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Cancer immunotherapy
Re-programming cells of the innate and adaptive immune systems

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Abbreviations: ACT, adoptive cellular therapy; MDSC, myeloid-derived suppressor cells; NKT cells, natural killer T cells; B/I, bryostatin 1/ionomycin; PKC, protein kinase C; ICAM, intracellular adhesion molecule

Cancers utilize multiple mechanisms to overcome immune responses. Emerging evidence suggest that immunotherapy of cancer should focus on inducing and re-programming cells of the innate and adaptive immune systems rather than focusing solely on T cells. Recently, we have shown that such a multifaceted approach can improve immunotherapy of breast cancer.

Major barriers/challenges to the advancement of cancer immunotherapy include: (1) immunological tolerance due to the fact that cancer cells originate from normal tissue to which cells of the adaptive immune system were tolerized; (2) tumor escape as a result of epigenetic changes in the tumor cells induced by immune responses, e.g., antigen loss, MHC class I loss or "missing" unknown, yet critical, target antigens; (3) tumor-induced immune suppression mediated by an increased population of myeloid-derived suppressor cells (MDSC) in cancer patients.[1-4] In fact, cancer cells utilize multiple strategies to survive in such an immunologically hostile environment, however many strategies used in cancer immunotherapy have been narrowly focused on a specific type of immune cell, particularly $CD8^+$ T cells. Innate immune cells such as NK cells or NKT cells are usually considered as a secondary source of immunotherapy when tumor cells escape from adaptive immune responses by losing their target antigen or MHC class I molecule. In addition, patients who participate in immunotherapy clinical trials have received chemotherapy and radiation therapy. Such conventional therapies affect their immune system. Therefore, an effective cancer immunotherapy is expected to overcome the above mentioned barriers, and to be designed based on an understanding of the role of conventional therapies in boosting or compromising the immune responses.

Despite recent advances in adoptive cellular therapy (ACT) of melanoma, no success has been achieved in ACT of breast cancer. This is in part due to low immunogenicity of breast cancer expressing self antigens compared with melanoma that expresses a variety of highly immunogenic antigens as suitable targets for immunotherapy,[5] as well as increased MDSC in breast cancer patients.[3,4] Using autologous T cells that are weakly and inefficiently reactive against the self antigens expressed by breast tumors would not generate objective responses. Therefore, re-programming of tumor-reactive immune cells toward the most effective phenotypes may be the only way to cure breast cancer and/or prevent recurrences immunologically. In addition, most ACT protocols have focused on T cells and ignored a critical role of the innate immune cells including NKT cells and NK cells in anti-tumor protection. Given the critical cross talk between cells of the innate and adaptive immune systems, a combined approach utilizing NKT cells, NK cells and T cells could result in highly effective anti-tumor immune responses.[6,7] Our recent findings suggest that such a multifaceted strategy can overcome MDSC mediated immune suppression as well as tumor escape in the FVBN202 mouse model of spontaneous mammary carcinoma.[8] Others have demonstrated that NKT cells play a key role in overcoming MDSC by converting them into antigen-presenting cells (APC), thereby rescuing T cells from suppression and improving their effector function.[9,10] It has been reported that T central memory (T_{CM}) phenotypes are more effective than T effector (T_E) phenotypes in generating long-lasting protection against tumor cells.[11,12] The presence of NKT memory cells has also been suggested to be protective against tumor cells.[13] Therefore, the most effective ACT strategy would be to re-program cells of both innate and adaptive immune systems and differentiate the T cells toward memory phenotypes, while at the same time overcoming tumor-induced immune suppression.

We have recently developed an antigen-free protocol by means of pharmacological agents, bryostatin 1 (B) and ionomycin (I), and combined common gamma chain (γ-c) cytokines for re-programming tumor-reactive cells of the innate (NKT cells and NK cells) and adaptive ($CD4^+$ and $CD8^+$ T cells) immune systems which displayed resistance to MDSC. Broystatin 1 is a naturally occurring antineoplastic drug which is also a potent modulator of protein kinase C (PKC). Short-term effects of

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bryostatin 1 include activation of classical and novel PKCs, whereas prolonged stimulation leads to lowered PKC activation. It was reported long ago and repeatedly that bryostatin + ionomycin (B/I) selectively stimulate tumor-sensitized T cells in vitro; when lymphocytes from sarcoma-bearing mice were activated with B/I and expanded in IL-2, tumor-specific T cell frequency increased by orders of magnitude compared with the starting population.[14] In fact, B/I selectively activated $CD62L^{low}$ (sensitized) T cells from mice bearing 4T1 mammary carcinomas.[15] Emerging interests in bryostatin 1 as an immune modulator have resulted in a better understanding of its role in the modulation of antigen presentation. For instance, it was reported that bryostatin 1 acts as TLR-4 ligand and activates dendritic cells (DCs),[16] and upregulates expression of IFNγ receptor in monocytes[17] as well as induction of IFNγ and T-bet transcripts.[18] These data suggest that B/I could be a potent modulator of the immune cells.

The common γ-c cytokines IL-2, IL-7 and IL-15 play a key role in homeostasis of the immune cells. An understanding of the distinct properties of these cytokines will lead to the development of an effective formulation or combination for the expansion of tumor-reactive immune cells ex vivo during a pharmacological reprogramming. In addition to being a T cell growth factor, IL-2 also supports differentiation of $CD8^+$ T cells toward effector and effector/memory phenotypes (T_E and T_{EM}) by downregulation of lymph node homing receptors CD62L and CCR7. This results in the trafficking of the IL-2-expanded T cells to the tumor site. Therefore, such cells may induce early anti-tumor responses but may not lead to a long-term memory response. IL-7 is crucial for the survival and homeostatic expansion of naive and memory $CD8^+$ T cells; it is secreted by stromal cells, epithelial cells and fibroblasts but is not produced by lymphocytes. The IL-7 R is expressed by T cells, pre-B cells and DCs. The receptor comprises two polypeptides, an affinity binding receptor IL-7 Rα or CD127 and a signaling γ-chain receptor CD132. Because of an important role of IL-7 in all stages of T cell development and maintenance, it has been used in clinical trials in an attempt to increase the replenishment of T cells. Injection of IL-7 resulted in the expansion of both $CD4^+$ and $CD8^+$ T cells as well as a relative reduction of $CD4^+$ Tregs.[19] IL-15 is produced by monocytes, DCs and epithelial cells. IL-15 R is expressed by T cells and NK cells, and consists of three polypeptide subunits: an IL-15 Rα chain, which determines its binding to IL-15, and shared IL-2 β (CD122) and γ (CD132) chains.

About 20% of the human $CD8^+$ T cell pool in peripheral blood has low expression of CD127. Although CD127 is a specific receptor for IL-7, its expression on T cells also determines responsiveness of T cells to common gamma chain cytokines other than IL-7. For example, IL-15 decreases activation induced cell death (AICD) in $CD8^+CD127^+$ T cells but not in $CD8^+CD127^-$ T cells while inducing comparable proliferation of the two subsets.[20] Such a differential effect was in part mediated by IL-15-induced expression of anti-apoptotic Bcl-2 as well as inhibition of the pro-apoptotic Bim in $CD8^+CD127^+$ T cells but not in $CD8^+CD127^-$ T cells. Although IL-15 can induce expression of Bcl-2 in naive and memory T cells, its best defined role is supporting memory T cells. It has been shown that naïve T cells and T_{CM} cells are $CD8^+CD127^+$ while T_E and T_{EM} cells are mostly $CD8^+CD127^-$.[21] Culture of antigen-experienced T cells with IL-15 ex vivo restores their ability to respond to the antigen.[21]

Due to the variable expression of the common γ-c receptors at different stages of immune cell homeostasis, a sequential use of the cytokines should be considered during the expansion and reprogramming of tumor-sensitized immune cells.

The role of NKT cells in tumor immunity has not been studied extensively. NKT cells are classified into three types which include: (1) type I or classical or invariant NKT cells (iNKT cells); (2) type II or non-classical or non-invariant NKT cells. Type I and II NKT cells recognize glycolipid antigens associated with MHC class I-like molecules CD1d; (3) NKT-like cells or CD1d-independent NKT cells.[22] Type I NKT cells express an invariant Vα24Jα18 chain paired with a Vβ11 in humans or an invariant Vα14Jα18 chain paired with a Vβ8.2, Vβ2, or Vβ7 in mice. In contrast, TcR Vβ regions used by type II NKT cells are highly diverse. Type I NKT cells produce IFNγ whereas type II NKT cells produce IL-13 that facilitates production of TGFβ by myeloid cells.[23] Recent studies have shown that iNKT cells conferred protection against lymphoma, whereas type II NKT cells facilitated immune suppression.[24] For instance, increased iNKT cells at the tumor site of patients with colorectal cancer were found to be associated with a favorable prognosis.[25] iNKT cells have been shown to act as adjuvants for anti-tumor T cell vaccines.[26,27] The precise mechanism responsible for such an adjuvant effect is not fully understood. A recent report suggested that iNKT cells were shown to increase expression of CD70 on DCs, following immunization with the glycolipid, α-galactosylceramide (α-GalCer), in mice, thereby supporting $CD8^+$ T cell responses through engagement with the CD27 receptor.[7] Most iNKT cells in mice are double negative ($CD4^-CD8^-$) and express CD28 as well as CD154 upon activation.[6,7] Ligation of these costimulatory molecules increases secretion of IFNγ by iNKT cells.

It was reported that MDSC loaded with α-GalCer on their CD1d showed enhanced immunostimulatory function through interaction with activated iNKT cells. Activated iNKT cells in turn converted MDSC into antigen-presenting cells (APCs), and supported antigen-specific proliferation of IFNγ producing CD8+ T cells.[9] In humans, activated iNKT cells have also been shown to direct monocytes to differentiate into immature DCs through the engagement of CD1d on monocytes.[28] These reports underscore a critical role of the activated iNKT cells in interactions with MDSC and tumor-reactive T cells. Very recently we made a similar observation in the FVBN202 transgenic mouse model of breast carcinoma, where anti-tumor efficacy of HER-2/neu-specific T cells, in vitro and in vivo, was influenced by the presence or absence of activated NKT cells.[8]

Based on these findings we propose a model to explain a cross talk between NKT cells and MDSC as well as with T cells during an effective anti-tumor immune response (**Fig. 1**). According to this model, double negative ($CD4^-CD8^-$) $CD25^+$ invariant NKT (iNKT) cells interact with CD1d on MDSC, resulting in the conversion of MDSC into DC by increasing the expression of CD80/86, CD70 and ICAM-1. Engagement of CD80 and CD70 on newly converted DCs with CD28 and CD27 on T cells

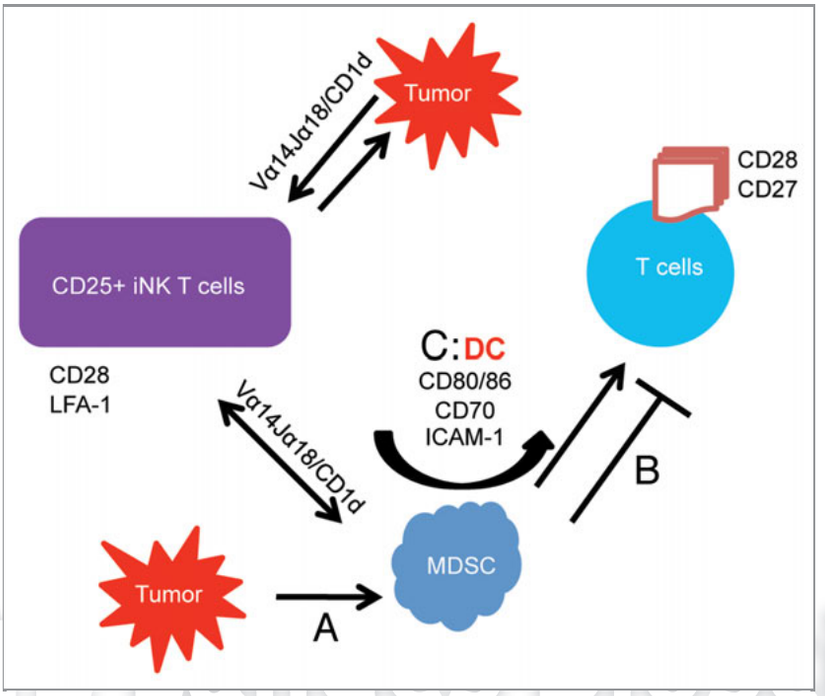

Figure 1. Mechanisms by which CD25⁺ iNKT cells interact with MDSC and rescue T cells from suppression. Tumor-derived soluble factors increase MDSC (A) which in turn suppress anti-tumor T cell responses (B). Activated CD25⁺ NKT cells interact with CD1d on tumor cells and MDSC and demonstrate enhanced anti-tumor responses (C). This will result in MDSCs increasing expression of CD80/86, CD70, ICAM-1 thus effectively converting to a DC phenotype, which then interacts with CD28 and CD27 on activated T cells, thereby enhancing T cell anti-tumor responses.

support T cell responses to the tumor cells and overcome MDSC suppression. CD25⁺ iNKT cells can also respond to tumor cells and MDSC in a CD1d-dependent manner. Extensive production of tumor-specific IFNγ by the expanded cells may also overcome tumor relapse that we found to be due to low levels of IFNγ production. This hypothetical model has been supported by our recent publication[8] showing that tumor-specific IFNγ production was significantly increased by NKT cells when MDSC were present. In addition, the presence of NKT cells was required in order to overcome MDSC-mediated T cell suppression.

The source of tumor-reactive immune cells that are used for ex vivo re-programming is critical. We showed that radiation therapy compromised phenotypic distribution of T cells such that re-programming of these cells did not yield T_{CM} phenotypes and failed to protect animals against the tumor cells.[8] Altogether, recent evidence demonstrates a shift from adaptive T cell responses to a multifaceted cellular immunity by means of immune cell expansion using γ-c cytokines utilizing cells harvested prior to radiation therapy for the treatment of human carcinomas.

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