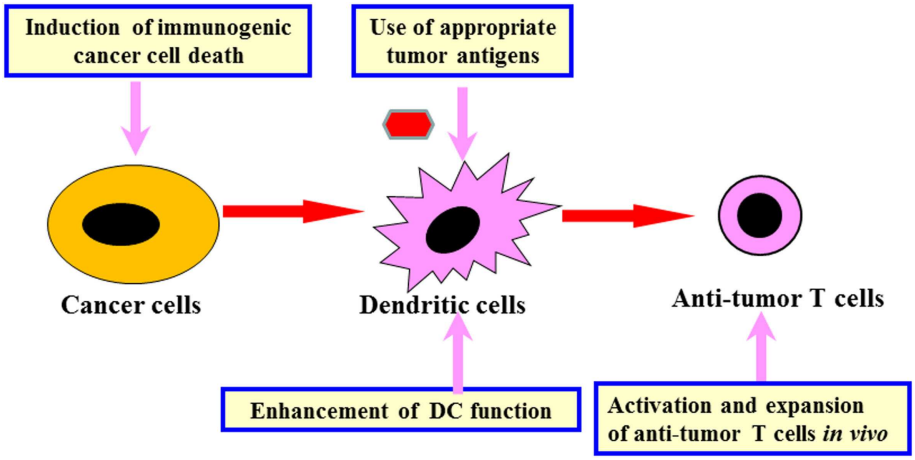

FIGURE 1 | Development of effective immunotherapy by comprehensive regulation of anti-tumor immune network. Comprehensive regulation of anti-tumor immune network, including induction of immunogenic cancer cell death, use of appropriate tumor antigens, enhancement of DC function, activation and expansion of anti-tumor T cells *as well as* reversal of cancer-induced immunosuppression (**Figure 2**) is important for development of effective cancer immunotherapy.

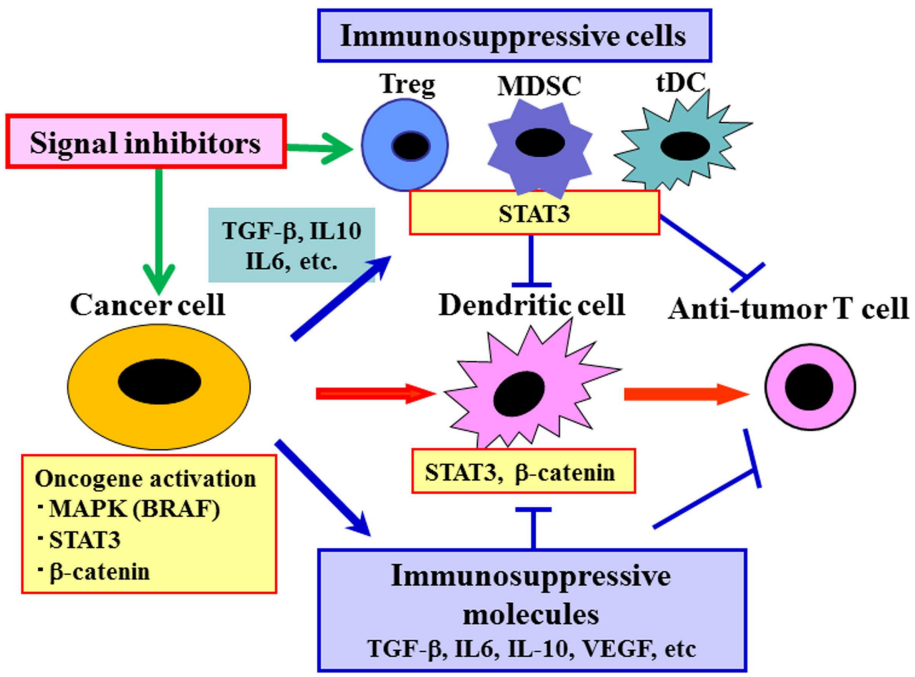

FIGURE 2 | Reversal of cancer-induced immunosuppression by targeting both cancer cells and immune cells using molecular targeted drugs. Cancer cells not only trigger anti-tumor immune responses but also induce various immunosuppressive molecules and cells through oncogene and signaling activation, leading to impaired anti-tumor immune responses. Molecular targeted drugs including various signal inhibitors may be useful for augmentation of anti-tumor immune responses by acting on both cancer cells and various immune cells such as DC, MDSC, and Treg.

signaling molecules in human melanoma cells (Davies et al., 2002). We have evaluated the role of mutant BRAF (V600E) in human melanoma cells by using mutant BRAF (V600E)-specific lentiviral shRNAs, and found that BRAF mutation was involved in enhanced cell proliferation and invasion (Sumimoto et al., 2004, 2005). We also found that inhibition of MAPK signaling pathway in human melanoma cells by genetic depletion of mutant BRAF or specific inhibitors reduced production of multiple immunosuppressive cytokines such as IL-6, IL-10, and VEGF, in most cases without affecting cell viability (Sumimoto et al., 2006). These cytokines

suppress DCs' ability to stimulate T cells through decreased production of IL-12 and TNF-α and increased production of IL-10 by DCs. Treatment of melanoma cells with BRAF (V600E)-specific shRNA or MEK inhibitors resulted in decreased immunosuppressive activity of melanoma cells on DCs, suggesting that MAPK signaling pathway in cancer is associated with impaired DC function in melanoma patients. MEK inhibitors were reported to increase susceptibility of melanoma cells to CTL lysis partly due to increased expression of melanosomal antigens such as MART-1/melan-A and gp100 (Kono et al., 2006; Boni et al., 2010). These results indicate that the BRAF-MAPK axis is important not only in classical malignant features such as cancer cell proliferation and invasion, but also in immunosuppression and immunoresistance. "Avoiding immune destruction" has recently been recognized as one of the "the hallmarks of cancer" (Hanahan and Weinberg, 2011).

The BRAF-MAPK axis may be a common attractive target for melanoma treatment, including immunotherapy. However, MAPK signaling pathway is also important for normal cell functions, such as T cell proliferation. Thus, administration of MAPK inhibitors may also suppress desirable anti-tumor T cell responses. Recently, two BRAF inhibitors that preferentially inhibit mutant BRAF in cancer cells have been developed, and their administration resulted in regression of melanoma in clinical trials (Chapman et al., 2011). These mutant BRAF-selective inhibitors can be particularly useful in combination with immunotherapies for melanoma. Melanoma cell death induced by BRAF inhibitors may lead to release of multiple endogenous tumor antigens including mutated antigens unique to each patient (Melanoma is known to have more frequent mutations than other cancers probably due to UV irradiation). This results in subsequent induction of autologous tumor-specific T cells. Decreased production of multiple immunosuppressive cytokines along with decreased number of melanoma cells may result in simultaneous inhibition of multiple immunosuppressive cascades, and reduce total immunosuppressive activity of melanoma without suppressing anti-tumor T cell expansion. Increased expression of melanoma antigens leads to enhanced susceptibility of cancer cells to CTL lysis (Kono et al., 2006; Boni et al., 2010). Suppression of melanoma cell proliferation and invasion may also enhance total anti-tumor activity of mutant BRAF inhibitors. In fact, it has recently been reported that administration of the mutant BRAF inhibitors alone resulted in the increased infiltration of granzyme positive $CD8^+$ T cells in tumors without inhibiting general immune responses, which was correlated with tumor reduction and necrosis (Wilmott et al., 2011; Hong et al., 2012). In a recent study, mutant BRAF-selective inhibitor and anti-CTLA-4 mAb were used in combination to treat transgenic mice with mutant BRAF and PTEN deletion that spontaneously developed melanoma. Despite their expectation, the combined therapy did not show enhanced anti-tumor effects compared with the treatment with either inhibitor or antibody alone. However, in B16 melanoma model using non-transgenic mice, the anti-CTLA-4 mAb augmented the effects of cancer vaccine (Hooijkaas et al., 2012). Further analysis revealed that BRAF inhibitor did not cause cell death in melanoma of transgenic mouse model, suggesting that *in situ* destruction of cancer cells is an essential step in the enhancement of anti-tumor T cell responses. The mutant BRAF inhibitors may also be useful for treating other cancers that are BRAF mutation positive, such as colon cancer, lung cancer, and thyroid cancer. Although MEK inhibitor is known to suppress proliferation of melanoma with either NRAS or BRAF mutation, it remains to be evaluated whether the inhibitor also has immunological effects, such as stimulating or suppressing activity on anti-tumor T cells (Flaherty et al., 2012).

JAK/STAT3 SIGNALING INHIBITORS

STAT3 is frequently activated in various human cancers including melanoma. Similar to the RAS/BRAF/MAPK signaling activation, down-regulation of STAT3 by lentiviral shRNA in STAT3-activated melanoma resulted in inhibition of multiple immunosuppressive cytokines, including IL-6, IL-10, and VEGF, indicating that STAT3 inhibitors may also be useful for immunotherapy (Sumimoto et al., 2006). These suppressive cytokines subsequently activate STAT3 in various immune cells including DCs, MDSCs, and Tregs, and render them immunosuppressive. For example, these cytokines generated low IL-12- and high IL-10-producing human DCs with reduced T cell stimulatory activity. DCs obtained from myeloid-specific STAT3-conditional knockout mice were found to be affected less by cancer-derived immunosuppressive factors (Iwata-Kajihara et al., 2011). In addition, these STAT3-depleted DCs produced high and sustained level of IL-12 possibly due to the involvement of STAT3 in a negative feedback mechanism of DC activation via IL-10. These STAT3-depleted DCs have higher T cell stimulatory activity than wild type DCs. When STAT3-depleted DCs were injected into immunosuppressive tumor microenvironment, stronger anti-tumor effects than wild type DCs were observed along with induction of stronger IFN-γ producing Th1 and CTL (Iwata-Kajihara et al., 2011). It has been reported that STAT3 is also involved in expansion of MDSCs (Wu et al., 2011), activation of $CD14^+HLA\text{-}DR^{negative/low}$ MDSCs in blood of cancer patients (Poschke et al., 2010), expression of immunosuppressive arginase-1 in human MDSCs (Vasquez-Dunddel et al., 2013), survival of Tregs (Pallandre et al., 2007), and anti-tumor activity of $CD8^+$ T cells (Kujawski et al., 2010). These reports suggest that constitutive activation of STAT3 in cancer cells triggers induction of various immunosuppressive immune cells. STAT3 inhibitors are currently being evaluated in clinical trials such as NCT00955812. In murine tumor model, STAT3 inhibitors have been shown to augment anti-tumor immunity (Kortylewski et al., 2005; Yu et al., 2007; Lee et al., 2011). It was recently reported that STAT3 inhibitors also restored drug sensitivity of melanoma cells which had acquired resistance to BRAF inhibitors (Liu et al., 2013). Therefore, STAT3 inhibitors may be useful for reversal of cancer-induced immunosuppression through acting on both cancer cells and various immune cells.

Besides direct inhibition of STAT3, inhibitors of the molecules regulating STAT3 activation may also be effective for the reversal of cancer-induced immunosuppression. An inhibitor of JAKs, upstream molecules of STAT3, was reported to augment anti-tumor effects in combination with immunotherapies such as IL-12 administration (Burdelya et al., 2002). In patients with renal cell cancer (RCC), administration of a multikinase inhibitor Sunitinib capable of suppressing downstream STAT3 signaling resulted in decrease of MDSCs and Tregs along with increase of IFN-γ

producing T cells (Ko et al., 2009; Ozao-Choy et al., 2009; Xin et al., 2009). Another multikinase inhibitor Dasatinib, which also inhibit downstream STAT3, increased response rate of the patients with $Ph1^+$ leukemia (CML and ALL) accompanied by LGL lymphocytosis and autoimmune like syndrome such as pleuritis and colitis (Mustjoki et al., 2009; Jalkanen et al., 2010), suggesting that Dasatinib has immunostimulatory activity partly through STAT3 inhibition. Therefore, various ways of STAT3 signal inhibition may be applicable in combination with various immunotherapies.

β-CATENIN-SIGNALING INHIBITORS

In some human cancers including colon cancer, liver cancer, and melanoma, activation of β-catenin pathway (suggested by nuclear staining of β-catenin) is observed. We found that β-catenin directly promote transcription of immunosuppressive cytokine IL-10 in human melanoma (Yaguchi et al., 2012), and protein expression of β-catenin was correlated with expression of IL-10 when evaluated by immunohistochemical analysis of melanoma tissues samples. Culture supernatant of human melanoma cells with accumulated β-catenin-induced high IL-10- and low IL-12-producing DCs in an IL-10 dependent manner. These DCs possessed low T cell stimulatory activity *in vitro*, and induced $FOXP3^+$ immunosuppressive Treg cells. The melanoma derived factors also inhibited the effector function of melanoma-specific CTLs in a β-catenin-dependent, but interestingly IL-10-independent manner, indicating that other immunosuppressive molecules are also involved in the β-catenin-induced immunosuppression. Melanoma cells pretreated with β-catenin-specific shRNA had reduced immunosuppressive activities on both DC and T cells.

When β-catenin-activated human melanoma cell lines were implanted in immunodeficient mice, human IL-10 in mouse serum was increased, and function of mouse DCs in spleens and tumors were impaired for T cell stimulatory activity probably due to increased human IL-10 which is capable of affecting mouse DCs (Yaguchi et al., 2012). Systemic administration of a β-catenin inhibitor restored T cell stimulatory function of the mouse splenic DCs along with decrease of human IL-10 in serum. β-catenin was also reported to be involved in generation of regulatory DC (Fu and Jiang, 2010; Manicassamy et al., 2010a) and survival of Treg (Ding et al., 2008). In addition, β-catenin inhibitor had a direct ability on DC to augment their T cell stimulatory activity partly due to decreased IL-10 production by DC (Manicassamy et al., 2010b). Therefore, β-catenin inhibitors may also be useful for reversal of cancer-induced immunosuppression by acting on both cancer and immune cells.

CONCLUDING REMARKS

As discussed in this article, altered activation of various oncogenes and signaling in both cancer cells and immune cells can be an attractive target to reverse immunosuppressive conditions in tumor-associated microenvironments of cancer patients. Signal inhibitors may augment current cancer immunotherapy, in addition to its possible direct anti-tumor effects through inhibition of cancer cell proliferation and invasion. However, its total *in vivo* activity should be carefully evaluated because it may also cause various adverse effects, including possible inhibition of anti-tumor immune responses. In this regard, mutated-molecule-specific inhibition such as that of the mutant BRAF-selective inhibitors is one of the promising strategies. Activation of STAT3 appears to shift immune response toward cancer's advantage, thus, its inhibition is attractive for possible improvement of anti-tumor immune responses. Altogether, combination therapy using molecular targeted drugs and various immunotherapies such as cancer vaccines and check point blockade is a promising strategy to treat cancer patients. Future clinical trials may demonstrate the proof of concept of this strategy.

However, there are several obstacles to overcome before the benefits of combination therapy can reach the patients. One such obstacle is scientific. Although quite a few signal inhibitors, immunotherapies, and combined therapies have shown promising results in experimental settings, mouse model, and human are different. A successful treatment in mouse models may not work in patients. Therefore, for the selection of appropriate molecular targets and inhibition methods, further understanding of human cancer immunopathology is deeply essential and urgently desired. Another obstacle is a pragmatic one, which may arise when individual therapies in a combination therapy are developed and/or owned by different companies. The issues of company regulations, patents, and logistics could become a barrier between research and clinical translation. The core idea of combination therapy is that by using multiple already-available therapies, cancer patients are able to gain greater-than-sum benefits. Therefore, it is crucial that institutions and companies to look beyond self-interests and work together to reach a common goal. Academic institution may mediate the cooperation between companies and provided combination therapies to patients.

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Expanding roles for CD4 T cells and their subpopulations in tumor immunity and therapy

Mark J. Dobrzanski *†

Department of Internal Medicine, Texas Tech University Health Sciences Center School of Medicine, Amarillo, TX, USA

Edited by:
Chao Ma, California Institute of Technology, USA

Reviewed by:
Chris Schmidt, Queensland Institute of Medical Research, Australia
Amedeo Amedei, University of Florence, Italy

****Correspondence:***
Mark J. Dobrzanski, Department of Internal Medicine, Texas Tech University Health Sciences Center School of Medicine, 1400 Wallace Blvd, Amarillo, TX 79106, USA.
e-mail: mark.dobrzanski@ttuhsc.edu

†Present address:
Mark J. Dobrzanski, Department of Internal Medicine, School of Medicine, Texas Tech University, 1400 Wallace Blvd, Room 214, Amarillo, TX 79106, USA.

The importance of CD4 T cells in orchestrating the immune system and their role in inducing effective T cell-mediated therapies for the treatment of patients with select established malignancies are undisputable. Through a complex and balanced array of direct and indirect mechanisms of cellular activation and regulation, this functionally diverse family of lymphocytes can potentially promote tumor eradication, long-term tumor immunity, and aid in establishing and/or rebalancing immune cell homeostasis through interaction with other immune cell populations within the highly dynamic tumor environment. However, recent studies have uncovered additional functions and roles for CD4 T cells, some of which are independent of other lymphocytes, that can not only influence and contribute to tumor immunity but paradoxically promote tumor growth and progression. Here, we review the recent advances in our understanding of the various CD4 T cell lineages and their signature cytokines in disease progression and/or regression. We discuss their direct and indirect mechanistic interplay among themselves and with other responding cells of the antitumor response, their potential roles and abilities for "plasticity" and memory cell generation within the hostile tumor environment, and their potentials in cancer treatment and immunotherapy.

Keywords: CD4 helper cells, memory T cells, T regulatory cells, immunotherapy, cytolytic CD4 T cells, cytokines, tumor immunity

INTRODUCTION

Cancer cells express antigens that differentiate them from their non-transformed counterparts (Van Der Bruggen et al., 2002). These antigens are often products of mutated cellular genes, aberrantly expressed normal genes, or genes encoding viral proteins. Categories and examples of human tumor antigens thus far identified include (a) the differentiation antigens encoded by genes that are only expressed in particular types of tissue such as those antigens expressed in melanocytes and melanoma and involved in melanin production, (b) mutational antigens that arise as a consequence of gene rearrangement and point mutations (i.e., p53), (c) cellular antigens that are over expressed in transformed cells when compared with their normal counterparts (i.e., HER-2), (d) molecules that display abnormal post-translational modifications (i.e., MUC1), (e) viral antigens derived from viral oncogenes (i.e., human papillomavirus proteins), and (f) cancer/testis (CT) antigens that are expressed in germ cells of testis and ovary but silent in normal somatic cells (i.e., MAGE and NY-ESO-1) (Cheever et al., 2009). It has been shown that tumors co-expressing such antigens can be recognized by effector T cells of the adaptive immune system and induce antitumor immune responses in both experimental animal and human systems (Dougan and Dranoff, 2009).

One of the earliest processes involved in the development of the adaptive immune response and tumor immunity is inflammation which functions to localize and eradicate tissue stressors induced by tumor growth and re-establish normal tissue homeostasis (Medzhitov, 2008). However, evidence obtained from various murine tumor models and clinical studies in cancer patients, have shown that chronic inflammation, mediated by ensuing adaptive immune responses, can contribute to tumorigenesis at all stages. For example, such responses may contribute to cancer initiation by generating genotoxic stress, to cancer promotion by inducing cellular proliferation, and to cancer progression by enhancing angiogenesis and tissue invasion (Grivennikov et al., 2010). In either instance, this forms the conceptual framework of the cancer immunoediting hypothesis, which stresses the dual host-protective and tumor-promoting actions of immunity on developing tumors (Schreiber et al., 2011; Vesely et al., 2011). In its most simplistic form, this model proposes that tumors develop through three successive and distinct phases termed "elimination," "equilibration," and "escape." The elimination phase entails the process where the innate and adoptive immune systems collaboratively detect the presence of a developing tumor and destroy it before it becomes clinically apparent. The next phase of equilibration entails the process where rare tumor cell variants survive immune-mediated elimination and enter a state of equilibrium with the adaptive immune response. In this state, the immune system maintains residual tumor cells in a functional state of dormancy, a term used to describe latent tumor cells that may reside in patients for decades before eventually resuming growth as either recurrent primary tumors or distant metastases (Aguirre-Ghiso, 2007). Aside from preventing tumor outgrowth, it is also believed that the immune response in this phase "fashion" the immunogenicity of the occult

tumors. Lastly, in the escape phase, tumor cells that have acquired the ability to circumvent immune recognition and/or destruction emerge as progressively growing and detectable tumors. It is further postulated that the course and progression through such phases are influenced and determined, in part, by tumor cell population changes in response to the immune system, host immune system changes in response to mechanisms re-establishing cellular homeostasis, or increased cancer-mediated immunosuppression and/or immune system decline. Such local and systemic environmental stressors are thought to be the major contributors to affect not only tumor outgrowth but also immunotherapeutic interventions and their efficacy in cancer patients. Thus, the tumor-promoting inflammation and protective tumor immunity processes appear dynamically interconnected where an imbalance can further result in the shaping of tumor immunogenicity which may either initiate and/or facilitate disease progression or regression.

At the earliest stages of the antitumor immune response, professional antigen presenting cells (APCs), most notably dendritic cells (DCs), encounter and capture tumor antigens that are released from either viable or dying tumor cells. This results in the activation and up-regulation of co-stimulatory molecules that facilitate and/or promote the migration of these cells to secondary lymphoid organs such as regional draining lymph nodes (Steinman and Mellman, 2004; Steinman and Banchereau, 2007). To develop into potent CD4 effector T cells that contribute to the antitumor immune response, naïve CD4 T cells need to recognize peptide antigens presented in an immunogenic context with HLA class II molecules on activated DCs. Additional co-stimulatory signals, such as DC-derived cytokines, then promote their differentiation into effector CD4 Th cell subsets characterized by distinct cytokine secreting profiles (O'Shea and Paul, 2010; Zhu et al., 2010). The best characterized of these effector cell subsets are the Th1 and Th2 cells, which are characterized by their production of IFN-γ and interleukin-4 (IL-4), respectively. More recently, the pro-inflammatory Th9 and Th17 cell subsets have also been shown to develop and/or reside in some tumors, along with TReg cells which are responsible for immune regulation and tissue homeostasis. Following recognition of a specific tumor-associated antigen presented by an appropriately activated APC, naïve CD4 T cells undergo several rounds of division and can become polarized into such distinct effector Th cell subsets that can differentially orchestrate antitumor immune responses, in part, through their production of signature cytokines. The differentiation of polarized CD4 effector T cells is controlled by unique sets of transcription factors, the expression of which is determined by multiple signals, in particular, by soluble factors that act on responding CD4 T cells during their activation. Subsequently, such differentiation results in distinct Th cell subsets characterized, in part, by select cytokine production that can initiate, facilitate, and influence distinct mechanistic arms of the immune response to tumors as determined in several murine tumor models as well as in evidence obtained from human studies. Although the production, function, and mechanistic interplay of these signature cytokines derived from the distinct Th cell subsets will be discussed in more detail below, it is becoming increasingly clear that considerable plasticity exists among the various subsets *in vivo*, especially during responses to tumors at various stages of development, progression, and/or regression. Moreover, certain cytokines such as IL-10, can be produced by nearly all subpopulations of cells within the multiple effector cell subsets further suggesting, that CD4 T cell responses are apparently convoluted and capable of initiating and maintaining quantitatively and qualitatively variable antitumor responses involved in facilitating either direct or indirect tumor cell killing or survival.

The CD4+ T cell represents a major component of the adaptive immune response and has been shown to be an integral part in the activation and regulation processes of the host response to many pathogens. Although the role of CD4 T cells in the antitumor response remains under investigated, it is becoming clear that effective immune responses to a developing or progressing tumor requires their activation, maturation, and active participation (Pardoll and Topalian, 1998; Blattman and Greenberg, 2004; Kennedy and Celis, 2008; Muranski and Restifo, 2009). As one of their primary emerging roles as "regulators" of the immune response to cancer, CD4 T cells have been shown to orchestrate and coordinate many facets of both the innate and adaptive immune responses to ensure optimal responses by other lymphocytes. CD4 T cells are necessary elements for priming tumor-specific CD8 T cells, influencing the differentiation and expansion of tumor antigen-specific CTLs and are essential for generating and maintaining long-term CD8 memory T cell responses (Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003; Sun et al., 2004). Moreover, several studies have defined additional roles for CD4 T cells, some of which are independent of other lymphocytes, that influence and/or contribute to tumor immunity during carcinogenesis. Paradoxically, several experimental and clinical observations have recently shown that these same CD4 effector cell subsets and their signature cytokines can not only contribute to antitumor responses but also tumor-promoting activities. We discuss what is known about the T cell subsets that develop during different stages of tumor growth and progression, how such diverse T cell subset responses contribute to disease progression and/or regression, their development into memory cells, and their potentials in cancer treatment.

CD4 EFFECTOR T CELL SUBSETS

The roles of polarized CD4 T cell subsets in the antitumor immune response are greatly influenced by their signature cytokines which arm the cells with distinct immunological functions. Such cellular polarization processes are dependent, in part, on their expression of specific transcription factors that are influenced by multiple cellular and soluble biological signals within the priming milieu of the tumor environment. Moreover, substantial proportions of effector T cell subsets that are found *in vivo* are often characterized by plasticity and heterogeneity in terms of their cytokine-producing potentials. Thus effective tumor immunity is often dependent on such complex CD4 T cell responses following polarization and their interactions with other Th cell subsets within the hostile tumor environment. In any instance, the most characterized CD4 Th cell subset is the *Th1* that can potentially produce large amounts of IFN-γ upon tumor antigen encounter and expresses the transcription factor T-bet. The Th1 developmental pathway is typically driven by IL-12 activation of the signal transducer

and activator of transcription 4 (STAT 4) and T-bet transcription factors during immune activation of naïve T cells (Szabo et al., 2000, 2003). As the "critical regulator" of the Th1 differentiation program, T-bet is responsible for the up-regulation of the IL-12 receptor β2 (IL-12β2R) subunit and confers IL-12 responsiveness and sustained T-bet expression (Lazarevic and Glimcher, 2011). In addition, it induces and upregulates IFN-γ (ifnγ) production but also induces the expression of genes encoding the chemokine receptor CXCR3 and the chemokines CCL3 and CCL4 (Jenner et al., 2009) which are responsible for enhancing the mobilization of select type 1-related immune cell responses to sites of tumor growth. In addition, T-bet suppresses commitment to the Th2 and Th17 lineage programs (Hwang et al., 2005). Although IFN-γ is considered the signature cytokine for this subset in both murine and human effector T cells, other cytokines have been shown to be produced by human Th1 cells and include IL-2, TNF-α, and IL-10. Interestingly, the importance of IL-10 production by Th1 effector cell subpopulations in the antitumor response is controversial. Several recent studies have suggested that IL-10 plays a role in inhibiting tumor development, growth, and metastases (Mocellin et al., 2005; Emmerich et al., 2012; Tanikawa et al., 2012). Whereas others have suggested that Th1 effector cell responses are autoregulated through a negative feedback loop via the co-induction and expression of IL-10 (Cope et al., 2011). Conceivably, the relative amounts and/or duration of IFN-γ and IL-10 produced by such double-positive cytokine secreting Th1 cell subsets and their ability for "cytokine switching" might define the inflammatory/immune response, tolerance induction, and/or prevention of excessive immunopathology within the tumor microenvironment.

Th2 effector cell subsets are characterized by the production of IL-4, IL-5, and IL-13 and are responsible for coordinating humoral immunity and allergic inflammatory responses. IL-4 is primarily accountable for the differentiation of Th2 cells through STAT 6 and the transcription factor GATA-3 (Kaplan et al., 1996; Zheng and Flavell, 1997; Kurata et al., 1999; Zhu et al., 2001). The Th1 and Th2 developmental pathways among naïve CD4 T cells are controlled by a delicate balance of positive feedback loops, as IFN-γ enhances further Th1 development and IL-4 supports continued Th2 differentiation. At the same time, cross regulation by IFN-γ and IL-4 suppresses Th2 and Th1 differentiation, respectively. In a murine lung metastases model, Th2 effector cells have shown some indirect antitumor activity through the eosinophil chemotactic factor, eotaxin and eosinophil tumor infiltration (Mattes et al., 2003). However, the role of Th2 effector cells in the antitumor immune response remains unclear with several studies suggesting that such CD4 effector cells are associated with carcinogenesis and tumor progression (Tatsumi et al., 2002; Ochi et al., 2012). Recent investigations have shown that in addition to IL-10, which is essentially produced by all Th cell subsets, a subpopulation within the Th2 subset can preferentially co-produce IL-24 (a unique member of the IL-10 cytokine family) (Schaefer et al., 2001; Ouyang et al., 2011). Although its detailed regulation in Th2 cells is currently unclear, IL-24 has been shown to lack immune repressive functions, suppress human ovarian cancer cell growth both *in vitro* and *in vivo*, and induce substantial "bystander antitumor" immunity in patients (Fisher, 2005; Lebedeva et al., 2007; Emdad et al., 2009; Dash et al., 2010). Further investigation into understanding the development and properties of IL-24-secreting Th2 cells may provide profound therapeutic benefits for cancer patients.

The expression of IL-17 characterizes a subset of CD4 helper T cells (Th17). This cell lineage represents a third effector arm of CD4-mediated immune response and complements, in part, the functions of the Th1 and Th2 cell lineages. In addition to IL-17A and IL-17F, human Th17 cells also produce other cytokines such as IL-21, IL-22, and IL-26. In addition, the chemokine receptor CCR6 [which binds to the chemokine CC ligand 20 (CCL20) that is present in many malignant pleural effusions of lung cancer patients] is highly expressed on Th17 cells thus further facilitating their recruitment to sites of tumor growth and inflammation (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007, 2012). Both human and murine Th17 cells express the transcription factors retinoic acid orphan receptor (ROR)γt (*Rorc*). ROR-γt is a critical regulator of Th17 cell differentiation and induces IL-17A, IL-17F, IL-26, and CCR6 expression while downregulating IFN-γ production in human naïve T cells (Manel et al., 2008). For induction and differentiation of murine Th17 cells, TGF-β and IL-6 are the most crucial cytokines for naïve CD4 cell differentiation (Murugaiyan and Saha, 2009; Gaffen, 2011). However, in the development of human Th17 cells, it has been shown that IL-6 and IL-1β, but not TGF-β, is essential for differentiation (Acosta-Rodriguez et al., 2007; Wilson et al., 2007). In any instance, IL-21 produced by Th17 cells further amplify Th17 generation in an autocrine manner and induce IL-23 receptor expression that enables cell responses to IL-23 stimulation. Subsequently, DC-derived IL-23 stabilizes the Th17 phenotype and helps Th17 cells to acquire effector functions. The induction of ROR-γt is dependent on STAT-3, which is preferentially activated by IL-6, IL-21, and IL-23. STAT-3 affects ROR-γt expression and binds to the IL-17 and IL-21 promoters. Thus, both STAT-3 and ROR-γt transcription factors regulate IL-17 production in a highly coordinated manner (Murugaiyan and Saha, 2009). In addition to characterizing Th17 cells by transcription factors and cytokine production, recent studies have shown that human Th17 cells originate from a CD4 precursor cell population that is present in both thymus and umbilical cord blood and co-expresses a member of the NK cell receptor-P1 family, CD161 (Cosmi et al., 2008). Although the precise function of CD161 is unknown at this time, it is considered a "hallmark" of human memory Th17 cells at sites of tissue inflammation (Cosmi et al., 2008; Kleinschek et al., 2009). Another marker that was identified to be specifically associated with human Th17 cells is the IL-4-induced gene 1 (IL-4I1) which encodes the enzyme L-phenylalanine oxidase. This enzyme is responsible, in part, for H_2O_2 production that can contribute to the inhibition of T lymphocyte proliferation (Boulland et al., 2007). As will be discussed below, Th17 cells are found in several human tumors. Although Th17-associated cytokines have been linked with carcinogenesis and tumor progression within the context of chronic inflammation and infection, it is becoming clear that this cell lineage can also contribute to the antitumor response in human malignancies of epithelial origin (Kryczek et al., 2007, 2009a; Zou and Restifo, 2010; Wilke et al., 2011).

Th22 CD4 T cells, which are thought to be a distinct Th subset that produce IL-22 independently of IL-17, were initially identified

in patients with inflammatory disorders of the skin, intestine, and joints (Eyerich et al., 2009; Sonnenberg et al., 2011). More recently, such cells have also been identified and suggested to contribute to immune responses and inflammation in malignant pleural effusions and other human cancers (Zhang et al., 2008; Jiang et al., 2011; Miyagaki et al., 2011; Ye et al., 2012). Such Th22 cells express the cutaneous lymphocyte antigen (CLA), a functional E-selectin ligand that is involved in lymphocyte rolling on the endothelial cells of cutaneous postcapillary venules, along with the chemokine receptors CCR6, CCR4, and CCR10 which together facilitate the constitutive migration potentials of these cells to sites of inflammation (Duhen et al., 2009). Although the role, if any, of Th22 cells in the antitumor immune response is unclear, it is believed that such IL-22 producing CD4 Th cells contribute to local immune homeostasis and inflammation (Duhen et al., 2009).

The latest addition to the list of subsets, termed Th9, secretes IL-9 as the signature cytokine and may play a role in several inflammatory disorders. Naïve human and murine CD4 T cells both acquire a Th9 phenotype *in vitro* with the combination of IL-4 and TGF-β (Houssiau et al., 1995; Veldhoen et al., 2008; Beriou et al., 2010; Chang et al., 2010; Putheti et al., 2010; Yao et al., 2011; Jabeen and Kaplan, 2012; Wilhelm et al., 2012). The transcription factors STAT6, interferon regulatory factor 4 (IRF4), and PU.1 contribute to Th9 differentiation (Jabeen and Kaplan, 2012). IL-4-activated STAT6 promotes expression of IRF4 whereas PU.1 activation occurs downstream of TGF-β signaling where it directly bind to the IL-9 (*il9*) promoter (Chang et al., 2005, 2009, 2010). Interestingly, polarized Th2 cells can be deviated into Th9 cells by exposure to TGF-β, which results in down-regulation of GATA-3 and loss of IL-4 and IL-5 production. Using various gene knockout and transgenic mouse systems, Th9 cells have been shown to contribute to autoimmune and allergic inflammation processes (Noelle and Nowak, 2009). However, one recent study identified Th9 cells in healthy human blood and skin and also in metastatic lesions of patients with stage IV melanoma (Purwar et al., 2012). Moreover, using a murine tumor model, these same investigators showed that adoptive transfer of tumor-reactive Th9 cells and administration of recombinant IL-9 can effectively reduce melanoma growth. This evidence from a single study suggests that IL-9 producing Th9 cells may play a role in the generation of effective antitumor responses.

The final CD4 Th cell lineage to be described here resides in proximity to B cells within germinal centers of lymphoid tissues. These cells, referred to as follicular helper T cells (T_{FH}), are responsible for providing B cell help and supporting B cell expansion and differentiation (Reinhardt et al., 2009; Ma et al., 2012). They are defined by expression of the transcription factor Bcl6 and the cytokines IL-21 (Liu et al., 2012b). Moreover, they express the chemokine receptor CXCR5 which facilitates migration to the B cell follicles after activation (Chtanova et al., 2004; Haynes et al., 2007). Studies have also shown that they possess key surface receptor molecules (PD-1, CD40 ligand, OX-40, CD84, and ICOS) that play critical roles in promoting B cell activation, differentiation, and survival (Ma et al., 2012). However, the role of T_{FH} cells in tumor immunity remains relatively undefined.

Accumulating evidence suggests that select CD4 effector T cell subsets may have a more "direct" role in inhibiting tumor growth and progression that are independent of their more "indirect" helper activities. As such, CD4 effector T cells have, in general, been shown to protect against both tumors and virally infected target cells through two distinct primary effector mechanisms. They include the production of cytokines, most notably IFN-γ and TNF (Hung et al., 1998; Pardoll and Topalian, 1998) and through direct cytolytic activity (Trapani and Smyth, 2002) that is mediated by degranulation of cytotoxic granules containing toxic effector molecules (i.e., perforin and granzyme) or ligation of the Fas (also known as CD95)/Fas Ligand (FasL; also known as CD95L) apoptotic killing pathway (Green and Ferguson, 2001). *Cytolytic CD4* T cells possessing such cytotoxic activity have been described in peripheral blood of both healthy and virally infected individuals (Feighery and Stastny, 1979; Appay et al., 2002; van Leeuwen et al., 2004; Casazza et al., 2006; Stuller and Flano, 2009; Nemes et al., 2010; Stuller et al., 2010). Phenotypic analysis has shown that they are $CD45RO^+$, $CCR7^-$, $CD27^-$, and $CD28^-$ and shown to possess high levels of the cytolytic effector molecules granzyme A, granzyme B and perforin (Appay et al., 2002; Casazza et al., 2006) suggesting that these cells are antigen-experienced and terminally differentiated CD4 effector cells. Moreover, as with CD8 effector T cells (Pearce et al., 2003; Intlekofer et al., 2005), there is evidence that expression of the eomesodermin (*Eomes*) transcription factor may be crucial in driving the development of cytotoxic CD4 T cells *in vivo* (Qui et al., 2011; Hirschhorn-Cymerman et al., 2012). Although T cell expression of eomesodermin has been linked to terminal differentiation and memory cell phenotype with the concomitant secretion of Th1 cytokines (Hirschhorn-Cymerman et al., 2012), others have suggested that cytotoxic activity of CD4 effector T cells does not depend on Th1 cell polarization (Brown et al., 2009). Thus suggesting that such cells constitute a unique and separate cell lineage from those already described. In either instance, more recent studies using a murine transgenic tumor model of advanced melanoma, showed that transfer of naïve tumor-reactive CD4 T cells into lymphopenic recipients can induce a substantial expansion and differentiation of Th1 cells with cytotoxic activity (Quezada et al., 2010; Xie et al., 2010). Moreover, induction of such cells correlated with class II-restricted tumor rejection that was dependent on the presence of IFN-γ which was believed to mediate the up-regulation of class II on tumor target cells. In more recent studies using both human and murine cells, generation of tumor-reactive cytotoxic CD4 Th1 cells were further shown to be induced, in part, by both the engagement of a specific co-stimulatory pathway of the tumor necrosis factor receptor (TNFR) family member, OX-40 (also known as CD134) and an intracellular mechanism relying on eomesodermin expression (Qui et al., 2011; Hirschhorn-Cymerman et al., 2012). Further identification and characterization of the mechanisms involved in the induction of tumor-reactive CD4 T cells with cytotoxic activities in cancer patients may offer significant advantages for future treatment strategies of human malignancies.

Lastly, the subpopulations of $CD4^+$ TReg cells can be classified into two main subsets according to their origin and suppressive activity. Natural $CD4^+$ TReg effector cells (nTRegs), constitutively expressing FoxP3 and the activation marker CD25, originate

in the thymus by high affinity interaction of the T cell receptor (TCR) with Ag expressed on the thymic stroma (Sakaguchi, 2008; Shevach, 2009; Buckner, 2010; Nishikawa and Sakaguchi, 2010; Sakaguchi et al., 2010; Miyara and Sakaguchi, 2011). Such cells suppress the proliferation of effector T cells in a contact-dependent, cytokine-independent manner. In contrast, other types of TReg cells can be induced from naive CD4 cells in the periphery, such as IL-10-producing TR1 cells and TGF-β-producing Th3 cells (Groux et al., 1997; O'Garra et al., 2004; Grazia-Roncarolo et al., 2006; Nishikawa and Sakaguchi, 2010). Such "induced" $CD4^+CD25^-$ TReg subpopulations (iTReg) exert suppression mostly through soluble factors and their suppressive function is not strictly associated with a high level of FoxP3 expression. Moreover, human TReg cell subpopulations have also been further divided into two subsets based on their expression of the "resting" CD45RA (a marker of naïve or antigen-inexperienced cells) or "activated" CD45RO (a marker for memory or antigen-experienced T cells) cell surface markers (Vukmanovic-Stejic et al., 2006; Miyara et al., 2009; Miyara and Sakaguchi, 2011; Duhen et al., 2012) further suggesting different levels of activation and/or differentiation among these CD4 subsets. More recently, another inducible subpopulation of the $CD4^+$ TReg cell subset have been reported in both human and murine systems that involve production of IL-35 and are thus referred to as iTreg35 cells (Collison et al., 2010; Chaturvedi et al., 2011). Notably, these cells are phenotypically and functionally distinct from other subpopulations of TReg cells described thus far in that they do not express FoxP3 and they mediate immunosuppression via IL-35 and seemingly independent of IL-10, TGF-β, the immunomodulatory receptor CTLA-4, or any other currently known TReg cell-associated suppressive molecule. Although it seems that human nTReg cells do not express IL-35 (Bardel et al., 2008), naïve human CD4 T cells can be induced to develop into iTReg35 cells in the presence of IL-35 or activated DCs (Collison et al., 2010; Seyerl et al., 2010). Alternatively, it has been suggested that human TReg subpopulations can be further classified by their expression of select chemokine receptors that correspond to Th cell lineage-specific immune responses (Duhen et al., 2012). For example, TReg subpopulations co-expressing CCR6 (Th17-associated responses), CXCR3 (Th1-associated responses), CCR4 (Th2-associated responses), and CCR10 (Th22-associated responses) enable human TReg cell subpopulations with unique specificities and immunomodulatory functions to target defined immune environments during different types of inflammatory responses so as to exert an "appropriate" regulatory process. Thus, suggesting that Th and TReg cells undergo functional specialization in parallel, resulting in the development of TReg cell subpopulations capable of co-localizing and effectively regulating different types of Th cell responses *in vivo* (Hall et al., 2011; Duhen et al., 2012). In any instance, the precise mechanisms by which these various subpopulations of TReg cells function to maintain the balance between protective tumor immunity and establishing or rebalancing immune cell homeostasis remains poorly understood. However, several mechanisms responsible for preventing inflammatory disease by restraining aberrant responses to self or innocuous antigens have been identified (Vignali et al., 2008; Shevach, 2009; Qureshi et al., 2011; Yamaguchi et al., 2011; Vignali, 2012; Wing and Sakaguchi, 2012). These include both cell contact and soluble factor-dependent mechanisms, such as production of IL-10; the production and surface expression of TGF-β; the production of IL-35; the release of cytolytic molecules such as granzyme and perforin; the consumption of IL-2 through the high density expression of cell surface CD25 (the alpha chain of the IL-2 receptor) which weans effector T cells from IL-2; the degradation of ATP through ectonucleotidases; and expression of the inhibitory receptors CTLA-4, which outcompetes receptor CD28 on effector T cells for access to the co-stimulatory molecules CD80 and CD86 on APCs.

CD4 MEMORY T CELL DEVELOPMENT AND THEIR ROLE IN THE ANTITUMOR RESPONSE

During the antitumor response a small population of tumor-specific CD4 effector T cells may develop into memory T cells that retain their previous effector functions and rapidly produce effector cytokines (McKinstry et al., 2010; Taylor and Jenkins, 2011; Strutt et al., 2012). Their ability to remember previously encountered antigens leads to faster responses to tumor antigen re-exposure and thus may play a role in preventing disease relapse in cancer patients. Alternatively, as discussed earlier, it can shape tumor cell immunogenicity and modulate immune response dynamics to influence disease progression (Schreiber et al., 2011; Vesely et al., 2011). In humans, different isoforms of the CD45 molecule are often used to differentiate naïve and memory cells with the former expressing CD45RA and the latter expressing CD45RO (Ahmed and Gray, 1996). Increased expression of other surface molecules such as the CD95 death receptor has also been shown to differentiate memory cells from naïve CD4 T cells. Moreover, memory T cells have been divided into two general subgroups based on their patterns of migration (Sallusto et al., 1999). Central memory T cells (T_{CM}) express the CC-chemokine receptor CCR7 and L-selectin CD62L following activation. Expression of these receptors enable the T_{CM} subgroup to recirculate through secondary lymphoid organs such as lymph nodes. Such circulation is beneficial since DCs from diverse tissue sites continuously bring antigen to the draining lymph nodes, thereby increasing the effective area of memory cell immunosurveillance for progressing tumor growth due to metastases and/or occult cell outgrowth. Alternatively, effector memory T cells (T_{EM}) lack expression of CCR7 or CD62L and thus have a propensity to migrate to peripheral tissues in response to localized inflammatory stimuli and bolster the process of immune surveillance at such sites. Moreover, expression of other surface chemokine receptors such as CCR5 have been associated with polarized Th1 memory T cell subsets (Loetscher et al., 1998; Sallusto et al., 1998; Kim et al., 2001; Luther and Cyster, 2001; Charo and Ransohoff, 2006). In either instance, retention and tissue tropism of memory CD4 T cell subsets and their diverse functional capacities are dependent, in part, on specific interactions between adhesion molecules and effector cell chemokine receptors that induce T cell subset localization and influence tumor environments. This has been shown in studies with cancer patients where increased intratumoral memory T cell levels were associated with longer disease free and overall survival rates (Pagès et al., 2005; Bindea et al., 2010). In another study involving colon cancer patients, histopathological analysis showed the presence of "patches" of T_{EM} cells located within either

the center or invasive margins of the tumor that further correlated with good clinical outcome (Galon et al., 2006). These investigators suggested that memory cell localization at select regions within the tumor mass may be associated with not only enhanced antitumor immune responses but also effective control of metastatic escape. However, as briefly mentioned earlier, such observations and effective antitumor responses may not only depend on memory cell phenotype and localization, but also their functional memory precursor phenotype and ability for "cellular plasticity" within the hostile tumor environment.

In addition to surface markers, differential expression levels of select transcription factors have been associated with promoting effector and memory T cell development. In studies using various genetically modified mouse strains, investigators have shown that under conditions of inflammation, elevated levels of the transcription factor T-bet (*Tbx21*) among responding CD8 T cells promoted the generation of terminally differentiated short-lived effector cells whereas lower levels facilitated long-lived, self renewing memory T cell development (Joshi et al., 2005; Lazarevic and Glimcher, 2011). In more recent studies, it was shown that IL-12 augmented activity of the kinase mammalian target of ramamycin (mTOR) (Rao et al., 2010; Cox et al., 2011) which is essential for sustained T-bet expression and the generation of effector CD8 T cells. Subsequently, inhibition of mTOR activity blocked T-bet expression and promoted elevated and sustained levels of the closely related T-bet transcription factor eomesodermin (*Eomes*) that is associated with the development of memory T cells (Pearce et al., 2003; Intlekofer et al., 2005, 2008; Joshi et al., 2005; Rao et al., 2010). Moreover, over-expression of eomesodermin or T-bet has been shown to be sufficient to induce expression of IFN-γ, perforin, and granzyme B in CD8 T cells (Pearce et al., 2003; Cruz-Guilloty et al., 2009). Thus suggesting that (i) the transcription factors T-bet and eomesodermin have cooperative and partially redundant functions in CD8 T cell differentiation and fate (Rao et al., 2010) and (ii) the balance between the two transcription factors, as "instructed" by mTOR kinase activity, can determine the CD8 effector cell fate verses memory cell fate (Araki et al., 2009; Rao et al., 2010). Although the transition of CD4 effector to memory T cell phenotype is less defined, evidence using a murine viral infection model has shown a similar correlation with decreased T-bet (Tbx21) expression levels and potential Th1 memory cell development (Marshall et al., 2011). Furthermore, in a mouse model of allergic airway inflammation, IL-5 production among a Th2 memory cell subpopulation was shown to be uniquely regulated by the expression of eomesodermin (*Eomes*) suggesting a role for this transcription factor in the regulation of polarized CD4 T cell functions (Endo et al., 2011). Whereas, in a study using peripheral blood from healthy human donors, expression of T-bet was shown to be up-regulated among specific Th1 memory cell subpopulations following TCR stimulation whereas elevated expression levels of eomesodermin (*Eomes*) were associated with a higher level of IFN-γ production during the recall response in a corresponding cell subpopulation (Narayanan et al., 2010). Lastly, similar results were observed in the murine system suggesting a role for Eomesodermin (*Eomes*) in the development of Th1 cell differentiation and memory phenotype under various stimulating conditions *ex vivo* (Suto et al., 2006; Yang et al., 2008; Hirschhorn-Cymerman et al., 2012). Collectively, it is unclear which responding CD4 effector T cells make the transition to a memory phenotype, but these recent studies suggest that differential expression levels and balance between transcription factors promote and/or facilitate the T cells potential to do so. In either instance, this "phenotypic progression" from effector to memory T cell provides a qualitative advantage in the antigen-specific antitumor response by enhancing immune response time, the need for less co-simulation and more vigorous proliferation especially at lower levels of tumor antigen exposure when compared to that of antigen-inexperienced T cells.

HELPER FUNCTIONS AND THE POTENTIALLY "GOOD" AND "BAD" SIDES OF CD4 EFFECTOR T CELL SUBSETS IN THE ANTITUMOR IMMUNE RESPONSE

Although the best studied pathways of CD4 T cell-mediated help are those that promote antibody production by B cells, such cells also enhance tumor-specific CD8 T cell responses during disease progression and contribute to the maintenance of a functional memory CD8 T cell pool (Pardoll and Topalian, 1998). Various CD4 T cell subsets can also alter the function of APCs (especially DCs) and innate immune cells (Hung et al., 1998). In addition to enhancing and/or regulating T cell-mediated responses, that include both promoting long-term immunity and establishing or rebalancing immune cell homeostasis, CD4 T cells can also have a direct role in tumor elimination (Pardoll and Topalian, 1998; Quezada et al., 2010; Xie et al., 2010). Paradoxically, several experimental and clinical observations have recently shown that such CD4 effector cell subsets and their signature cytokines can not only contribute to effective antitumor responses but also facilitate tumor-promoting activities. In this section of the review, we will discuss these points and focus on the three most studied and potentially most promising CD4 effector T cell subsets involved in antitumor immunity and therapy, namely the Th1, Th17, and TReg cell subsets.

THE TH1 AND IFN-γ PARADOX

Th1 cells are potent inducers of cell-mediated immunity and inflammation. Through studies using various murine tumor models, Th1-mediated immune responses have been shown to participate and facilitate in the elimination of established tumors and reduce tumor development and susceptibility to carcinogenesis. Moreover, it has been observed in studies of patients with various cancer types that favorable clinical outcomes, as assessed by disease free and overall survival, can be attributed to an enhanced and coordinated Th1 effector cell infiltration within the tumors of these patients (Fridman et al., 2011). IFN-γ is produced predominantly by the Th1 CD4 effector T cell subset. Tumor antigen-specific Th1 cells control tumors, in part, through the secretion of IFN-γ that can have both direct and indirect effects on immune activation and modulation (Mumberg et al., 1999; Zaidi and Merlino, 2011). IFN-γ derived from Th1 cells can induce a cascade of events involving the priming and maturation of cytolytic CD8 T cells through activation of DCs at the sites of tumor growth and further induce tumor elimination through activation of NK cells and type 1 macrophages (Corthay et al., 2005; Quezada et al., 2010; Palucka and Banchereau, 2012). Moreover, IFN-γ can induce

development of the Th1 cell lineage, rather than the potentially tumor-promoting Th2 lineage, and further promote expression of the chemokine receptor CXCR3 and its ligands CXCL9, CXCL10, and CXCL11 that can specifically attract and enhance Th1 cell localization to sites of tumor growth and inflammation (Rotondi et al., 2003). Other studies have suggested that IFN-γ actually inhibits the generation and/or activation of naturally occurring TReg cell subsets (Nishikawa et al., 2005; Caretto et al., 2010). Similarly, another group showed IFN-γ signaling caused cell cycle arrest in TReg cells suggesting that this IFN-γ-dependent mechanism could counteract the ability of TReg cells to protect tumors in cancer patients (Cao et al., 2009). Aside from its immune stimulatory roles and affects on various T cell subpopulation dynamics, IFN-γ can up-regulate HLA class I and class II molecules on tumor cell populations that aid in facilitating cytolytic T cell recognition and elimination of tumors. Studies in both human and murine systems have shown IFN-γ to inhibit cancer cell proliferation (Bromberg et al., 1996; Chin et al., 1996; Hobeika et al., 1999; Platanias et al., 1999; Zaidi and Merlino, 2011), promote tumor cell apoptosis through effects on the expression of caspases, FAS (also known as CD95), and TRAIL (Takeda et al., 2002; Chin et al., 1997; Xu et al., 1998; Meng and El-Deiry, 2001), and inhibit angiogenesis within the tumor environment (Luster and Leder, 1993; Coughlin et al., 1998; Ruegg et al., 1998. Beatty and Paterson, 2001). With respect to angiogenesis, IFN-γ is a potent inducer of several angiostatic chemokines, such as CXCL9 and CXCL10, from a variety of cells, including monocytes, macrophages, fibroblasts, endothelial cells, and tumor cells (Luster and Ravetch, 1987; Farber, 1990; Arenberg et al., 1996; Cole et al., 1998). This may contribute to a shift the local biologic balance between angiogenic and angiostatic chemokines that results in anti-angiogenesis and tumor-associated vascular inhibition. Of course, the different IFN-γ-inducible processes and effects, that are responsible for directly limiting tumor growth and progression, are not only dependent on tumor type, but also cytokine concentration and expression of the extracellular domains of the IFN-γ receptor subunits and their intracellular signaling transmission pathways among the various cells within a dynamic tumor environment (Ealick et al., 1991; Boehm et al., 1997).

Alternatively, a dual role for IFN-γ in the context of malignancy has been reported and associated with contributing to enhanced tumor growth and metastases (Dunn et al., 2006; Schreiber et al., 2011). Several reports have suggested that IFN-γ, possibly derived from Th1 cells, can up-regulate the surface expression of the immunoinhibitory molecule B7-H1 on tumor-associated APCs (Dong et al., 2002; Liu et al., 2007; Zou and Chen, 2008; Wu et al., 2009a; Kondo et al., 2010). Under such conditions, cross-talk between these APCs and T cell expressing the corresponding ligand, PD-1, could result in a coordinated suppressive and tolerogenic environment. In addition, it has been shown that T cell-derived IFN-γ can interact with tumor cells and tumor-associated APC to induce the expression of indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades the essential amino acid tryptophan that leads to the suppression of T cell immunity (Carlin et al., 1987; Munn et al., 2002; Fallarino et al., 2003; Zou, 2005; Sharma et al., 2007; Mellor and Munn, 2008; Muller et al., 2008). It has been shown that IFN-γ can enhance the presence of myeloid-derived suppressor cells (MDSC), in an IFN-γ-dependent manner, within the tumor microenvironment resulting in the suppression of T cell responses (Ostrand-Rosenberg and Sinha, 2009; Gabrilovich et al., 2012). Lastly, it has been reported that IFN-γ can facilitate and/or mediate either contraction of the CD4 T cell population via induction of apoptosis (Berner et al., 2007) or up-regulate and induce the development of TReg cells (Agnello et al., 2003; Liu et al., 2009; Campbell and Koch, 2011). Such IFN-γ-mediated activity derived from Th1 cells infiltrating sites of tumor growth can conceivably undermine the antitumor immune response by negatively affecting T cell population dynamics *in vivo*. For example, Th1 cells may possess homeostatic functions under select conditions within the tumor that can influence the generation, survival and balance of CD4 and CD8 effector and memory T cell subpopulation pools necessary for effective antitumor responses. Consequently, such immune-mediated effects on the local tumor environment could be responsible for the promotion of tumor cell dormancy and contribute to the maintenance, potential progression and/or re-emergence of occult tumor cells or cancer-related stem cells (Mellor and Munn, 2008; Schreiber et al., 2011).

Finally, with regards to clinical application, recombinant IFN-γ was initially used to treat chronic myelogenous leukemia, alone and in combination with IFN-α, but failed to show any significant positive outcome (Kurzrock et al., 1987; Kloke et al., 1992). Since then, recombinant IFN-γ has been used in the clinical management of a variety of malignancies including bladder carcinoma, colorectal cancer, ovarian cancer, and adult T cell leukemia; however, the results have been mixed (Miller et al., 2009). In melanoma patients, early small scale clinical trials were largely inconclusive (Creagan et al., 1987; Ernstoff et al., 1987; Kowalzick et al., 1990; Kopp et al., 1993). In another trial for adjuvant application in patients with melanoma, studies were prematurely terminated due to the observations that IFN-γ-treated patients fared worse than the untreated population (Meyskens et al., 1990, 1995). Although the clinical application and therapeutic effects of directly administered recombinant IFN-γ in cancer patients appears marginal, there is limited experience in investigations focused on therapies utilizing direct transfer of tumor-reactive CD4 T cells secreting IFN-γ. Several earlier clinical studies utilizing various T cell transfer therapies have suggested that the incorporation of CD4 T cells would heighten therapeutic efficacy and improve clinical outcome (Walter et al., 1995; Dudley et al., 2002; Ho et al., 2002; Levine et al., 2002; Kershaw et al., 2006; Bollard et al., 2007). One of the initial clinical studies showing autologous IFN-γ producing CD4 T cell transfer as an effective therapeutic agent in cancer patients was performed by Hunder et al. Using an autologous CD4 T cell clone with specificity to the melanoma-associated antigen NY-ESO-1, these investigators showed that transferred tumor-reactive IFN-γ-producing CD4 T cells mediated a durable clinical remission and promoted endogenous responses against melanoma antigens other than NY-ESO-1 in a melanoma patient (Hunder et al., 2008). Furthermore, the patient experienced a complete response with persistence of the transferred cells and a concomitant induction of melanoma antigen-reactive CD8 T cells. In our adoptive T cell therapy studies with late stage ovarian cancer patients, using peripheral

blood-derived MUC1 peptide-stimulated Th1-like effector T cells, we reported that autologous T cell re-stimulation and subsequent intra-peritoneal re-infusion modulated endogenous T cell-mediated immune responses and systemic T cell subpopulation dynamics that were associated with enhanced patient survival (Dobrzanski et al., 2011). In spite of the limited numbers of such studies investigating CD4 T cell based immunotherapy in cancer patients, it is becoming apparent that Th1 cells possess the capacity to modulate the immune response and potentially enhance tumor immunity in the clinical setting.

THE IMMUNOMODULATION PARADOX OF IL-10 DERIVED FROM CD4 EFFECTOR T CELLS

Several studies support the view that IL-10 may diminish the immune response against cancer by directly inhibiting cell activation of select human T cell subpopulations (de Waal Malefyt et al., 1993; Taga et al., 1993; Joss et al., 2000). IL-10 can also act as a negative mediator in the cross-talk between innate and adaptive antitumor immunity. For example, it has been reported that the IL-10 immunosuppressive activity on T cells is mainly indirect and is functionally linked to other immune cells such as TReg cells, MDSC, and APCs. In the case of APCs, IL-10 restrains antigen presentation via its inhibition of MHC and co-stimulatory B7 family member molecules (Vicari and Trinchieri, 2004; O'Garra and Murphy, 2009), stimulates up-regulation of inhibitory B7 family members (Curiel et al., 2003; Kryczek et al., 2006; Zou and Chen, 2008), down-regulates IL-12 production, and inhibits DC maturation and differentiation (Moore et al., 2001). *In vitro* studies have shown that T cells can be anergized toward melanoma-associated antigens when stimulated with IL-10 conditioned DCs (Steinbrink et al., 1999), and DCs which infiltrate progressing melanoma metastases in humans, have been characterized to express low levels of CD86 and IL-12 but possess an enhanced capacity to produce IL-10 (Enk et al., 1997). Moreover, IL-10 producing monocytes and select populations of the myeloid lineage, which inhibit T cell proliferation, have been isolated from the ascites of patients with ovarian carcinomas (Loercher et al., 1999). These cells and their subpopulations, such as MDSCs, can promote the local clonal expansion and/or induce conversion of naïve CD4 T cells into TReg cell populations (Gabrilovich et al., 2012). Moreover, it has been reported that the $CD4^+$ Tr1 regulatory cells produce antigen-driven IL-10 that is responsible for peripheral immune tolerance through the impaired activation and regulation of CTL, Th1, and other effector Th cell subsets that further facilitate elevated tumor growth through immune escape mechanisms (Seo et al., 2002). It is now clear that IL-10 can not only mediates inducible TReg cell immunosuppressive activity but also plays a direct role in their genesis (Roncarolo et al., 2001). *In vitro* studies have shown that IL-10 treatment can convert different types of tumor cells, such as melanoma and lymphoma, to a CTL-resistant phenotype by decreasing the expression of HLA class I molecules on their surface (Petersson et al., 1998; Kurte et al., 2004). Similarly, IL-10 production by human basal and squamous cell carcinoma prevents *in vitro* lysis of autologous malignant cells by cytolytic T lymphocytes (Kim et al., 1995). Lastly, recent studies have shown that endogenous IL-10 can potentially limit the pro-tumor and/or antitumor effects of Th17-mediated inflammation either indirectly by promoting the regulatory functions of both $FoxP3^+$ and $FoxP3^-$ cells or directly by interacting with IL-10 receptors on Th17 cells in an IL-10 signaling-dependent manner (Chaudhry et al., 2011; Huber et al., 2011). Collectively these studies in both the animal and human systems suggests that IL-10 is involved in both direct and indirect tumor immunosuppressive networks that can promote and facilitate tumor immune tolerance resulting in malignant progression.

Although IL-10 is generally regarded as an anti-inflammatory and immunosuppressive cytokine that favors tumor escape from immune surveillance, evidence is accumulating that IL-10 also possesses immunomodulatory properties that support antitumor immunity. For example, transfection of tumor cells with IL-10 or systemic administration of exogenous IL-10 significantly suppressed tumor growth and led to tumor rejection in several different murine tumor models *in vivo* (Giovarelli et al., 1995; Berman et al., 1996; Fujii et al., 2001; Mumm et al., 2011). Moreover, such antitumor effects of IL-10 were dependent on CD8 T cells. *In vitro* studies have further shown that IL-10 can induce proliferation and cytotoxic activity of human CD8 T cells and function as a chemoattractant for CD8 T cells (Chen and Zlotnik, 1991; Jinquan et al., 1993; Groux et al., 1998; Santin et al., 2000). In more recent studies using IL-10 and IL-10 receptor knockout and transgenic mouse strains, investigators reported that IL-10 directly mediated intratumoral activation and expansion of resident tumor-reactive CD8 T cells that independently rejected established tumor growth and progression (Emmerich et al., 2012). Alternatively, another group using IL-10 knockout mice showed that IL-10 indirectly hindered tumor development, growth, and progression by impeding the development of both MDSCs and $CD4^+$ TReg cells which presumably contributed to immune suppression, carcinogenesis, and tumor pathology (Tanikawa et al., 2012). Lastly, recombinant IL-10 has been associated with stimulating pro-inflammatory responses, such as IFN-γ production, when administered to humans (Lauw et al., 2000; Tao et al., 2001; Tilg et al., 2002). Since it is clear that nearly all CD4 effector T cell subsets can potentially produce IL-10, it is conceivable that endogenous IL-10 (such as IL-10 derived from CD4 helper/effector T cells infiltrating sites of tumor growth) may exhibits both antitumor and pro-tumor activities (Mocellin et al., 2001). Under both scenarios, IL-10 may influence tumor cells through the development, recruitment, and/or activation of various immune response cells, including tumor-reactive CD8 and other CD4 effector T cell subsets. Alternatively, a body of both clinical and pre-clinical data is emerging showing that IL-10 can influence tumor growth and progression by non-immune-related phenomena such as the inhibition of angiogenesis and induction of tumor cell apoptosis (Mocellin et al., 2005). In either instance, the role of endogenous IL-10 as a mediator of either tumor escape or successful immune surveillance may depend upon the conditions of initial carcinogenesis and tumor type, level of tumor progression, and the presence of responding immune cell populations at the sites of tumor growth.

Many CD4 Th cell subsets can potentially produce IL-10, as well as their hallmark cytokines, following engagement of their TCR with antigen (Sariava and O'Gara, 2010). The presence of reactive tumor infiltrating CD4 helper T cells have been associated with good clinical outcomes in patients with select cancer types (Pagès

et al., 2005). Interestingly, this broaches the possibility that IL-10-derived from such Th cells may act, in part, as an immunological adjuvant in the antitumor response to cancer. A subset of Th1 cells has been identified in both murine and human systems and found to either stably or transiently produce both IL-10 and IFN-γ during periods of chronic inflammation and disease (Jankovic et al., 2010; Cope et al., 2011). Several signals have been found to stimulate the generation of such dual cytokine-expressing effector cell subpopulations, including high levels of antigen, soluble factors such as IL-12 and IL-27, and co-stimulatory signals such as ICOS (Jankovic et al., 2010). More recently, it has been suggested that such cytokine switching and/or co-expression in both human and mouse cells of the Th1 lineage may be linked to the role of the complement regulator and T cell co-stimulatory molecule, CD46 with the addition of either TCR engagement or high amounts of IL-12 (Meyaard et al., 1996; Cardone et al., 2010). In either instance, it has been suggested that Th1 effector cell responses are auto-regulated through not only extrinsic but also intrinsic negative feedback loops via the co-induction of IL-10 and IFN-γ. Thus, it is conceivable that the relative amounts and/or duration of IFN-γ and IL-10 produced by such double-positive cytokine secreting effector cell subsets might define the antitumor and/or inflammatory immune response within the tumor microenvironment that results in either tumor eradication or tolerance induction and disease progression. In our recent studies investigating the therapeutic role of adoptively transferred Th1-like effector cells in patients with ovarian cancer, we reported that autologous IFN-γ-secreting CD4 effector cells used in the treatment of long-term surviving patients co-produced higher levels of CD4 effector cell-derived IL-10 when compared to that of short-term survivors. We suggest that such heightened or variable levels of effector cell-derived IL-10, either in combination with IFN-γ or alone, may contribute, in part, to enhancing patient antitumor responses by modulating select effector T cell subsets, such as $CD4^+$ TReg cells and their subpopulations. Conceivably, such modulation in effector cell population dynamics could affect the balance between effective and ineffective antitumor responses and patient survival. Although the molecular mechanisms and roles underlying the effects of IL-10 have not been well characterized, the biological activities of IL-10 in tumor immunity and pathology appear highly context-dependent.

THE TH17 AND IL-17 PARADOX

IL-17 secreting Th cells (Th17) have been implicated in promoting inflammation responsible for immunopathology in both cancer and several autoimmune disorders. Studies in various murine tumor models have suggested that Th17 cells may be associated with tumor initiation and growth in the context of chronic inflammation (Kawakami et al., 2009; Wang et al., 2009; Wu et al., 2009b). In patients with hormone-resistant prostate cancer, an inverse correlation has been reported between pretreatment circulating levels of Th17 cell numbers and time to disease progression suggesting that Th17 cells may accelerate tumor development in such patients (Derhovanessian et al., 2009). Alternatively, others have suggested that Th17 cells may contribute to protective antitumor responses in select human malignancies whereas Th17-associated cytokines may be the contributing factors related to tumor initiation and growth. In studies utilizing various genetically modified murine tumor models, investigators have shown that endogenous IL-17 (such as IL-17 derived from Th17 cells infiltrating sites of tumor growth) could promote tumor growth by inducing tumor vascularization, suggesting that the cellular targets of IL-17 in the tumor microenvironment can be vascular endothelial cells, stromal cells, and cells of the tumor itself (Numasaki et al., 2003; Wilke et al., 2011). Later studies showed that IL-17 induced a wide range of angiogenic mediators (Numasaki et al., 2004; Takahashi et al., 2005; Honorati et al., 2006), including vascular endothelial growth factor (VGEF), that markedly promotes inflammation and tumor angiogenesis. Alternatively, IL-17 has been shown to induce IL-6 production from tumor cells and tumor-associated stromal cells, which in turn activate STAT-3, an oncogenic transcription factor that upregulates pro-survival and pro-angiogenic gene levels in transformed cells (Wang et al., 2009). Furthermore, IL-17 has been shown to selectively enhance the production of angiogenic chemokines such as CXCL1, CXCL5, CXCL6, and CXCL8 in tumor cells and epithelial cells (Numasaki et al., 2005; Lee et al., 2008). Thus, the biological activities and tumor-promoting effects of endogenous IL-17-mediated inflammatory responses appear to be dependent on differences in local cytokine concentrations, bioavailability, and presence of select responding target tissues. Moreover, IL-17 appears highly context-dependent with respect to tumor type, stages of development, and host immune status as cytokine-mediated effects have been shown to be heightened in immunocompromised animals (Murugaiyan and Saha, 2009).

Alternatively, pre-clinical murine tumor studies have correlated the presence of intratumoral Th17 cells with reduced tumor growth and effective antitumor immunity (Muranski et al., 2008; Martin-Orozco et al., 2009). Polarized Th17 cells have been observed in distinct human cancer types, including colon, melanoma, pancreatic, hepatocellular, and ovarian (Kryczek et al., 2009a). In clinical studies, patients with advanced ovarian cancer were observed to possess elevated levels of both intratumoral Th17 cell numbers and IL-17 concentrations within patient ascites that correlated with improved survival (Kryczek et al., 2009a; Wilke et al., 2011). Similar results have been observed in patients with other malignancies suggesting a beneficial role for Th17 cells in cancer (Sfanos et al., 2008; Ye et al., 2010; Chen et al., 2011). Moreover, Th17 cells have been observed to be negatively correlated with TReg cells and positively correlated with effector immune cells including IFN-γ-secreting Th1 cells, cytotoxic CD8 T cells, and NK cells within the tumor microenvironment (Kryczek et al., 2009a; Zou and Restifo, 2010; Wilke et al., 2011). However, their roles and mechanisms of action in the antitumor response are not well understood. It has been suggested that possible protective mechanism(s) mediated by Th17 cells include their capacity to secrete multiple and functionally distinct cytokines such as IL-17A, IL-17F, IL-22, and IL-21. For example, IL-21 production has been shown to sustain CD8 T cell responses (Moroz et al., 2004; Zeng et al., 2005; Frederiksen et al., 2008). Moreover, Th17 cells have been shown to produce the chemokine CCL20 which can promote DC trafficking to the sites of tumor growth in a CCL20-CCR6 dependent manner. Recruitment of such DCs can effectively result in the priming and activation of CD8 T cells that result in enhanced CTL activity. In addition, IL-17 has been shown to promote maturation of DC progenitors (Antonysamy et al., 1999)

and induce IL-12 production from macrophages (Jovanovic et al., 1998). Thus, these studies suggest that the combined cellular products from both Th17 and additional immune cells infiltrating the tumor, and the interaction between these cell types, may play a role in the balance between effective antitumor immunity and pro-tumor responses.

Investigations on the association between Th17 cells and promising clinical outcomes in cancer patients, have suggested a link and close interplay between the Th17 and Th1 cell lineages (Kryczek et al., 2009a; Marshall et al., 2012). Within this positive association between intratumoral Th17 and IFN-γ-secreting Th1 cells, human Th17 cell populations producing both IL-17 and IFN-γ have been identified (Annunziato et al., 2007; Hamaï et al., 2012). It has been reported that these dual cytokine secreting cell populations are exclusively derived from Th17 cells and not initially differentiated Th1 cells (Hirota et al., 2011). These "converted" and/or "re-differentiated" Th17 cells express the Th1 related transcription factor T-bet in addition to the IL-17-related transcription factor ROR-γt (Annunziato et al., 2007). Stimulation by IL-12 rapidly down-regulated IL-17 production and induced expression of IFN-γ through enhanced T-bet expression and the subsequent down-regulation of ROR-γt expression (Annunziato et al., 2007; Annunziato and Romagnani, 2010; Lazarevic et al., 2011). Moreover, this shift in phenotype appeared to be facilitated by the low but constitutive expression of IL-12Rβ2 among Th17 cells (Annunziato et al., 2007; Lee et al., 2009). Thus these findings provided a molecular basis to explain Th17 cell plasticity and/or conversion to the Th1 cell lineage and further supports the concept that such events can occur within portions of the Th17 cell population under inflammatory conditions such as that of a hostile tumor environment *in vivo* (Muranski et al., 2008; Bending et al., 2009; Martin-Orozco et al., 2009b; Annunziato and Romagnani, 2010; Murphy and Stockinger, 2010; Nistala et al., 2010; Hamaï et al., 2012; Marshall et al., 2012). This process of Th17 cellular plasticity appears highly context-dependent and can be influenced, in part, by the cytokine milieu produced by innate immune cells within the inflammatory environment. Consequently, such conversion of the Th17 cell population into the Th1 cell lineage, can have important biological implications in tumor immunity and disease progression. As mentioned earlier, both effector cell-derived IL-17 and IFN-γ can potentially promote or suppress the generation of effective immune responses through a myriad of different mechanisms (Xiao et al., 2009; Tosolini et al., 2011). Although the role of Th17 cells co-producing IL-17 and IFN-γ is not clear, it has been suggested that both cytokines can either synergistically or independently induce the production of functionally diverse chemokines within the tumor environment which in turn can recruit and promote distinct types of effector T cells and/or other immune cells that can influence antitumor immune responses and mediate tumor regression or progression (Kryczek et al., 2009b; Martin-Orozco et al., 2009; Kesselring et al., 2010). Thus, it is conceivable that the relative quantity and/or duration of either IL-17 or IFN-γ produced by such double-positive cytokine secreting Th17 cell subpopulations may define the antitumor immune response. Moreover, the type of tumor, the cells within its microenvironment, and their responsiveness to the various tumor-associated cytokines may further promote and influence an imbalance between pro-tumor verses antitumor effects. For example, recent studies in the murine system by Huber et al., have shown that both IL-17- and IL-17/IFN-γ-producing Th17 cells express higher surface levels of the IL-10Rα when compared to that Th1 cells and that the potential antitumor or pro-tumor effects of the Th17-mediated inflammatory response can be more readily suppressed by endogenously produced IL-10 (Huber et al., 2011). Since many lineage-related tumor types can initially possess and generally favor similar microenvironments that can induce and selectively affect specific effector T cell-mediated responses, this may partially explain why Th17 cells have been observed and associated with protective tumor immunity in only some cancers but not all (Kryczek et al., 2009a). The pro-tumor verses antitumor effects of such Th17 effector cell subpopulations may thus represent a "balance" between IL-17 and IFN-γ cytokine production that can facilitate either tumor promotion or regression. Further identification and characterization of the mechanisms involved in the induction of tumor-reactive Th17 effector cells and their activities in cancer patients may offer significant advantages for future treatment strategies of human malignancies.

CD4$^+$ TREG CELL SUBPOPULATIONS IN IMMUNE REGULATION AND THE ANTITUMOR RESPONSE

The immunoregulatory roles of CD4$^+$ TReg cell subsets have been associated with the prevention of immunopathology during excessive and/or unwanted inflammation and prevention of autoimmune disease. However, in the context of cancer, such cells have been associated with facilitating the suppression of the antitumor response through various tolerance induction and tissue homeostatic mechanisms. However, the role and prognostic value of TReg cells in cancer has recently been disputed (Wilke et al., 2010; Tosolini et al., 2011; deLeeuw et al., 2012). It has initially been reported that high TReg cell frequencies infiltrating the tumor environment correlate with more advanced disease and poor prognosis in patients. In ovarian, pancreatic, and breast cancer patients, either systemic or local FoxP3$^+$ TReg cell expression has been associated with both a poor prognosis and diminished survival rates (Woo et al., 2001; Liyanage et al., 2002; Wolf et al., 2003, 2005; Curiel et al., 2004; Li et al., 2005; Bates et al., 2006; Merlo et al., 2009). Curiel and colleagues reported that the presence of high numbers of CD4$^+$FoxP3$^+$ T cells in malignant ascites of patients with ovarian carcinomas correlated with advanced tumor staging and reduced survival. Alternatively, in colorectal cancer, several investigators did not find any differences between patients with high or low TReg cell infiltration (Loddenkemper et al., 2006) whereas others have found an improved survival associated with a high density of local and systemic FoxP3$^+$ cells suggesting no major immunosuppressive role of TReg cells in colorectal cancer (Salama et al., 2009). Moreover, it has been suggested that the presence and levels of various TReg cell subsets in cancer patients may be beneficial to survival (Alvaro et al., 2005; Erdman et al., 2005; Grivennikov et al., 2010; Wilke et al., 2010; deLeeuw et al., 2012). None-the-less, in early clinical studies investigating adoptive T cell transfer therapies in patients with select cancer types, it was observed that TReg cell depletion prior to therapy can enhance clinically relevant immune responses to such treatments (Muranski et al., 2006; Wrzesinski et al., 2007; Dudley et al., 2008; Porter

et al., 2011; Rosenberg et al., 2011; Le and Jaffee, 2012; Yao et al., 2012). These findings fit with the general notion that TReg cells suppress adaptive immune responses and led many groups to pursue various cytoablative strategies to deplete such cells from cancer patients receiving immunotherapy as a means to enhance clinical responses. In contrast, others have observed the induction of effective antitumor responses following administration of various immunotherapeutic strategies in the absence of cytoablative treatments, and have suggested that such responses are likely due to the balance between effector T cells (i.e., either CD4 or CD8) and TReg cells within treated cancer patients (Alvaro et al., 2005; Quezada et al., 2006; Hunder et al., 2008; Le and Jaffee, 2012; Liu et al., 2012a). Along these lines, in our clinical study investigating adoptive T cell therapy using autologous Th1-like effector cells in the treatment of ovarian cancer patients, we observed enhanced T cell-mediated immune responses in long-term surviving patients that appeared to correlate with differences in their ratios of "inducible" verses "natural" TReg cell subpopulations when compared to that of short-term survivors receiving similar treatments (Dobrzanski et al., 2009, 2011). We suggest that such patient responses did not appear to be dependent on TReg cell numbers but upon their subpopulation ratios within responding patients. Although the precise mechanisms by which these regulatory cells and their various subpopulations (and those that have yet to be defined) potentially function to establish or rebalance immune cell homeostasis and sustain the "balance" between tumor immunity, suppression, and tolerance remains poorly understood, it could be speculated that a collaboration and cross-talk among these various TReg subpopulations are required for the maintenance and control of effective immune responses. Thus, a conceivable role for co-therapeutic approaches targeting modulation, and not depletion, of the TReg cellular network in patients with select tumor types may be an alternative and potentially effective therapeutic approach to treating cancer patients.

Select chemokines and their corresponding receptors have been shown to play a role in the recruitment of specific T cell subsets into tumors and sites of inflammation (Sallusto et al., 1997, 1998; Bonecchi et al., 1998; Loetscher et al., 1998; Hirai et al., 2001; Iellem et al., 2001; Muthuswamy et al., 2012). Among human TReg cells, the chemokine receptor CCR4, and its ligands CCL22 and CCL17, are believed to be the most predominant chemokine-related mechanism responsible for TReg cell trafficking to tumors (Iellem et al., 2001). It has been reported that production of the chemokine CCL22 is associated with human ovarian cancer (Iellem et al., 2001; Curiel et al., 2004) and has also been observed in other types of malignancies, such as gastric cancer (Haas et al., 2008, 2009), Hodgkin's lymphoma (Ishida et al., 2006), and breast cancer (Menetrier-Caux et al., 2009). Blockade of CCL22 *in vivo* significantly reduced human TReg cell trafficking to ovarian carcinomas (Curiel et al., 2004). In a study on gastric cancer, CCL22 and CCL17 appeared to be both important in recruiting TReg cells to such tumors as demonstrated by *in vitro* migration assays (Mizukami et al., 2008). Additional observations in this same study further indicated that the levels of intratumoral CCL22 and CCL17 appeared to correlate with increased levels of TReg cell localization within these tumors at early stages of development. Besides the CCR4 chemokine receptor/ligand interaction, CCR5/CCL5 may also selectively recruit TReg cells to pancreatic tumors as shown in both human and murine systems (Tan et al., 2009). In addition, the chemokine CCL20 shows high affinity to TReg cells expressing CCR6 and has been shown to mediate selective $CCR6^+$ TReg cell trafficking (Kleinewietfeld et al., 2005). In any instance, both naturally occurring (nTReg) or inducible (iTReg) $CD4^+$ TReg cell subpopulations may become enriched within tumors, through a variety of different chemokine receptor/ligand interactions. Furthermore, cytokines and chemokines produced, in part, by either tumor cells, tumor infiltrating lymphocytes, and/or APCs within the tumor milieu may preferentially support such TReg cell expansion, retention, survival, and in some cases, their "further" differentiation and/or change in phenotype (Campbell and Koch, 2011). Following recruitment to sites of inflammation, one of the major functions of $CD4^+$ TReg cell subsets is to maintain and/or restore local immune cell homeostasis during polarized Th1, Th2, and Th17 cell-mediated immune responses. This led to the identification and observation that human Th cell subsets and TReg cells appear to undergo functional specialization in parallel resulting in the development of functionally distinct TReg cell subsets capable of co-localizing with and effectively regulating different types of Th cell responses *in vivo* (Hall et al., 2011; Duhen et al., 2012). Although the precise mechanisms by which these various TReg cell subpopulations maintain or restore immune homeostasis at sites of inflammation and/or tumor growth is unknown, it is conceivable that such interactions that involve the local modulation of the naïve, effector, and memory Th cell pools can further influence antitumor responses that may favor either disease progression or regression. In addition, evidence is accumulating in several pre-clinical murine experimental models that a portion of $CD4^+$ TReg cells can down-regulate FoxP3 expression and their associated regulatory properties and in some cases acquire an effector cell phenotype that expresses IFN-γ and/or IL-17 (Gavin et al., 2007; Strauss et al., 2007; Miyara et al., 2009; Oldenhove et al., 2009; Martin et al., 2010; Whiteside, 2010; Miyao et al., 2012). Although this concept and process of cellular conversion and/or plasticity among $CD4^+FoxP3^+$ TReg cells remains controversial (Rubtsov et al., 2010), it is clear that the biological properties of CD4 TReg cells and their subpopulations are heterogeneous and influenced by the tumor environment in which they infiltrate (Hamann, 2012; Sainz-Perez et al., 2012).

Alternatively, $CD4^+$ TReg cells may possess other underappreciated anti-cancer functions. For example, such cells may have the ability to limit the extent and potential of inflammatory responses to induce tumor development and carcinogenesis. Using a murine herpes viral model, investigators observed that TReg cell-mediated down-modulation of inflammatory responses in secondary lymphoid tissues can actually optimize ensuing immune responses to local infection by more effectively redirecting it to sites of initial infection (Lund et al., 2008). These investigators further suggested that this down-modulation in inflammation within distal secondary lymphoid tissues following local viral infection can facilitate efficient effector T cell migration to sites of primary infection and more effectively promote disease eradication. Conceivably, this can be a concept that can also be applied to local sites of carcinogenesis and primary tumor growth. Moreover, others have suggested that, in colorectal and gastric cancers, $CD4^+$

TReg cells may inhibit tumor-promoting inflammatory responses induced by local microbes, which may help explain their presence and favorable association with outcomes to these cancers (Haas et al., 2009; Zamarron and Chen, 2011). Thus the initial views on the role of TReg cells in carcinogenesis, tumor pathology, and tumor immunity appear oversimplified and in fact appear highly context-dependent with respect to their interactions with different components of a dynamic tumor environment.

SUMMARY AND OVERALL THOUGHTS

The immune system has the capacity to either obstruct tumor development and deter established tumors, or to promote carcinogenesis and tumor progression. Here, we reviewed how CD4 T cells and their various functionally distinct subpopulations contribute to the antitumor immune response and potentially influence this process. Several key principles emerge. Distinct effector and memory CD4 T cell subsets have important roles in the antitumor response. Key among these roles is the provision of orchestrating and/or regulating other immune cells that result in promoting tumor eradication, long-term tumor immunity, and establishing or rebalancing immune cell homeostasis within the tumor environment. Such roles are mediated, in part, by the production of signature cytokines by specific CD4 T cell lineages and through direct cytotoxic effects on tumor cells. The efficacy of such CD4 effector T cell responses may not only depend on memory cell generation, phenotype, and their functional capacity to interact with other immune cell populations, but also in some cases, on their ability to evolve through "cellular plasticity" within the hostile tumor environment. Conceivably, this trait of "cellular Darwinism" by the various CD4 T cell lineages could endow them with considerable flexibility to procure effective tumor immunity. Alternatively, it appears that such CD4 T cell subsets and their signature cytokines can also contribute and facilitate tumor-promoting activities. This may be due, in part, on the abilities of select CD4 T cell lineages and their subpopulations to modulate effector cell population dynamics and thus affect the balance between effective and ineffective antitumor responses. Achieving an "appropriate cellular balance" appears highly context-dependent. Further studies on the biological activities and mechanisms of how the various polarized CD4 effector and/or memory T cell subsets influence and/or facilitate the immune response as a whole in patients with different types of cancers should further enhance the development of more effective cancer treatment strategies.

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Nano-curcumin inhibits proliferation of esophageal adenocarcinoma cells and enhances the T cell mediated immune response

Francesca Milano[1,2]*†‡, Luigi Mari[1,2‡], Wendy van de Luijtgaarden[1,2], Kaushal Parikh[1,2], Silvia Calpe[1,2] and Kausilia K. Krishnadath[1,2]*

[1] *Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam, Netherlands*
[2] *Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, Netherlands*

Edited by:
Chao Ma, California Institute of Technology, USA

Reviewed by:
Nadege Bercovici, CNRS, France
Alex Y. Huang, Case Western Reserve University School of Medicine, USA

****Correspondence:***
Francesca Milano, Department of Internal and Experimental Medicine, Section of Hematology and Clinical Immunology, S. Maria della Misericordia Hospital, San't Andrea delle Fratte, building B, 06132 Perugia, Italy
e-mail: f.milano@amc.uva.nl;
Kausilia K. Krishnadath, Department of Gastroenterology and Hepatology, Center for Experimental and Molecular Medicine, Academic Medical Center, Meibergdreef 9, Amsterdam 1105AZ, Netherlands
e-mail: k.k.krishnadath@amc.uva.nl

†Present address:
Francesca Milano, Department of Internal and Experimental Medicine, Institute of Hematology and Clinical Immunology, University of Perugia, Perugia, Italy.

‡Francesca Milano and Luigi Mari have contributed equally to this work.

In Western countries the incidence of the esophageal adenocarcinoma (EAC) has risen at a more rapid rate than that of any other malignancy. Despite intensive therapies this cancer is associated with extreme high morbidity and mortality. For this reason, novel effective therapeutic strategies are urgently required. Dendritic Cell (DC)-based immunotherapy is a promising novel treatment strategy, which combined with other anti-cancer strategies has been proven to be beneficial for cancer patients. Curcumin (diferuloylmethane), is a natural polyphenol that is known for its anti-cancer effects however, in it's free form, curcumin has poor bioavailability. The aim of this study was to investigate whether using a highly absorptive form of curcumin, dispersed with colloidal nano-particles, named Theracurmin would be more effective against EAC cells and to analyze if this new compound affects DC-induced T cell response. As a result, we show efficient uptake of nano-curcumin by the EAC cell lines, OE33, and OE19. Moreover, nano-curcumin significantly decreased the proliferation of the EAC cells, while did not affect the normal esophageal cell line HET-1A. We also found that nano-curcumin significantly up-regulated the expression of the co-stimulatory molecule CD86 in DCs and significantly decreased the secretion of pro-inflammatory cytokines from *in vitro* activated T cells. When we combined T cells with nano-curcumin treatment in OE19 and OE33, we found that the basic levels of T cell induced cytotoxicity of 6.4 and 4.1%, increased to 15 and 13%, respectively. In conclusion, we found that nano-curcumin is effective against EAC, sensitizes EAC cells to T cell induced cytotoxicity and decreases the pro-inflammatory signals from T cells. Combining DC immunotherapy with nano-curcumin is potentially a promising approach for future treatment of EAC.

Keywords: curcumin, nano-curcumin, theracurmin, esophageal adenocarcinoma, dendritic cell vaccines, T cell responses

INTRODUCTION

Esophageal adenocarcinoma (EAC) has the most rapidly increasing incidence compared to other malignancies (Gamliel, 2000; Brown et al., 2008). Overall EAC patients have a rather poor prognosis with a 5-year survival rate of <15% (Gee and Rattner, 2007; Shimada et al., 2008). Therefore, more effective treatments are urgently required. Recently, Dendritic Cell (DC)-based therapeutic cancer vaccines have proven to be a promising therapy for the treatment of cancer. For the preparation of DC-based cancer vaccines, DCs are loaded *ex vivo* with tumor antigens, and then given back to patients. After activation by the DCs, T cells become effector cytotoxic T lymphocytes (CTLs), which can recognize and lyse tumor cells (Boczkowski et al., 1996; Nair et al., 1998; Milano et al., 2007). Despite the promising advances in DC vaccination, the outcomes of patients treated with DC immunotherapy as a monotherapy are still below expectations and several critical hurdles have to be resolved to improve its effectiveness (Fox et al., 2011). It has been shown that an unfavorable tumor microenvironment, that inhibits the development and function of DCs and CTLs, plays a major role in this phenomenon (Zou, 2005, 2006). It has become clear that using DC-based therapeutic vaccines in combination with agents that modulate the tumor microenvironment, sensitize the tumor cells, or diminish the tumor bulk prior to DC treatment, would highly enhance the efficacy of this approach (Milano and Krishnadath, 2008; Kamrava et al., 2009; Dougan et al., 2010). Therefore, it is necessary to find new combinatorial approaches, which tilt the balance in favor of tumor immunity and enhance DC-induced T cell response in cancer patients. In this respect, the natural substance Curcumin 1,6-Heptadiene-3,5-dione, 1,7-bis(4-hydroxy-3-methoxyphenyl), (1E,6E)-, a derivate of the plant *Curcuma longa*, has recently gained interest. Curcumin is known to have beneficial effects against several types of cancers,

such as colon, colorectal, pancreatic, and esophageal cancer (Kunnumakkara et al., 2009; O'Sullivan-Coyne et al., 2009; Sahu et al., 2009; Sandur et al., 2009; Jutooru et al., 2010). Several studies have shown that curcumin can suppress nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation. Up-regulation of NF-κB is known to be a key event for carcinogenesis. Curcumin also down regulates the expression of NF-κB regulated gene products that play a role in anti-apoptosis, proliferation, invasion, angiogenesis, and metastasis (Singh and Aggarwal, 1995; Aggarwal et al., 2006; Rafiee et al., 2009; Hartojo et al., 2010). Furthermore, curcumin can down-regulate the expression of various pro-inflammatory cytokines such as TNF-α, IL-1, IL-2, IL-8, IL-12 (Xu et al., 1997; Hidaka et al., 2002; Kunnumakkara et al., 2009; Epstein et al., 2010). Importantly, it sensitizes pancreatic tumor cells to the chemotherapeutic drug Gemcitabine, by suppressing proliferation and angiogenesis (Kanai et al., 2010). In addition, curcumin has been proven to be remarkably safe in animal studies and in phase I/II clinical trials even in dosages as high as 12 g per day (Shankar et al., 1980; Sharma et al., 2004; Lao et al., 2006; Dhillon et al., 2008). Curcumin is classified "generally recognized as safe" (GRAS) by the US Food and Drug Administration. In spite of all the proven beneficial effects of curcumin, the major problem limiting the effect in patients, is its poor solubility in water and consequently poor bioavailability (Anand et al., 2007; Yang et al., 2007). In the present study, an effective preparation of curcumin, a nano-particle colloidal dispersion, with highly improved bioavailability and water solubility, named Theracurmin, was used (Sasaki et al., 2011). This compounds is composed of 10% curcumin, 2% other curcuminoids (demethoxycurcumin and bisdemethoxycurcumin), 46% glycerin, 4% gum ghatti, and 38% water. Because of a superior solubility in water, Theracurmin allows easier *in vitro* testing, and eventual *in vivo* administration as compared to free curcumin (Bisht et al., 2007; Anand et al., 2010). In this study, we first evaluated the direct effects of Theracurmin (nano-curcumin) on EAC cell lines. Secondly, we evaluated the direct effects of nano-curcumin on activated T cells and DCs. Finally, we tested whether nano-curcumin would sensitize the tumor cells to DC-mediated cytotoxic T cell response and would more effectively induce lysis of esophageal cancer cells.

MATERIALS AND METHODS

CELL CULTURE

OE19 and OE33 esophageal Barrett cancer cell lines were purchased from ECACC (Porton Down, Wiltshire, SP4 DJG, UK), and cultured in RPMI 1640 (Invitrogen, NY, USA) supplemented with 10% fetal calf serum (FCS) (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 2 mmol/l L-glutamine (Invitrogen). HET-1A esophageal squamous cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in MCDB-153 medium (Sigma, St. Louis, MO, USA) modified as previously described (Milano et al., 2007). All cells were cultured in a 5% CO_2 incubator at 37 °C. The cells were maintained with twice weekly passage/refreshing medium and were harvested with trypsin-ethylenediamine tetra-acetic acid (EDTA).

CELL TREATMENT WITH NANO-CURCUMIN

Nano-curcumin (Theracurmin) was a kind gift by S. Guha (MD Anderson Cancer Center, Huston, TX, USA), and was provided by Theravalues Corporation (Tokyo, Japan). Nano-curcumin was dissolved in sterile water. After establishing the IC50 using MTS assay (data not shown), the final concentration of 50 μM nano-curcumin at the time point of 48 h was chosen. For the experiments, cells were either left untreated or exposed to 50 μM nano-curcumin for 48 h and subsequently harvested for different types of analysis.

BrdU ASSAY FOR MEASUREMENT OF CELL PROLIFERATION

To measure cell proliferation, OE19, OE33, and HET-1A cells were plated in quadruplicate in a black 96 well microplate. After treatment with nano-curcumin, cell proliferation was measured using a BrdU incorporation assay (Roche, Almere, The Netherlands). Briefly, cells were labeled with 10 μM BrdU for 4 h at 37 °C and the labeling solution was subsequently removed. The cells were fixed and the DNA was denatured by adding FixDenat solution for 30 min at room temperature, then the anti-BrdU POD antibody was added and the plate was incubated for 90 min at RT. Next, the plate was washed 3 times and the developing substrate was added and incubated for 3 min. Finally, chemiluminescence was measured using a Synergy multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA). Supernatants were collected and used to measure the effect of nano-curcumin on the cytokine production of the different cell lines by performing CBA (see description below).

WESTERN BLOT ANALYSIS

The expression of caspase-3 and procaspase-9 was detected using Western blot. After treatment with nano-curcumin, OE19, OE33, and HET-1A cells were harvested in M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, USA) and the protein concentration was determined using a BCA assay (Thermo Scientific, Rockford, USA). Equal concentrations (25 μg) of proteins were fractionated by SDS-polyacrylamide gel electrophoresis, electro-transferred to Polyvinylidene fluoride (PVDF) membranes and blocked in 5% low fat milk in TBST. For the detection of caspase-3 and caspase-9, membranes were probed using primary anti-human rabbit polyclonal caspase-3 antibody (Abcam, Cambridge, UK, ab90437) or primary anti-human rabbit polyclonal caspase-9 antibody (Santa Cruz, CA, USA, 556585). Blots were then washed with TBST and incubated for 1 h at room temperature in 1:1000 Horse Radish Peroxidase (HRP) conjugated secondary antibody in 5% low fat milk in TBST. After a final wash with TBST, blots were incubated for 5 min in Lumilight (Roche, Almere, The Netherlands) and chemiluminescence was detected using a Image-Quant LAS 4000 biomolecular imager (GE Healthcare). Band intensity from electronic images of western blots was calculated by densitometry using the public domain Java image processing program Image J (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

FLUORESCENCE MICROSCOPY FOR NANO-CURCUMIN UPTAKE IN EAC CELLS

Curcumin is naturally fluorescent in the visible green spectrum (Bisht et al., 2007). In order to study intracellular uptake of

nano-curcumin, the cells were plated in an 8-well culture glass slide (BD Biosciences), and allowed to grow to sub-confluent levels. Thereafter, the cells were incubated with 50 μM nano-curcumin for different time points ranging from 0 to 48 h. The slides were mounted in Vectashield Mounting Medium (Vector laboratories) with DAPI (4′, 6-diamidino-2-phenylindole) in order to visualize the nucleus of the cells and visualized in the Green channel using confocal laser scanning microscope (CLSM) coupled to an inverted microscope (Olympus IX81, Japan).

RNA ISOLATION

RNA of the different esophageal cell lines was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Briefly, the cells were disrupted in RLT buffer and homogenized, ethanol was added and the mixture was applied to an RNeasy mini spin column. Total RNA bonded to the membrane while contaminants were washed away using buffer RW1 and buffer RPE, containing ethanol. Finally, the total RNA was eluted in RNase-free water. Quality was determined using NanoDrop (Type ND-1000, Wilmington, USA) to measure 260/280 and 260/230 ratios. When ratios were >1.9 and >1.7, respectively, the RNA was used for further experiments.

DENDRITIC CELL CULTURE AND IMMUNOPHENOTYPING

Peripheral blood mononuclear cells (PBMCs) were isolated from a fresh buffy coat (Sanquin blood bank North West, Amsterdam, The Netherlands). These were obtained from healthy volunteers that were HLA-A2 positive to obtain HLA-A2 positive PBMCs matched with HLA-A2 positive cell lines. The PBMCs were isolated by density gradient centrifugation using Ficoll. The monocytes and lymphocytes were then separated by a density separation gradient as previously described (Milano et al., 2007). The monocytes were cultured in 24 wells plates (Greiner Bio-one, Alphen aan de Rijn, The Netherlands) at a density of 5×10^5 cells/ml in CellGro medium (CellGenix, Freiburg, Germany) supplemented with 800 U/ml IL-4 and 1000 IU/ml GM-CSF. At day 3, the immature DCs were stimulated for 3 days with 5 μg/ml monophosphoryl lipid A (MPLA) (Invivogen, San Diego, USA) and 1000 IE/ml IFN-γ, to obtain mature DCs, which were harvested and used for stimulation of T cells as described before (ten Brinke et al., 2007). Mature DCs were then incubated with 0 or 50 μM nano-curcumin, and then harvested after 48 h to evaluate the effect of nano-curcumin on their immunophenotype by FACS analysis as described previously (Milano et al., 2007). Briefly, DCs were washed and incubated with primary anti-human antibody for CD80, CD86, and CCR7 or isotype control in PBA (PBS containing 0.5% sodium azide). After 30 min of incubation on ice in the dark, the cells were washed, re-suspended in PBA and analyzed on a FACSCalibur (BD Biosciences). The data were analyzed by using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Supernatants were as well collected to measure the effect of nano-curcumin on cytokine levels performing CBA, as described below.

T CELL CULTURES AND IMMUNOPHENOTYPING

T lymphocytes were isolated from PBMC obtained from buffy coats. PBMC were isolated by centrifugation on Ficoll (GE Healthcare Bio-Sciences) and the T cell fraction was immediately cryopreserved. On the day of the experiment, T cells were thawed and cultured in RPMI 1640 (Invitrogen, NY, USA) supplemented with 10% FCS (Invitrogen). The T cell Activation/Expansion kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to activate the T cells. Briefly, cells were incubated with anti-biotin MACSiBead particles loaded with CD2, CD3, and CD28 antibodies, using one loaded anti-biotin particle per two T cells, for 48 h at 37 °C. Resting or activated T cells were incubated with 0 or 50 μM nano-curcumin, and then harvested after 48 h to evaluate the effect of nano-curcumin on their immunophenotype by FACS analysis as previously described (Milano et al., 2007). Briefly, either resting or activated T cells were washed and incubated with primary anti-human antibody for CD4, CD8, or isotype control in PBA (PBS containing 0.5% sodium azide). After 30 min of incubation on ice in the dark, the cells were washed, re-suspended in PBA and analyzed on a FACSCalibur (BD Biosciences). The data were analyzed by using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Supernatants were as well collected to measure the effect of nano-curcumin on cytokine levels performing CBA, as described below.

TRANSFECTION OF DC WITH TUMOR RNA AND CO-INCUBATION WITH T CELLS

Mature DCs were harvested at day 6 of culture and after washing they were electroporated using the Amaxa cell line Nucleofector Kit V (Amaxa GmbH, Germany). DCs were mixed with Nucleofector transfection solution V and 4 μg of total RNA of OE19 or of OE33 or HET-1A cells. The program U16 of the Amaxa transfection device was used to electroporate the cells. After this, electroporated DCs were co-cultured with T lymphocytes at a ratio of 1:4 in a 24 wells plate. After 1 week the T cells were harvested and again co-cultured with freshly electroporated DCs for a second stimulation. The different populations of T cells, namely CTLs specific for the different cell line antigens, were used in the cytotoxicity assay to determine their killing capacity against EAC cancer cell lines.

EFFECT OF DC-MEDIATED T CELL RESPONSES AND NANO-CURCUMIN TREATMENT ON CELL LYSIS OF TUMOR CELLS

The OE19, OE33, and HET-1A cell lines were pre-treated with 0 or 50 μM nano-curcumin for 48 h. Subsequently, 10,000 target cells were harvested, washed, and co-incubated with different amount of effector cells i.e., the above mentioned HLA-matched specific CTLs that were stimulated with tumor RNA electroporated DCs. DC and CTLs were co-cultured at an effector:target ratio of 10:1 to 0,625:1 in 100 μl of medium in a 96 wells plate for 4 h at 37 °C. The percentage of cytotoxicity was measured by using CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega, Madison, USA) following the manufacturer's instructions and as previously described (Milano et al., 2010). This assay quantitatively measures lactate dehydrogenase (LDH), which is a stable cytosolic enzyme released upon cell lysis. The released LDH in culture supernatants is measured with a 30-min coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color is proportional to the number of lysed cells. Visible wavelength absorbance data was collected using a multi-well scanning spectrophotometer (ELISA reader). The percentage of specific cytotoxicity was calculated

using the formula: % cytotoxicity = (Experimental − Effector Spontaneous − Target Spontaneous)/(Target Maximum − Target Spontaneous) × 100.

CBA ASSAY

Supernatant collected from the cytotoxicity assays, and from the cultures of DC, T cells and the EAC cell lines, untreated or treated with nano-curcumin were analyzed for the simultaneous measurement of different pro- and anti-inflammatory cytokines using the cytometric bead enzyme-linked immunosorbent assay systems (CBA, BD Biosciences). Specifically, the Human Th1/Th2 cytokines kit and the Human Inflammatory kit were used according to the manufacturer's protocol, as previously described (Milano et al., 2008).

STATISTICS

Data is represented as mean ± SD. Comparison between groups was carried out with Student's *t* test. Values of $P < 0.05$ were considered as statistically significant. Asterisks indicate the level of significance. All the experiments were carried out at least 3 times ($n = 3$) with 2–4 technical replicate.

RESULTS

NANO-CURCUMIN UPTAKE ASSAY IN ESOPHAGEAL ADENOCARCINOMA CELL LINES

To confirm that the structural changes in the preparation of this nano-curcumin, do not affect its cellular uptake in esophageal cell lines, we monitored its intracellular accumulation using CLSM coupled to an inverted microscope (Olympus IX81, Japan). In **Figure 1** it is shown that after 1 h of incubation with 50 μM nano-curcumin, all the cell lines show a green signal, which increased after 2 and 4 h, as compared to the negative untreated cells. At 6 h the fluorescent signal of nano-curcumin decrease and at 48 h it could not be visualized anymore by fluorescent microscopy. Our results are in line with previous findings, where it was shown that once taken up by cells, nano-curcumin is rapidly (within 7 h) metabolized and becomes invisible after 48 h (Bisht et al., 2007; Kunwar et al., 2008; Mathew et al., 2012). No major differences were observed between the esophageal cancer (OE33 and OE19) and normal (HET-1A) cell lines.

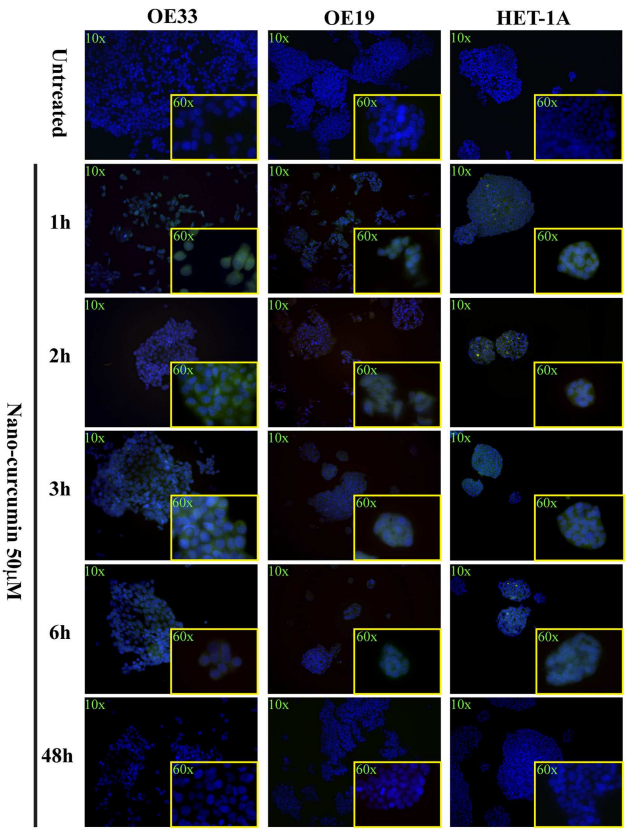

FIGURE 1 | Uptake of nano-curcumin in EAC cell lines compared to normal esophageal cell lines. Fluorescence microscopy analysis shows that nano-curcumin, which is green autofluorescent is rapidly (within 1 h) taken up by cells. The uptake seems to increase with time and it is still observed after 6 h. Micrographic pictures are representative of three independent experiments.

EFFECT OF NANO-CURCUMIN ON CELL PROLIFERATION OF EAC CELL LINES

The functional effect of nano-curcumin on cell proliferation of the EAC cell lines, OE19, OE33, and the normal squamous cells, HET-1A, was investigated using a BrdU incorporation assay. **Figure 2A** shows that treatment of the cells with 50 μM nano-curcumin for 48 h, significantly decreased cell proliferation in the EAC cell lines OE19 and OE33, but not in the normal esophageal squamous cell line HET-1A, indicating that nano-curcumin selectively affects the proliferation of cancer cells leaving the normal squamous epithelial cells unaffected.

EFFECT OF NANO-CURCUMIN ON APOPTOSIS OF EAC CELL LINES

To test whether the inhibition of proliferation correspond to an increase in apoptosis, we set out to investigate if the apoptotic signaling pathways are affected after treatment with nano-curcumin. By performing Western blot on the lysates of the cell lines, we could not detect a significant up-regulation of cleaved caspase-3 (the activated form of caspase-3) nor a significant decreasing levels of procaspase-9 after treatment with nano-curcumin, as shown by the densitometry of the western blots indicating that it does not induce apoptosis in these cell lines (**Figures 2B,C**). Levels of several other pro- and anti-apoptotic proteins and a Nicoletti apoptosis assay confirmed these findings (data not shown).

EFFECT OF NANO-CURCUMIN ON THE IMMUNOPHENOTYPE, CYTOKINE PRODUCTION AND APOPTOSIS OF DCs AND T CELLS

In previous studies it was shown that curcumin negatively affects the immunophenotype of DCs (Kim et al., 2005). To determine whether nano-curcumin has detrimental effects on cells of the immune system, we first studied the changes in the immunophenotype, cytokine profile and apoptosis level of DCs before and after exposure to nano-curcumin. Expression levels of CD80 and CCR7 in DCs did not change before and after exposure to nano-curcumin. Thus unlike previous reports on free curcumin, nano-curcumin leaves the functional phenotype of DCs intact (**Figure 3A**). Also, we observed that nano-curcumin significantly

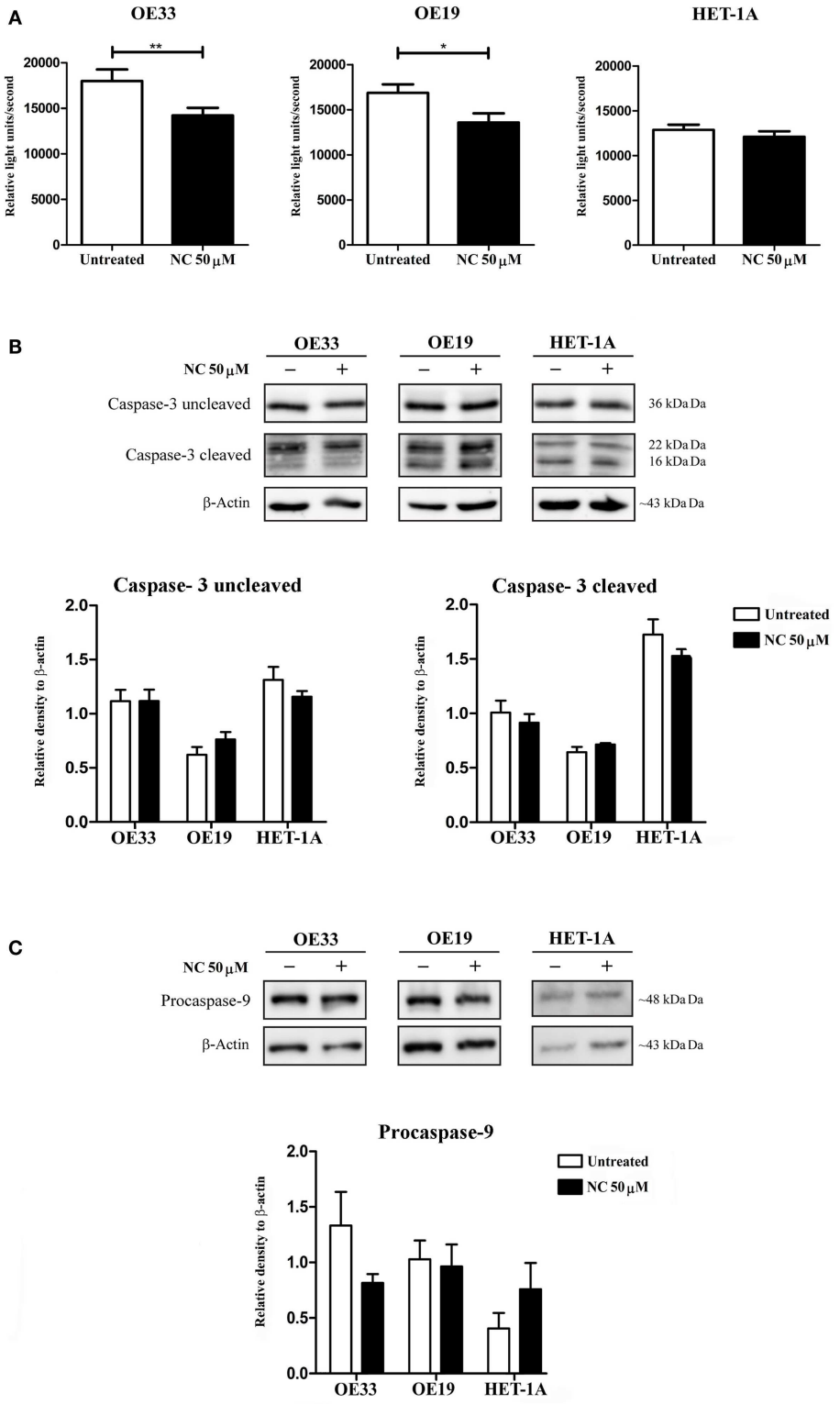

FIGURE 2 | Nano-curcumin inhibits cell proliferation and does not induce apoptosis in EAC cell lines. **(A)** BrdU assay results show that treatment of the EAC cell lines OE33 and OE19 with 50 μM nano-curcumin (NC) induces a significant decrease in cell proliferation. This is not the case for the normal esophageal cell line HET-1A (student's two tailed paired *t* test, $*P < 0.05$, $**P < 0.01$, $n = 3$). **(B,C)** Western blot analysis to check levels of cleaved caspase-3 and procaspase-9 shows that treatment of the EAC cell lines OE33 and OE19 with 50 μM nano-curcumin does not affect the levels of cleaved caspase-3 and procaspase-9. This is as well shown by densitometry data showing the ratio of cleaved caspase-3 and procaspase-9 to the loading control β-actin using Image J software (student's two tailed paired *t* test, $n = 3$). Gels are representative of three independent experiments.

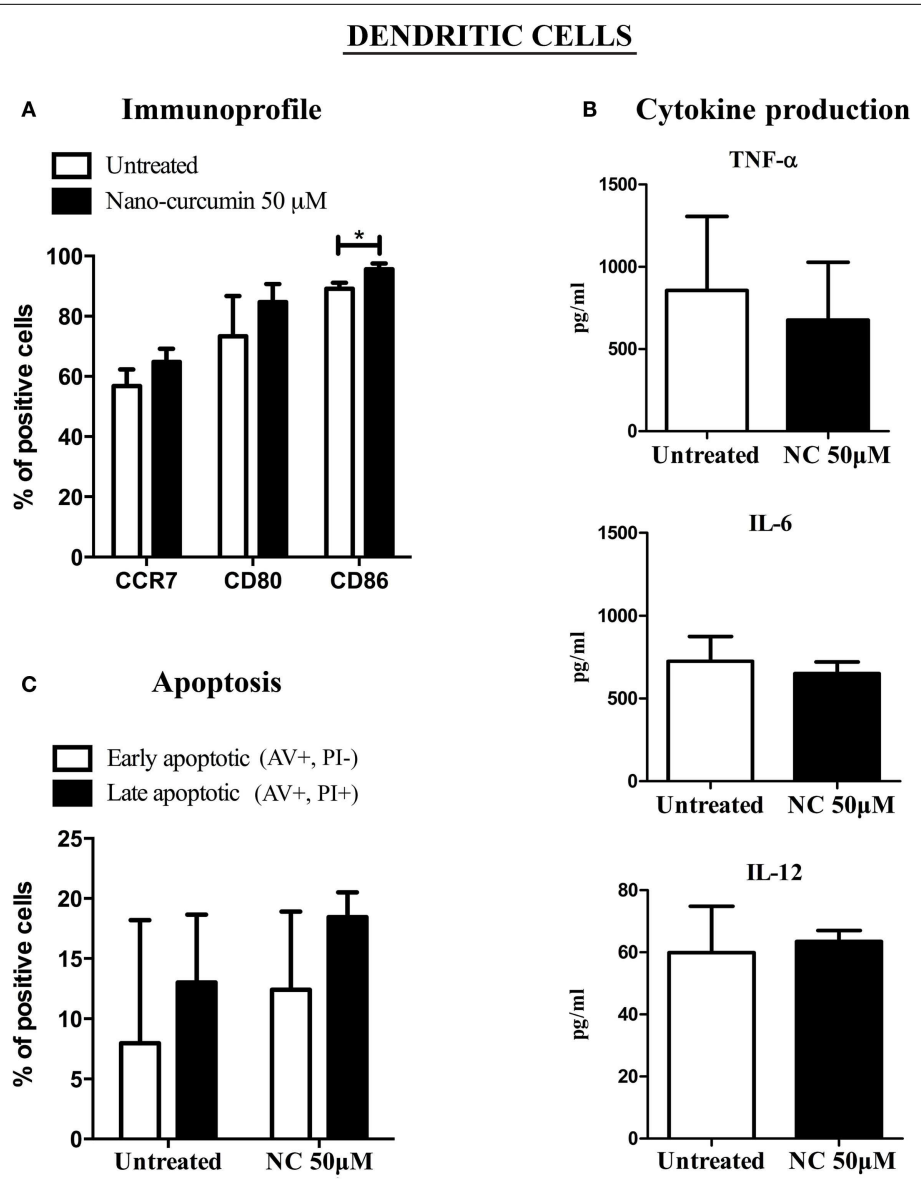

FIGURE 3 | Nano-curcumin does not interfere with the function of dendritic cells (DC), and increases expression of the co-stimulatory molecule CD86. **(A)** Fluorescence activated cell sorting (FACS) analysis shows that nano-curcumin does not change the expression of CD80 and CCR7 in DC, but it does significantly increase the expression of CD86 on DCs (student's two tailed paired *t* test, $*P < 0.05$, $n = 4$). **(B)** Cytometric bead array show that nano-curcumin does not change the cytokine production profile of the DCs (student's two tailed paired *t* test, $n = 3$). **(C)** Flow cytometric detection of annexin V (AV) and propidium iodide (PI) shows that nano-curcumin does not induce apoptosis in DCs. Data are showing the percentage of early apoptotic (annexin V+/PI−) and late apoptotic/necrotic (annexin V+/PI+) populations (student's two tailed paired *t* test, $n = 3$).

increased the expression level of the co-stimulatory molecule CD86 in DCs, indicating that it drives DCs to mature toward a functional phenotype. We also observed that nano-curcumin has no effects on the cytokine profile of these DCs (**Figure 3B**) and that it did not induce apoptosis of DCs (**Figure 3C**).

Next, we investigated the effect of nano-curcumin on T cell phenotype and function. We showed that nano-curcumin did not change the phenotype of resting nor activated T cells as observed by unchanged expression levels of CD4 and CD8 after treatment (**Figures 4A,D**). In activated T cells, nano-curcumin significantly decreased the amount of early apoptotic cells (**Figure 4F**) but had no effect on resting T cells (**Figure 4C**). Accordingly, nano-curcumin did not affect the production of cytokines of resting T cells (**Figure 4B**), but it did significantly reduce the secretion of TNF-α, IL-8, IL-6, IL-10, and IL-1β in activated T cells. This indicates that nano-curcumin modulates the cytokine profile of activated T cells toward a profile that negatively affects tumor cell growth and migration (**Figure 4E**).

From our results we can conclude that nano-curcumin has no negative effects on the immune-profile of both DCs and T cells

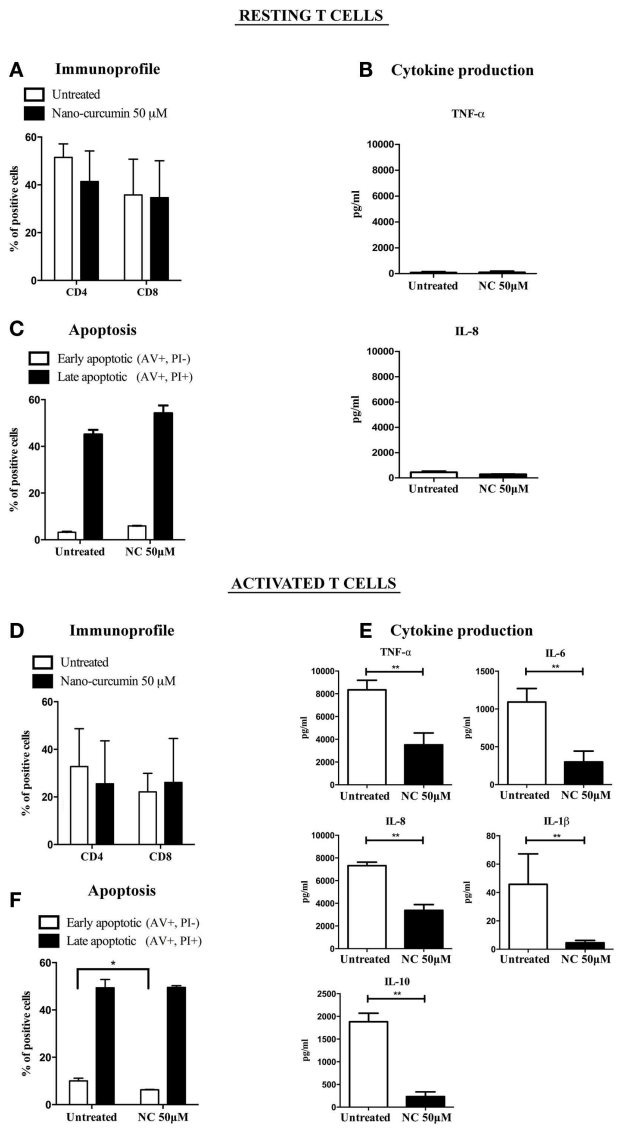

FIGURE 4 | Effect of nano-curcumin on resting and activated T cells. (A) As measured by FACS, the CD4/CD8 ratio of resting T cells does not change after nano-curcumin treatment (student's two tailed paired *t* test, $P > 0,05$, $n = 3$). **(B)** Nano-curcumin does not affect the release of TNF-α and IL-8 of resting T cells (student's two tailed paired *t* test, $P > 0,05$, $n = 3$). **(C)** The fractions of early apoptotic (annexin V+/PI−) and late apoptotic/necrotic (annexin V+/PI+) populations as measured by FACS analysis show no difference in apoptosis between resting T cells that were left untreated or treated with nano-curcumin (student's two tailed paired *t* test, $^*P < 0.05$, $n = 3$). **(D)** As measured by FACS, the CD4/CD8 ratio of activated T cells does not change after nano-curcumin treatment (student's two tailed paired *t* test, $P > 0,05$, $n = 3$). **(E)** Nano-curcumin reduces the production of TNF-α, IL-8, IL-6, IL-10, IL-1β in activated T cells (student's two tailed paired *t* test, $^{**}P < 0.01$, $n = 3$). **(F)** There is a decrease of the early apoptotic (AV+/PI−) fraction of the activated T cells after treatment with nano-curcumin (student's two tailed paired *t* test, $^*P < 0.05$, $n = 3$).

and does not negatively affect their function. Instead, these results for the first time show that nano-curcumin supports the function of DCs and T cells in inducing anti-tumor immune responses.

EFFECT OF NANO-CURCUMIN ON DC-MEDIATED T CELL-INDUCED CYTOTOXICITY

To determine whether nano-curcumin enhances DC-mediated T cell induced cytotoxicity, we tested the effects of nano-curcumin pre-treatment of EAC cell lines in CTL cytotoxicity assays. Through electroporation DCs were loaded with the RNA of OE19 and OE33 cell lines. Specific anti-cancer CTL populations were obtained through stimulating T cells with the RNA loaded DCs. Using an effector (CTLs) to target (EAC cells) ratio of 10:1, in OE19 and OE33 cells, the CTLs induced a mean cell lysis of 6.4 and 4.06%, respectively (**Figure 5A**). Pre-treatment of the tumor cells with 50 μM nano-curcumin significantly increased the mean cell lysis to 15 and 13%, respectively. This indicates that pre-treatment with nano-curcumin sensitizes EAC cells to specific CTL-induced cytotoxicity. In a similar experiment we found no cytotoxicity against the normal esophageal cell line HET-1A (**Figure 5A**). We also evaluated the production of cytokines after 4 h of incubation of the EAC target cells with the effector cytotoxic T cells with and without pre-treatment of nano-curcumin. We found that in the co-culture of OE19 with CTLs there were no changes in the cytokine production profile (**Figure 5C**). When CTLs where incubated with OE33, however, nano-curcumin pre-treatment significantly increased the production of IFN-γ, while the production of IL-8 significantly decreased. No significant changes were observed for TNF-α and IL-2 (**Figure 5B**). This is interesting considering that IL-8 was found to be highly expressed in OE33 and OE19 (data not shown) and was previously reported to be highly expressed in EAC (Milano et al., 2008). Reduced levels of IL-8 may reduce the migratory functions of esophageal cancer cells, while higher levels of IFN-γ support the function of DCs and T cells, again indicating that nano-curcumin seems to enhance the function of the immune system against tumor cells.

DISCUSSION

Curcumin is a natural substance that is known to have anti-carcinogenic and anti-inflammatory effects against several types of cancers (Kunnumakkara et al., 2009; O'Sullivan-Coyne et al., 2009; Jutooru et al., 2010; Yallapu et al., 2012). A disadvantage of free curcumin is that it is highly hydrophobic and is poorly absorbed after oral administration (Li et al., 2005). Due to this low biological activity, high doses of free curcumin are necessary to obtain significant responses (Bhawana et al., 2011). Various curcumin nano-formulations have been developed to improve its solubility, bioavailability, and pharmacokinetic properties, allowing easier *in vitro* and preclinical *in vivo* testing. Sasaki et al. (2011), recently formulated an innovative colloid-based preparation of curcumin named Theracurmin (nano-curcumin) and demonstrated its oral bioavailability and safety in healthy subjects. The toxicity of this type of nano-curcumin is currently being tested in a clinical trial in patients with advanced malignancies (ClinicalTrials.gov identifier: NCT01201694). So far, besides one single event of diarrhea, no adverse events have been recorded, again indicating that this form of nano-curcumin is safe and well tolerated (Kanai et al., 2012).

Although these studies deem nano-curcumin as a safe substance, its biological function, has yet to be confirmed. Because other forms of nano-curcumin have the same biological effects as

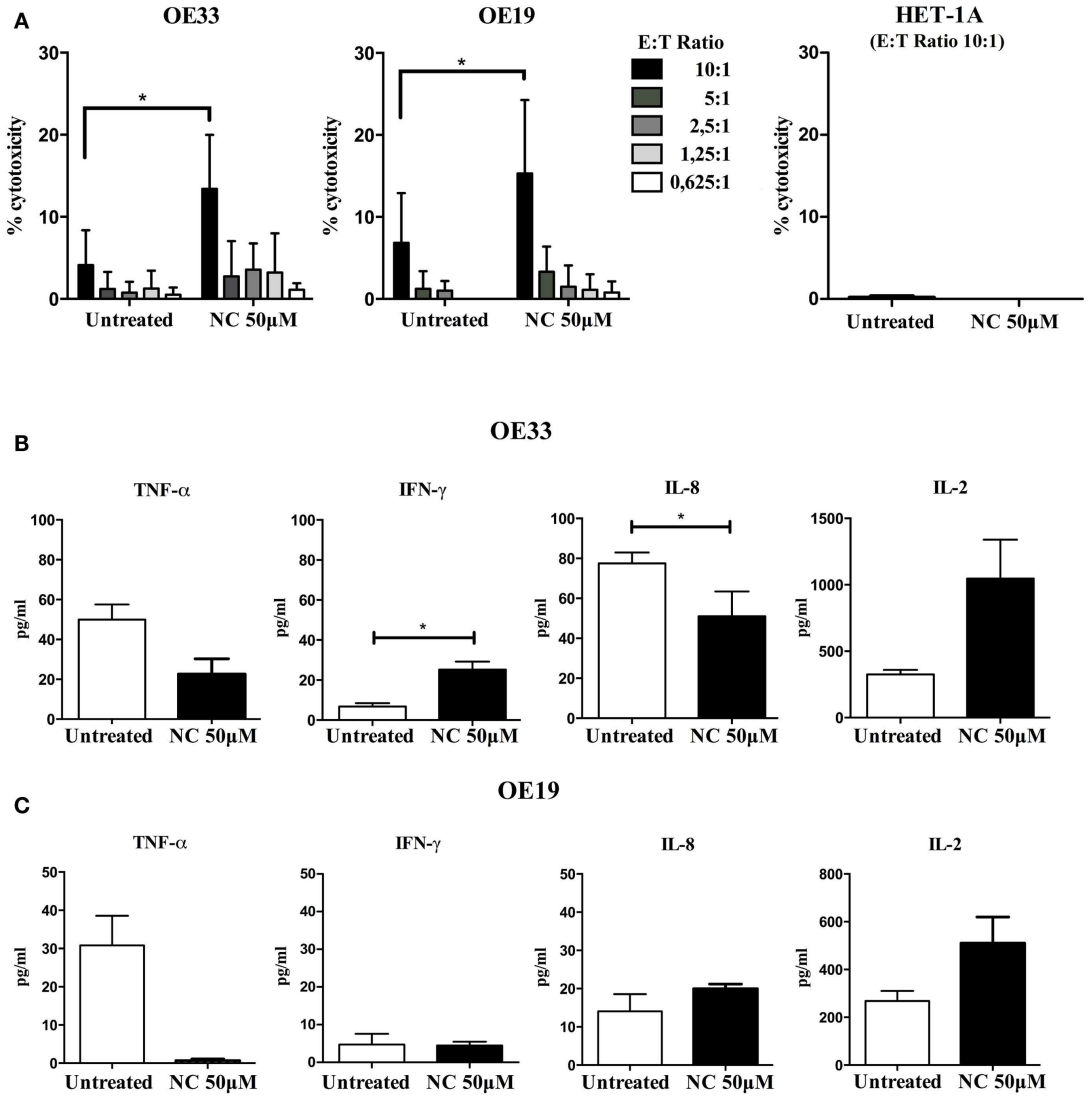

FIGURE 5 | Nano-curcumin enhances T cell mediated cancer cell lysis. **(A)** Cytotoxicity assay showing different E:T ratios of OE19 and OE33 co-culture with CTLs. Cell lysis induced by CTLs at the E:T ratio of 10:1 is 6.4 and 4.06%, respectively. Pre-treatment with nano-curcumin, significantly increases the CTLs induced lysis to 15 and 13% respectively (E = effector = CTLs, T = target = EAC cells; student's two tailed paired *t* test, $*P < 0.05$, $n = 3$). At the E:T ratio of 10:1 there is no cytotoxicity induced by CTLs on the HET-1A cells, which also does not change after nano-curcumin treatment. **(B)** The cytokine profile as measured in the supernatant after the cytotoxicity assay shows that in the co-culture of CTLs and OE33, pre-treatment with nano-curcumin increases IFN-γ production and decreases the production of IL-8 (student's two tailed paired *t* test, $*P < 0.05$, $n = 3$). **(C)** In the supernatant of the co-culture of OE19 cells with CTLs, pre-treatment with nano-curcumin did not change the cytokine profile (student's two tailed paired *t* test, $n = 3$).

free curcumin in pancreatic and prostate cancer (Bisht et al., 2007; Yallapu et al., 2012), we set out to demonstrate that nano-curcumin retains the anti-carcinogenic and anti-inflammatory properties attributed to free curcumin, and thus be used as a possible adjuvant for the treatment of EAC.

We show that nano-curcumin has a direct anti-proliferative effect on EAC cell lines, in line with previous results showing that free curcumin decreases the proliferation and survival of esophageal cancer cells (Subramaniam et al., 2012). It is worth mentioning the specific anti-proliferative effect of nano-curcumin toward cancer but not normal cell lines. This characteristic is not due to differential cellular uptake, as we have proved that intracellular accumulation of nano-curcumin is equal in both types of cell lines. Instead, it could be speculated that divergences in signaling pathways between cancer and normal cells account for the selective proliferative effects of the nano-curcumin. For example, the signaling pathways affected by nano-curcumin, might be more activated in esophageal cancer cells as compared to normal cells, rendering the cancer cells more susceptible to the effects of nano-curcumin. Indeed, one of the most important pathways involved in cellular proliferation and aberrantly activated in cancer stem cells, the Notch signaling pathway, has been shown to be inhibited

by free curcumin in esophageal cell lines (Subramaniam et al., 2012). Therefore, it is tempting to speculate that by inhibiting this pathway, nano-curcumin might selectively suppress proliferation of the EAC cell lines.

Although nano-curcumin has been demonstrated to also induce apoptosis in a variety of cells including pancreatic cancer cells (Sahu et al., 2009; Jutooru et al., 2010), we did not observe an effect on apoptosis of esophageal cell lines. Several mechanisms for curcumin-mediated apoptosis have been suggested. Shankar et al. (2007), for instance demonstrated that curcumin upregulates the expression of pro-apoptotic members of the Bcl-2 family like Bax and Bak and inhibits the anti-apoptotic Bcl-2 proteins, such as Bcl-X_L and Bcl-2. Also curcumin was found to affect several caspases such as caspase-8 (Anto et al., 2002). We tested the levels of the above mentioned pro- and anti-apoptotic pathways, including Bcl-2 and Bcl-X_Ls, but could not see any significant change in any of the apoptotic pathways after treating the EAC cells with nano-curcumin. The discrepancy between our results and the above mentioned studies could be attributed to differences in signaling pathways between the different cancer cells or to the intrinsic resistance of EAC cells to apoptosis, as it has been previously reported (O'Sullivan-Coyne et al., 2009).

Another level at which Theracurmin exerts its anti-carcinogenic effect on esophageal tumor cells is by increasing their susceptibility to be killed by cytotoxic T cells. CTLs were stimulated *ex vivo* with DCs loaded with tumor-derived RNA, and were used in cytotoxic assays to determine their ability to recognize and lyse EAC cells. We found that nano-curcumin has a sensitizing effect on DC-mediated T cell cytotoxicity by increasing cell lysis on EAC cells. We also observed that nano-curcumin increased the IFN-γ secretion and decreased the TNF-α secretion in the co-culture of OE33 with CTLs and nano-curcumin.

One earlier report showed that curcumin has a detrimental effect on the immune-phenotype of DCs (Kim et al., 2005). In our study, however, we found that neither DCs nor T cells are negatively affected by nano-curcumin. Instead, we found that nano-curcumin up-regulated the expression of the co-stimulatory molecule CD86 and reduced the levels of anti-inflammatory cytokines in activated T cells, asserting the role of nano-curcumin in anti-inflammatory processes. This is as well in line with previous findings were it was shown that nano-curcumin reduces the levels of multiple pro-inflammatory cytokines (Bisht et al., 2007; Anand et al., 2010).

It has become clear that combining anti-cancer treatments potentiates the effect of anti-cancer agents (Vanneman and Dranoff, 2012). For instance, the combination of curcumin with Gemcitabine for the treatment of pancreatic cancer, leads to increased apoptosis *in vitro* and reduced cell proliferation *in vivo* (Buckanovich et al., 2008; Dhillon et al., 2008).

One important direction in the field of oncology is to combine conventional (chemo) therapeutical agents with strategies that enhance the immune system (Ramakrishnan et al., 2010). Our results confirm that nano-curcumin not only directly affects EAC cancer cell proliferation but also potentiates the immune response to the tumor cells, making this compound extremely attractive to be used in immune combinatorial therapies for EAC.

However, further *in vivo* evaluations are warranted to confirm its efficacy as a novel and more efficacious adjuvant therapy for this aggressive cancer.

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