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Characterization of CD8 T cell responses after immunotherapy: The role of antigen specificity in anti-tumor effects

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Cellular and Molecular Biology

by

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Abstract

We have previously demonstrated that immunotherapy with an agonist CD40 antibody in combination with IL-2 results in synergistic CD4-indenpendent anti-tumor effects but actually impairs the ability to generate an antigen-specific response. In the present study, our goal was to examine the role of antigen specificity in the efficacy of cytokine-based immunotherapy. Due to the massive CD8 T cell expansion that occurs after these regimens in both normal and tumor-bearing mice, we hypothesized that the anti-tumor effects resulting from immunotherapy are due not only to the induction of antigen specific T cells, but also to the increased activation and non-specific killing capability of CD8 T cells. This hypothesis was supported by the observations that CD8 T cells did not upregulate surface molecules that are indicative of recent TCR ligation following immunotherapy, and that these cells were highly lytic.

To specifically determine if TCR engagement was necessary for the expansion of CD8 T cells following immunotherapy, we performed adoptive transfer studies with T Cell Receptor (TCR) transgenic (Tg) OT-1 mice and observed an increase in proliferation following anti-CD40 and IL-2 immunotherapy in the absence of antigen. Direct treatment of OT-1 mice with immunotherapy resulted in the increased functional activation and lytic capability of CD8 T cells against irrelevant tumor targets. These effects were observed despite a surprising lack of proliferation after immunotherapy.

After determining that CD8 T cells were mediating anti-tumor responses in the absence of TCR ligation, we wanted to determine a possible tumor recognition mechanism for CD8 T cells following immunotherapy. NKG2D ligands are upregulated on several tumor types, and we observed an increase in the expression of NKG2D on CD8 T cells isolated from immunotherapy treated OT-1 and wild type mice. In light of the increased NKG2D expression with therapy, we further hypothesized that signaling through NKG2D on CD8+ T cells could be one mechanism by which non-specific recognition and killing occurs. We found that when treated with an NKG2D blocking antibody, CD8 T cells isolated from immunotherapy treated mice exhibited decreased lysis of NKG2D sensitive targets. The role of NKG2D in CD8 T cell killing following immunotherapy was further established by the observation that anti-tumor effects were diminished in mice that were treated with immunotherapy in the presence of an NKG2D blocking antibody. These data demonstrate that immunotherapy with anti-CD40 and IL-2 results in the expansion of antigen specific CD8 T cells, despite its deleterious effects on CD4+ T cells, and that anti-tumor responses may be generated through the increased lytic function of CD8+ T cells in an NKG2D assisted manner. Furthermore, these mechanisms may play an important role in the anti-tumor responses observed with this therapy.

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Introduction

The Immune System and Cancer: The Immunosurveilance Hypothesis

The role of the immune system in the development, progression, and regression of cancer has long been disputed. Over 100 years ago, Paul Ehrlich was the first to propose that the immune system might play a role in the prevention and control of cancer by suggesting that immune mechanisms might be capable of repressing a number of carcinomas (1-3) This observation served as a base for later work by Macfarlane Burnet and Lewis Thomas, who in the 1950s independently suggested that the unaltered immune system was composed of cellular components, whose duty was to circulate the body in search of nascent neoplastic or transformed cells. These collective suppositions became the "cancer immunosurveilance" hypothesis, which suggests that transformed cells are perpetually arising, but that their growth and progression are held in check by circulating "sentinel" lymphocytes (2, 4-8).

Initially, the tumor surveilance hypothesis was widely disregarded due to early tumor induction studies. In initial studies, the chemical carcinogen methylcholanthrene (MCA) was used to induce tumors in CBA/H wildtype or nude mice, however, the investigators did not observe an increase in the incidence of spontaneous or MCA induced sarcomas between the immunodeficient nude mice and their wildtype CBA/H counterparts (2, 9, 10). It wasn't until the 1990s that studies were published which demonstrated that targeted immunodeficiency can

result in an increase in the incidence of chemically inducible tumors, as well as a decrease in the immunogenicity of MCA induced tumors (2, 11-14). These studies were performed under the ablation or absence a variety of innate and adaptive immune cell types and effector molecules which include but are not limited to NK Cells, γ/δ T cells, α/β T cells, NKT cells, perforin, IFNγ, and IL-12 (2, 3, 9, 15).

Evidence for the importance of the immune system in warding off cancer is not limited to controlled animal studies. Indeed, long-term clinical observations of immune suppressed transplant recipients and AIDS patients have demonstrated an increase in the development of cancers with known viral etiology such as Kaposi's sarcoma and non-Hodgkin's lymphoma (2, 16, 17). Interestingly, higher rates of non-virally induced cancers such as malignant melanoma and cancers of the lung, colon, pancreas, kidney, ureter, and endocrine system have also been observed in renal transplant recipients compared to the general population (2, 17). In addition to these studies, several reports have emerged that describe the presence of tumor infiltrating lymphocytes (TILs) within the microenvironments of tumors. The type and functional capabilities of these cells determine whether they are tumoristatic or tumorigenic in nature; however, the observation stands that immune cells are capable of entering tumor, and depending on their location, can be a prognostic factor for patient survival (15, 18).

The immunosurveilance hypothesis has evolved to include the concept of immunoediting. The immunoediting hypothesis posits that tumor-immune mediator interactions occur in three distinct phases: Elimination, Equilibrium, and Escape (9). In the elimination phase, both the innate and adaptive arms of the immune system act as tumor suppressors which can recognize and kill transformed cells as they arise and prevent the development of clinically relevant disease. The second phase is equilibrium, which occurs if the immune system is not able to mediate the complete elimination of tumor cells. In the equilibrium phase, tumors develop but are held in check and are constantly sculpted by the immune response generated against them (3, 9, 15). The best example of the equilibrium phase was demonstrated clinically in the context of an allograft transplant. In this case, two kidney allograft recipients developed melanoma 1-2 years post transplant. Upon further investigation, it was found that the donor had been treated for melanoma 16 years before her death, and was considered to be disease free. However, upon transfer of her kidneys into the new immunosuppressed hosts, the tumor cells harbored within the kidneys were able to grow freely, whereas they had been held in equilibrium by the donor's intact immune system (9, 19).

The final principle in immunoediting is escape, which occurs when the tumor is no longer susceptible to immune control, and progresses into clinically detectable disease. Mechanisms that have been identified that contribute to immune escape include the downregulation of MHCI or cytokine receptors, as

well as tumor antigens (1, 2, 9). In clinical melanoma studies where patients received CTLs targeted against tumor specific antigens, it was observed that some of the tumors were able to downregulate the expression of the MART-1/MelanA or gp100 antigens, and in some cases, the MHCI molecules that expressed the antigens thus making them invisible to the adoptively transferred cells (20-22). Preventing the development of antigen loss variants continues to be a challenge for ACT.

Mouse models of carcinogenesis have also proven beneficial for examining the phenomena of immune surveillance and immunoediting. Additional evidence for immunoediting has was elegantly demonstrated by Koebel and colleagues who used a model of MCA-induced sarcomas in immune-deficient mice to examine the impact of immunoediting on the immunogenicity of tumors. They found that tumors which developed in the absence of pressure and "editing" from the immune system were dramatically more immunogenic than tumors which arose in immune-competent hosts (23). Conversely, tumors that developed under the pressure of immunoediting were highly invasive when transferred into immune-deficient hosts (23).

Immunotherapy: Activating the immune system to fight cancer

Immunotherapy has been used to best effect for the treatment of four particular types of cancer: renal cell carcinoma, melanoma, prostate cancer, and leukemias/lymphomas. The reasons behind this efficacy have yet to be

determined, however, it has been suggested that these types of cancer possess greater levels of tumor antigens, which can make them attractive targets for the immune system. These are also types of cancer which were observed early on to possess an immune component. For example, immunotherapy was first considered for application in renal cell carcinoma after it was observed that a very small sub-population of patients who had disseminated disease went into full remission after receiving radical nephrectomies to remove the primary tumor (24). The disappearance of the metastases in these patients led clinicians and investigators to postulate that there might be an immune component in the recession of RCC, and that immunotherapy might be a good treatment option for patients with disseminated disease (25).

Early attempts at immunotherapy involved the generation of a non specific immune response with agents such as *bacillus Calmette-Guerin*, and *Corynebacterium parvum* (26, 27). *Bacillus Calmette-Guerin* is a non-invasive bacteria that elicits robust immune responses at the site of infection and has been used for the treatment of bladder cancer (27). These practices, however, have been abandoned and replaced with reagents and adoptively transferred cells that are capable of invoking a more targeted immune response. The goal of contemporary immunotherapy regimens is the breaking of self tolerance. Cancer develops over a prolonged period of time, in the presence of an intact immune system. As such, the immune system becomes tolerized to the presence of tumor cells. In order to induce immune responses against tumors,

immunotherapy regimens are targeting a variety of tumor antigens in combination with methods to diminish the suppressive environments that are present within tumors. The identification of novel tumor antigens which allow for the selective targeting of tumor cells and not health tissues is crucial for the continued development of targeted immunotherapies.

A great deal of time and study has been dedicated to the identification of tumor antigens which are specific to transformed cells, or are aberrantly expressed on tumor cells. Tumor specific antigens (TSA) are molecules that are unique to the tumor and are not expressed on healthy tissues. Examples of tumor specific antigens include the melanoma specific MART-2 antigen, and the renal cell carcinoma specific antigen, HSP70-2/m*a* (28). Additional tumor specific antigens are listed in Table 1. Tumor specific antigens are usually the byproduct of point mutations in the genome of tumor cells, which lead to the aberrant expression of novel proteins and peptides (223). This is in contrast to tumor associated antigens (TAA), which are expressed on both healthy and neoplastic tissues, but are over-expressed on the transformed tissues. Examples of TAAs include the melanocyte marker Trp-1, as well as the epidermal growth factor, HER-2 (28, 29). These antigens are at a functional disadvantage compared to TSAs, as these are endogenous proteins to which immunological tolerance has already been developed. As such, immune targeting of these antigens is more likely to result in the development of Tregs and other peripheral tolerance mechanisms, compared to the novel TSAs to which the immune system is not

tolerized. To circumvent these regulatory mechanisms, some studies have utilized depleting and blocking antibodies against CD25 and CTLA-4, respectively in combination with TAA targeting. CD25 is constitutively expressed on Tregs, and CTLA-4 provides and inhibitory signal to activated T cells. As such, their combination with TAA targeting has proven beneficial for the targeting of tissues expressing the TAA, but due to the shared expression of TAAs with normal tissues, autoimmune effects such as vitiligo have been observed (29).

Passive versus active vaccination

Passive immunity refers to the administration of antibodies or immune cells to a host to help with the eradication of a disease or a toxin. The adoptive cell transfers described in later sections are an example of passive immunity. Active immunity involves the generation of a de novo immune response by priming with an infectious agent and an adjuvant to initiate immunity. Recently, two examples of active immune vaccines received FDA approval to target cancer. Gardasil® is a quadrivalent vaccine against 4 strains of human papilloma virus, two of which, (HPV16 and HPV18) are known to cause as many as 75% of cases of cervical cancer. By inducing active immune responses against these viruses, this vaccine can prevent many cases of cervical cancer (30, 31). Another example of the use of vaccination to induce active immunity in cancer, is the new autologous cellular immunotherapy, Provenge®. Provenge is the first therapy of its kind to receive FDA approval and is generated by culturing autologous

dendritic cells with GM-CSF and the prostate acid phosphatase (PAP) antigen, which is present on many prostate cancer cells [www.provenge.com (32)]. Provenge differs significantly from Gardasil in that it is targeting a self-antigen and not a virus. There is also the matter that while Gardasil is a true vaccine and is administered before infection, Provenge works to generate immune responses in an environment where the immune system has already failed to control tumor progression. This fact, combined with the low antigenicity of tumors and suppressive environment induced by self-antigens make the success of Provenge particularly remarkable.

Cell-based Immunotherapy

Adoptive cell therapies

Adoptive cell therapy (ACT) generally consists of the transfer of T cell subsets into a cancer patient in the hope that the T cells can mediate sustained anti-tumor effects. Adoptive cell therapies have also been used in the context of infectious disease for the treatment of cytomegalovirus (CMV), human immunodeficiency virus (HIV), and Epstein-bar virus (EBV) (33-35). CD8 T cells have been the primary target for adoptive cell therapies due to their specificity for tumor antigens and their ability to maintain their longevity in vivo; however, there is new evidence emerging that CD4 T cells may also be effective killers in this scenario (36-38).

The first hurdle facing the successful application of ACT in cancer is identifying a suitable tumor antigen. Once an antigen has been identified, another difficulty facing adoptive cell therapies is the *ex vivo* generation of suitable numbers of T cells, and maintaining their numbers and function *in vivo*. Clinically, two approaches have been described to expand cells for ACT *ex vivo*. The first technique involves the polyclonal expansion of tumor infiltrating lymphocytes (TILs) or peripheral blood mononuclear cells (PBMCs) with antibodies that activate CD3 and CD28, followed by secondary treatments to enrich effector or memory populations, to deplete Tregs, or to perform genetic manipulation, such as the transduction of tumor antigen specific TCRs (39-41). This method relies on the assumption that initial responses have been generated against tumor antigens and has only been reproducibly achieved with samples from patients with melanoma (42). The second method involves the activation and expansion of TILs or PBMCs by culturing them with antigen presenting cells pulsed with tumor antigens. This method is advantageous because it allows for the expansion of tumor antigen specific T cell clones; however, this method is labor intensive, cost-prohibitive, and technically difficult to achieve, thus making it unrealistic for large-scale use and regulatory approval (40, 41).

It has been observed that the co-transfer of CD8 T cells with antigen specific CD4 T cells can result in augmented anti-tumor effects (43-45). The inclusion of CD4 T cells has become problematic in light of the identification of regulatory T cell (Treg) and inflammatory IL-17 producing Th17 populations. In

fact, it has been proposed that early adoptive cell therapies may have been limited in their efficacy due to the inclusion of large numbers of Tregs in the transfusions (40, 41). CD4 T cells are thought to provide essential cytokine support to the transferred CD8 T cells, mostly in the form of IL-2, IL-21, and CD40L (46-48). Infusion of IL-2 or co-transfer of CD4 T cells has been found to increase the potency and longevity of adoptively transferred CD8 T cells (22, 44). Other cytokines such as IL-15 have also been shown to augment T cell cytotoxicity *in vitro* (40, 41) and *in vivo* (49), which makes this cytokine an attractive target for increasing the survival and longevity of adoptively transferred CD8 T cells.

The activation and memory status of CD8 T cells can also have a profound effect on the function of adoptively transferred cells. It has been demonstrated that upon transfer, memory T cells function better than naïve T cells due to their increased precursor frequency and their rapid responses to antigen encounter (50-52). It has also been observed that of the memory subsets, central memory cells expand more efficiently *in vitro* and mediate more potent antitumor effects in a non-human primate model than effector memory cells (53, 54). This is in contrast to a recent report which claims that naïve T cells produce more efficient effector T cell responses than central memory cells (55).

Recent studies are establishing the impact that preparative regimens can have on the outcome of adoptive cell therapies. Several studies have shown that lymphodepletion by total body irradiation can contribute to the efficacy to ACT in

three ways: 1) Lymphodepletion induces a cytokine sink so that there is less competition for cytokines and growth factors, and allows for the homeostatic proliferation of the transferred cells; 2) irradiation can induce tumor damage and the release of tumor antigens which can be presented by innate immune effectors and augment the recognition of the tumor by the adoptively transferred T cells; 3) Lymphodepletion can decrease the number of myeloid suppressor cells and Tregs, allowing for greater function of the transferred cells (56-59). It has long been established that T cells will undergo homeostatic proliferation in cases of extreme lymphopoenia and recent studies are also showing that the higher the intensity of lymphodepletion, the better the outcome of ACT (57, 60). Lymphodepletion in combination with the transfer of *ex vivo* expanded and IL-2 activated TILs has shown remarkable response rates in patients with melanoma. Response rates as high as 70% have been observed using this technique; however, despite these responses, very few patients demonstrated increases in the overall survival (22). Additionally, outcomes such as these have been limited to melanoma and attempts to extrapolate this technique into patients bearing other tumor types such as those of the breast, ovary, and prostate have been largely unsuccessful (61). Other immunotherapeutic strategies are still needed to elicit responses against less immunogenic tumors.

As with most cancer therapies, ACT has been associated with several toxicities. These toxicities can be the byproduct of culturing techniques used to expand the cells for transfer, the side-effects of exogenous cytokines that are co-

transferred with the cells, or direct cell-mediated toxicities which are generated by the transferred cells themselves. The transfer of TILs has been associated with cytokine release syndrome, which has been observed to occur within 4-6 hours of transfusion (22, 62). Autoimmunity has also been a common occurrence in studies using cells targeted against TAA such as gp100 and MART-1 (40). Vitiligo and skin depigmentation are common occurrences after the transfusion of TILS specific for shared melanotcyte antigens, and additional cases of retinitis, uveitis, hypothyroidism, and hepatitis have also been reported (40, 60, 63-66).

Toxicities tend to occur less frequently in patients receiving genentically unmodified autologous T cell transfers. The risk for toxicities is much higher when allogeneic T cells are transferred. Allogeneic T cell transfer following allogeneic hematopoietic stem cell transplantation (HSCT) is associated with graft-versus-host disease (GVHD) (67). The development of GVHD is crucially dependent upon the method and extent of pre-conditioning the patient is subjected to before transfer, as well as the dose and timing of T cell transfer following HSCT (40, 68). Toxicities are also more common with the transfer of T cells that have been genetically engineered to express tumor specific TCRs or chimeric antigen receptors (CAR) (40). A recent case study was published, outlining the events concerning a lethal adverse event which occurred in a patient receiving a transfusion of autologous CAR-bearing T cells which had been transduced with the ERbB2-specific antibody, herceptin, along with signaling

domains for CD28, 4-1BB, and CD3ζ (69). The patient experienced a rapid influx of CAR T cells to the lungs, which presumably interacted with low levels of ERbB2 expressed by the lung epithelium, thus mediating tissue damage and inducing cytokine relsease syndrome. This sad case is an example of the caution that must be taken when targeting shared tumor associated antigens for immune mediated destruction.

Lymphokine-Activated Killer (LAK) Cells

Lymphokine-activated killer (LAK) cells are generated by removing peripheral blood cells from a patient and activating them with IL-2 to expand the NK and T cell compartments, and the re-infusing the expanded LAK cells back into the patient. This regimen in combination with IL-2 has resulted in response rates that vary from 9 to 33 percent in patients with renal cell carcinoma (25). Interestingly, when the response rates of patients who receive the combination LAK/IL-2 treatment are compared with the response rates of patients who only received the IL-2 treatment, there is little difference; however, of the patients that show a response, more of the LAK/IL-2 treated patients show a complete clinical remission than those treated with IL-2 alone (70). One drawback for the use of LAKs in cancer treatment is their dependence on exogenous IL-2, which has been found to induce dose-limiting toxicities in human subjects (70, 71).

A subset of LAK cells are referred to as cytokine induced killers (CIK). CIKs are polyclonally expanded autologous T cells isolated from PBMC and activated

ex vivo with a combination of cytokines, usually consisting of IFN-gamma, IL-2, and an agonist CD3 antibody. CIKs have proven efficacious in both clinical and mouse models of cancer (72-75) and have been used clinically for the treatment of hepatocellular carcinoma (76), Hodgkin disease, non-Hodgkin lymphoma (77), and chronic myeloid leukemia (74). What separates these cells from the traditional antigen specific adoptive cell therapies are that these cells possess the markers of both T cells and NK cell (CD3+ CD56+), and are able to mediate cytolytic functions in an antigen independent manner. (75, 77-81). When administered after hematopoietic stem cell transplant, CIK have been found to kill tumor cells that make-up minimal residual disease. These killing mechanisms are primarily dependent upon the perforin/granzyme pathways and occur independent of signaling through CD3, once again demonstrating their antigen independent function (77, 81). Interestingly, CIKs have been found to express the NK cell activation marker NKG2D, which appears to be crucial for the cytolytic capabilities of these cells (82). Studies where NKG2D signaling was silenced or blocked resulted in the attenuation of the killing function of CIKs, thus suggesting that NKG2D-mediated cell activation plays a role in the cytolytic function of CIK T cells (80, 82).

Primary Immunotherapy Effectors: Cytotoxic CD8 T cells

Most of the immunotherapy regimens discussed in the previous sections sought to harness and expand the power of antigen specific cytotoxic CD8 T cells. The

next section will address the generation of CD8 T cell responses and memory formation.

CD8 T Cell Responses and Memory

Traditional understanding of CD8 T cell responses is that they are robust and highly specific. MHC restriction holds that CD8 T cells can only recognize antigen through their TCR if that antigen is presented by an APC in the context of self MHC I (83-87). As a fail-safe for T cell activation, the peptide/MHC I bearing APC must also present the proper co-stimulation in the form of B7 molecules, which engage CD28 receptors on the CD8 T cell and allow for proliferation and effector differentiation and function. Without proper co-stimulation, these cells become anergic and unresponsive. Concurrent with this initial activation, CD8 T cells also need to receive help in the form of cytokine support from CD4 T-helper cells (88). In the absence of CD4 signaling, primary CD8 T cell responses can still occur, but these cells are unable to perform later as effective memory cells in response to secondary antigenic challenge (46, 88-90). After naïve T cells become effector cells, they travel to sites of infection and mediate their killing functions. These effector functions are primarily in the form of perforin and granzyme release, but may also involve the production of cytokines such as IFN-g and the triggering of death pathways through interactions between Fas and TRAIL-receptor expressed on target cells with Fas ligand and TRAIL expressed on the cytolytic CD8 T cell. As the levels of antigen

decrease within the tissues, >90% of the effector T cells die off, however, a small subset of them will differentiate to become long lived memory cells (91).

A hallmark of CD8 T cell memory is the ability to respond faster and more vigorously to stimulation with cytokine and antigen. Two subsets of memory T cells have been described. Central memory T cells are characterized by the expression of CD44, CD62L and CCR7, and are noted for their rapid proliferation but diminished effector functions (92-94). The expression of CD62L and CCR7 allows these cells to migrate and reside in lymphoid tissues where they lie in wait for re-encounter with their cognate antigen. Effector memory T cells are characterized by their expression of activation markers such as CD44, LY6c, and CD69, however they do not express high levels of the lymph node homing chemokines CD62L and CCR7, which allows them to reside in the peripheral blood and tissues (92, 94-96). When resting, these effector memory cells possess a phenotype similar to that of effector T cells ($CD44^{hi}$) except they express low levels of the high affinity IL-2Rα, CD25 (97). Upon re-encounter with antigen, CD25 is rapidly upregulated and these cells are able to mediate immediate effector functions such as IFN-gamma production and the expression of cytolytic molecules such as perforin and granzyme B (92, 93, 95, 98, 99).

There has been much debate about how memory T cell differentiation occurs. Until recently, two models have dominated. The "asymmetric division" model posits that naïve CD8 T cells are capable of differentiating into either an effector T cell precursor or a memory T cell precursor. In this model, the

effector T cell precursors are only capable of a primary response and terminally differentiate as effectors, whereas memory precursor T cells respond only to a secondary encounter with antigen (100-102). In the "linear" model of memory T cell differentiation, effector T cells are capable of differentiating into either cytolytic effector memory T cells, or prolific central memory T cells. Evidence supporting the linear model of memory T cell differentiation has recently emerged. Bannard and colleagues demonstrated that CD8 T cells which have acquired an effector phenotype as determined by granzyme B expression, are indeed capable of secondary replicative function in response to antigenic rechallenge (101, 102). In a concurrent study, Teixero and colleagues found that in addition to the necessity for helper CD4 T cells and IL-2 at the time of naïve to effector cell priming, the TCR signaling requirements for the generation of a primary repsonse are qualitatively different from those required for a secondary memory response (46, 47, 88, 89, 101, 103, 104). Collectively, these studies demonstrate that effector cells are capable of differentiation into either central or effector memory cells, and that the putative requirements for TCR signaling in the generation of secondary responses differ from those needed to induce a primary CD8 T cell response.

Tissue Resident Memory CD8 T cells

Large numbers of effector memory T cells can be found in virtually every tissue in the body (94, 98, 105-107). The rapid and potent function of effector memory CD8 T cells combined with their localization in peripheral tissues gives these cells a distinct functional advantage over naïve and central memory T cells, which require trafficking to the lymph nodes. Tissue-resident effector memory T cells have been found to respond more efficiently to secondary antigenic challenge than cells of the same specificity residing in lymphoid tissues, and it has been further proposed that the extravasation of memory T cells from the blood vessels into peripheral sites programs these cells to have greater lytic function (94, 108).

The increased function of tissue-resident memory T cells raises an interesting point. Traditional immunological dogma holds that the priming of adaptive immunity and memory T cell responses occurs in the secondary lymphoid tissues; however, it has recently been proposed that T cell priming can occur outside of these restricted sites (109). Indeed, current studies have demonstrated that tissue-resident dendritic cells and CD4 T cells can prime secondary CD8 T cell responses within peripheral tissues (109). It has also been found that naïve and memory T cell activation can occur in the complete absence of secondary lymphoid tissues (110). This is supported by the observation that the lymph node-independent secondary activation of tissue-resident memory T cells contributes to the localized control of latent herpes simplex virus (HSV)

infections (111). These studies suggest a possible role for tissue-resident memory CD8 T cells in the localized control of latent and secondary infections, as well as tumor immunity.

Homeostatic Proliferation and "Virtual" Memory Cells

While it is traditionally accepted that most T cells that possess a memory phenotype have progressed through an effector phase of activation and differentiation, it has also been observed that naïve T cells can adopt a memory phenotype without undergoing an activation and effector phase in response to antigen (112-118). It has been demonstrated that under extreme lymphopoenic conditions, CD8 T cells can undergo homeostatic proliferation (HP) and upregulate memory cell marker such as CD44, LFA-1, CD122, and LY6C (117, 119). HP of naïve T cells does not require TCR engagement; however, it has been found to require interactions with self-MHC (120, 121). What is interesting about these cells is that the adoption of a memory phenotype is not permanent, as it has been found that when cell numbers normalize within the system, homeostatic proliferation derived "memory" cells can return to their naïve phenotype (117, 120). These cells are functionally active and have been found to mediate antitumor effects, bactierial immunity, as well as autoimmunity and allograft rejection (113, 118, 122-124).

An additional population of HP driven cells have also been identified. Haluszczak and colleagues have described a population of memory cells which

they refer to as "virtual memory" T cells. These subsets of memory CD8 T cells develop through homeostatic proliferation and are defined by their CD44hi phenotype and their responsiveness to cytokines. The observation that these cells are present in germ-free mice and are responsive to a variety of nominal antigens suggests that they are not responding to environmental or commensal antigens, and that these cells represent a repertoire of specificity similar to that of naïve CD8 T cells. However, these cells are unique from antigen stimulated memory CD8 T cells, and those that are generated through homeostatic proliferation in that they are unable to gain effector function in response to their cognate antigen. In contrast, these cells are unable to respond to TCR ligation, but remain acutely sensitive to cytokine stimulation via IL-12 and IL-18 and respond in a manner similar to conventional antigen stimulated memory cells (118). The origin of virtual memory cells remains unknown (ie. Homeostatic proliferation or reaction to environmental antigens); however, in light of their frequency in unprimed animals (10-30% of CD8 T cell populations) and their sensitivity to cytokine stimulation, it has been suggested that they may play a role in the generation of both innate and adaptive immune responses.

Bystander Proliferation: Cytokine Sensitivity

Early studies observed that T cell responses to viral infections were robust and characterized by the rapid proliferation of T cells at sites of infection. However, it was also observed that of all of the CD8 T cells expanding during the

peak of certain viral infections, these populations were found to be comprised of less than 1% viral specific CD8 T cells. These findings suggested that in some viral infections, the majority of the CD8 T cells that were expanding were doing so in an antigen independent manner, likely through bystander proliferation (125-128). Sprent and colleagues were the first to show that antigen independent bystander proliferation of CD8 T cells *in vivo* is the product of cytokine stimulation (125, 128-130). Subsequent studies by Sprent and several other groups elaborated on this concept and observed that CD44hi memory CD8 T cells were responsible for the expansion, which occurred in response to cytokine stimulation, and that type I IFN can trigger this expansion by inducing the production of IL-15 (131). Bystander proliferation has since been found to occur in response to several types of viral, bacterial, and parasitic infections such as influenza, LCMV, *listeria*, and *leishmania* (132-137). While the phenomena of bystander proliferation is well documented in response to pathogenic challenge, the effector function of these cells has yet to be clearly addressed. Memory CD8 T cells that expand in response to bystander proliferation are cytokine competent and produce IFN-gamma in response to stimulation with their cognate antigen and can lyse cells expressing their antigen after their cytokine induced expansion (125, 129). However, the question remains about the role these cells might play in the initial immune response that produces the cytokine which induces their activation and proliferation.

Cytokine based immunotherapies

Thus far, the majority of immunotherapy regimens involving CD8 T cells have focused on the generation of antigen-specific responses; however, there is evidence that cytokine activation and memory T cells can play a role in anti-tumor immunity in the absence of TCR engagement. The next few sections will address the antigen independent activation of cellular immunity by cytokine stimulation.

Interferons

Interferons are a class of cytokines that are produced in response to viral infections, and play an important role in the modulation of the immune system. Interferons are separated into two classes. Type I interferons include interferon-α and interferon-β, both of which bind to the type I IFN receptor and mediate strong anti-viral responses. IFN-α is produced by leukocytes, whereas IFN-β is predominantly produced by fibroblasts. IFN-α also plays a crucial role in the expansion of memory T cells via bystander proliferation (129). IFN-γ is the only member that binds to the type II IFN receptor, and is known mostly for its immunomodulatory effects, and is produced by activated T and NK cells (26, 27). The mechanisms by which interferons can mediate anti-tumor effects include: direct effects on tumor proliferation, the upregulation of major histocompatibility complexes (MHC) and tumor associated antigens, and the activation of immune components such as macrophages, NK cells and T cells (26, 27).

Interleukins and cancer immunotherapy

Interleukin-2 (IL-2) is a common gamma chain cytokine produced by activated T cells which acts as a potent activator of T and NK cells. IL-2 was the first cytokine observed to stimulate anti-tumor effects *in vivo* and received FDA approval for therapeutic use in the treatment of RCC in 1992 (26, 27). As a single agent, IL-2 therapy induced responses in both melanoma and RCC patients (70); however, high dose IL-2 therapy is associated with severe dose limiting toxicities, which has limited it's use (25, 70, 138, 139). Combination therapy with IFN-α and IL-2 has been tried as a method to allow a reduction in the dose of both reagents, thus lowering toxicities, and allowing outpatient treatment regimens. These treatment studies resulted in a 26% response rate in RCC, and demonstrated similar responses to high dose IL-2 therapy with a reduction in the resulting toxicities (25).

The IL-2 Receptor is made up of three subunits: IL-2Rα (CD25), IL-2Rβ (CD122), and the common gamma chain receptor γc (CD132) (140). The association of all three receptor subunits is required for signaling through the high affinity receptor, whereas the combination of CD122 and CD132 make up the intermediate affinity receptor. CD25 does not possess a cytoplasmic tail, and therefore is incapable of signaling independently of CD122 and CD132. However, CD25 rapidly binds low levels of IL-2, which leads to further association with CD122 and CD132. Upon receptor ligation, IL-2, CD122, and γc

are rapidly internalized and destined for lysosomal degradation, whereas CD25 is quickly recycled to the cell surface. It has been suggested that CD25 has a very rapid association rate for IL-2, which when coupled with the slow dissociation rate of CD122, increases the opportunity for full CD25/CD122/CD132 complexes to form and prolonged signaling to occur (140, 141). These receptors are differentially expressed on T cells depending on their activation status. CD25 is rapidly, but transiently expressed on effector T cells after TCR engagement (140, 142), whereas CD122 is expressed by memory T cells and CD132 is constitutively expressed by all T cells. While CD25 traditionally requires TCR engagement for upregulation, CD122 and CD132 are both inducible by autocrine or paracrine IL-2, which renders cells that constitutively express these receptors uniquely sensitive to the effects of this cytokine (140).

IL-12 is a cytokine that has been examined for efficacy both as a single agent, and in combination with other cytokines. IL-12 is produced by activated APC and has potent effects on T cells and NK cells. IL-12 and IL-2 combination therapy induces the reciprocal activation of each cytokine's receptors and can promote the activation of an antigen specific response. IL-12 stimulation in combination with CD28 ligation has also been shown to induce IFN-g production by human T cells (143, 144). IL-12 also synergizes with another common-gamma chain cytokine in IL-15, and has been found to induced anti-tumor effects when used in combination with IL-18 as well (143, 145).

IL-15 is another common gamma chain cytokine that is crucial for the maintenance of T cell and NK cell populations and has been used in combination with an agonist CD40 antibody to mediate antitumor effects in murine models of colon cancer (146). While IL-15 has demonstrated some efficacy in mouse models of cancer, its use as a single agent has been limited due to the fact that it must be crosslinked by the IL-15Rα on the surface of monocytes or dendritic cells and presented to T cells and NK cells. In the absence of another stimulus to upregulate the expression of the IL-15Rα on antigen presenting cells, the long-term efficacy of IL-15 alone may be limited (146-148). Newer studies are circumventing the need for APC expression of IL-15Rα by co-administering IL-15 along with soluble IL- IL-15Rα to aid in crosslinking and presentation. In these models, IL-15 has been shown to augment vaccine responses, improve immunity against some infections, and augment tumor death in a variety of mouse and primate tumor models (49, 149-152). IL-15 has also been investigated as an alternative to IL-2 administration due to lower toxicities, and due to the fact that although IL-15 shares the IL-2Rβ and γc receptor, it does not signal through the IL-2Rα CD25, and therefore it does not induce the expansion of Tregs.

IL-7 is another common gamma chain cytokine that is crucial for the generation of memory T cells. IL-7 is required for the homeostatic proliferation of naïve T cells, and when used in combination with IL-6 has been shown to induce the antigen independent activation of CD8 T cells and augment their cytolytic and proliferative capabilities when later stimulated with antigen (153).

However, as a single agent, IL-7 has not been found to support the proliferation of naïve T cells following stimulation with anti-CD3 (153). This is in contrast to IL-2 or IL-15, both of which support the robust proliferation after TCR engagement when used as single agents or in combination with IL-6 or IL-21 (153).

Dose-limiting toxicities are a common occurrence with singular cytokine and combination therapies. The most common toxicity associated with cytokine immunotherapy is cytokine release syndrome. It has been observed that treatment with a combination of IL-18 and IL-12 can result toxic levels of NK cell-produced IFN-gamma (143, 154). The best documented cytokine immunotherapy associated toxicities are those induced by IL-2. Systemic administration of high dose IL-2 has been associated with hypotension and vascular leak syndrome, both of which can contribute to multi-system organ failure (140, 155). These effects have been observed to be dependent on the activation of neutrophils and NK cells, which can attack and compromise the integrity of vascular endothelium, thus leading to the extravasation of fluids into the lungs (155). Based on these observations, combination therapies not only provide the best opportunity for therapeutic effects, but also allow for the dosing of both reagents to be decreased, thereby circumventing serious toxicities.

Anti-CD40 and Interleukin-2 Immunotherapy: A model of cytokine activation in cancer

CD40-CD40L interactions are crucial for the development of normal innate and adaptive immune responses. CD40 is expressed predominantly on B cells and professional antigen presenting cells (APC) such as dendritic cells, and plays a major role in the survival, development, and activation of these cell types (156). On APC, CD40 is expressed as a co-stimulatory molecule. Upon the interactions between a T cell receptor and an MHC/antigen complex presented on the surface of an APC, CD40-CD40L binding acts to activate the T cell and induce cytokine secretion by the APC. This interaction also occurs between T cells expressing CD40L and resting B cells, which express low levels of CD40, but which upon ligation become activated and enter the cell cycle (157). CD40 is also expressed on monocytes. Upon ligation of CD40, it has been found that macrophages can mediate T cell independent anti-tumor effects (158), and will increase their production of nitric oxide, thus leading to increased tumoricidal activity (159, 160).

CD40 is crucical for the generation of normal B cells. It has been observed that in the absence of CD40 signaling, there is a loss in memory B cell function, the formation of germinal centers, and an inability of immunoglobulins to isotype class switch (161). In the absence of CD40 signaling in humans due to a mutation in the CD40L gene, X-linked hyper IgM syndrome results, which is characterized clinically by severe susceptibility to bacterial infections, increased

occurrence of carcinomas and lymphomas, and an over-abundance of IgM antibody (162, 163). IgM accumulates due to the lack of CD40/CD154 signaling and the inability of B cells to immunoglobulin class switch.

Many preclinical studies have adopted the use of agonist anti-CD40 antibodies to simulate the interaction between CD40 and CD40L. CD40 stimulation has been investigated for use as an adjuvant in coordination with DC vaccines and cytokine therapies to generate antigen specific T cell responses (164). In addition to these studies, an effective form of immunotherapy has been developed by combining CD40 stimulation via agonist antibodies, with the cytokine interleukin-2 (IL-2). As independent agents, both of these treatments have proven beneficial for the treatment of renal cell carcinoma and melanoma (70, 165-167); however, it has become apparent that cytokines and receptor agonist antibodies work better when used in concert with each other, rather than independently(168-171).

In previous studies, our laboratory used an agonist murine CD40 antibody, which when administered in combination with IL-2, resulted in synergistic anti-tumor effects in an orthotopic model of renal cell carcinoma (168-171). We hypothesized that these anti-tumor responses were the result of the coordinated activation and maturation of antigen presenting cells, namely DCs by anti-CD40, and the induction of T cell proliferation and survival by IL-2. We observed that stimulation with IL-2 or anti-CD40 alone resulted in moderate increases in survival, as well as T cell and DC proliferation, but only the combination therapy

resulted in optimal anti-tumor responses. Studies using depleting antibodies, and knockout mice have shown that the anti-tumor effects generated with the combination therapy were $CD8^+$ T cell mediated, and were dependent on the expression of Fas ligand and interferon gamma (IFNγ) (168, 169, 171).

Interestingly, we later found that IFNγ, which is crucial for the development of primary anti-tumor effects after therapy, resulted in high levels of $CD4^+$ T cell death. It has been previously established that stimulation through CD40 can bypass the need for $CD4^+$ T cell help in the generation of primary $CD8^+$ T cell responses; however, it is widely accepted that $CD4^+$ T cell help is crucial for the generation or maintenance of secondary $CD8^+$ T cell memory responses (46-48, 88, 103, 172). In our studies, we found that the IFNγ mediated death of $CD4^+$ T cells after anti-CD40 and IL-2 immunotherapy resulted in the loss of subsequent secondary anti-tumor responses. It is also interesting to note that the negative effects of anti-CD40/IL-2 on $CD4^+$ T cells is not unique to this therapy, as we have also observed this phenomena in mice that received other immunotherapeutic regimens such as anti-CD40/IL-15, and CpG/IL-12 (168).

After the induction of strong immune activation, such as generated with anti-CD40/IL-2 immunotherapy, there are several inhibitory mechanisms that come into play to prevent undo damage to the host. Another observation we have made with anti-CD40/IL-2 immunotherapy is a marked expansion in $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) (168, 169). It is interesting to note

that the cell death we observed in the conventional $CD4^+$ T cell population was largely absent from the Treg population. We found that this was due to the preferential upregulation of programmed death 1 (PD-1) on the surface of the conventional $CD4^+$ T cells, which was not seen on the Treg cell population. We hypothesized that the mechanisms of the IFNγ mediated $CD4^+$ T cell loss after immunotherapy might be due to the upregulation of B7H-1 on the surface of various cells types, in coordination with the upregulation of PD-1 on the surface of the conventional $CD4^+$ T cells (169).

The effects of anti-CD40 and IL-2 are not limited to the generation of immune responses. In contrast to our studies, Hamzah and colleagues utilized the expression of CD40 on endothelial cells as a target for the treatment of spontaneously arising islet cell carcinomas. In a spin off of the combination therapy reported by our group, they created a fusion protein of an anti-CD40 antibody and IL-2, which preferentially homed to the sites of tumor vasculature. The anti-tumor effects that resulted from treatment with the fusion protein were greater than those obtained with the unconjugated anti-CD40 antibody and IL-2 (173). In contrast to our studies, they concluded that the anti-tumor effects after intratumor injection of the fusion protein were due to the disruption of the vasculature, and were not immune mediated. It is interesting to note that they did not see a loss of anti-tumor effects when mice were depleted of $CD4^+$ and $CD8^+$ T cells. In fact, the abrogation of anti-tumor effects were seen when they treated tumors in mice whose endothelium lacked CD40 expression. In keeping

with the variable expression of CD40 on transformed cells, immunohistochemistry studies in these mice also showed that CD40 expression was only upregulated on the vascular endothelium of tumors, and was not observed on normal pancreatic parenchyma and endothelium. This study demonstrates the versatile roles of cytokine immunotherapy, and suggests that use of these reagents may function beyond non-specific immune activation.

Based on these studies, it is clear that the global activation of the immune system by high dose cytokine therapy results in both desired anti-tumor responses, as well as the activation of toxicities and regulatory mechanisms which might be deleterious to the generation of tumor immunity. The following dissertation will examine primary CD8 T cells responses generated with anti-CD40 and IL-2 immunotherapy. While the majority of the data presented herein utilizes the combination of anti-CD40 and IL-2, it is important to note that other cytokine regimens have produced similar results ie: CPG/IL-12 and CD40/IL-15 (168), and that we are using anti-CD40 and IL-2 as a model for strong systemic cytokine immunotherapy. What is novel about these studies, is that they test the notion that antigen specificity is required for the generation of efficient anti-tumor responses, and propose a new role for memory CD8 T cells in the development of tumor immunity.

Part One

CD8 T cell responses following anti-CD40 / IL-2 immunotherapy: The role of memory T cells

Introduction

CD40 is a member of the TNF receptor superfamily and is predominantly expressed on B cells and professional antigen presenting cells such as monocytes and dendritic cells (DC). Upon engagement by its ligand (CD154), CD40 plays a major role in the survival, development, and activation of these cell types. Dendritic cell maturation via CD40 signaling has been found to be crucial for the generation of effective cytotoxic T lymphocyte responses, which has made CD40 stimulation via CD154 or agonist antibodies an attractive target for immunotherapy. Interleukin-2 (IL-2) is a cytokine that has been used clinically to augment immune mediated anti-tumor effects for the last 30 years. Because of the role it plays in the augmentation, maintenance, and survival of T cell responses and its clinical use, IL-2 also made an attractive target for use in combination immunotherapy with anti-CD40. Supplemental Figure 1 outlines the standard regimen of anti-CD40 and IL-2 used throughout the course of these studies. The doses of both reagents were reduced in BALB/c mice due to toxicities observed in those mice at the higher dose that proved tolerable to C57BL/6 mice.

We have previously shown that cytokine immunotherapy consisting of an agonist CD40 antibody in conjunction with IL-2 leads to synergistic anti-tumor

effects in a variety of tumor models (168-171). These anti-tumor effects are CD8 T cell mediated and dependent upon the expression of FasL and the production of IFN-gamma (171). The goal of this study was to examine the role of antigen specificity in the efficacy of anti-CD40 and IL-2 immunotherapy. This therapy is characterized by a large expansion in CD8 T cells, an observation which has been made in both naïve and tumor bearing hosts. In light of the massive expansion in CD8 T cells in the presence or absence of antigen, we hypothesized that the anti-tumor effects generated with this therapy are the result of the activation and augmented antigen independent lytic capabilities of CD8 T cells.

Results

Anti-CD40 and IL-2 immunotherapy leads to CD8 dependent anti-tumor responses in 3LL bearing C57BL/6 mice

Previous studies in our lab have shown that combined immunotherapy with anti-CD40 and IL-2 immunotherapy results in anti-tumor effects in IV and orthotopic models of Renal cell carcinoma in BALB/c mice. These models were well established and through depletion studies, we had demonstrated that the anti-tumor effects generated with anti-CD40 and IL-2 immunotherapy were dependent on CD8 T cells, but were NK cell independent. We observed that anti-CD40 and IL-2 immunotherapy was effective in mediating anti-tumor effects in a subcutaneous model of the 3LL Lewis lung carcinoma; however, we had not established if CD8 T cells were the primary mediators of these effects (Figure 1a). To determine this, we inoculated C57BL/6 with 2×10^6 3LL tumor cells subcutaneously in the right flank, monitored them for tumor engraftment, and then initiated immunotherapy. As expected, we found that anti-CD40 and IL-2 was able to cure 3LL bearing mice of their tumors (Figure 1a); however, when CD8 T cells were depleted at the initiation of immunotherapy, the anti-tumor effects were diminished (Figure 1b). A possible explanation for the incomplete abrogation of anti-tumor effects following CD8 depletion with immunotherapy is that mice only received one administration of CD8 depleting antibody. This was due to unforeseen toxicities which presented directly following treatment of the mice with the CD8 depleting antibody and anti-CD40 and IL-2, which prevented

further administration of the CD8 depleting antibody. Despite the incomplete effects, these data suggest that CD8 T cells are the primary mediators of anti-tumor effects against subcutaneous 3LL tumors following immunotherapy. We did not perform depletion studies for NK cells in this study because previous studies by our laboratory observed that NK depletion during immunotherapy does not diminish, but in fact enhances anti-tumor effects after immunotherapy. Furthermore, the tumoricidal effects of NK cells are predominantly limited to the blood stream and are most effective against metastases and blood-borne tumors. As such, their contribution to any anti-tumor effects generated against this tumor were likely minimal.

Memory CD8 T cells preferentially expand in response to immunotherapy

We originally hypothesized that combined anti-CD40 and IL-2 immunotherapy was arming both the innate and the adaptive arms of the immune system by inducing dendritic cell maturation and stimulating T cell proliferation. It was initially presumed that these responses would result in the expansion of CD8 T cells that were specific for unknown tumor antigens. Curiously, we observed a significant expansion of CD8 T cells after immunotherapy in naïve mice that had not received tumor (Figure 2). We found that CD8 T cells begin to expand in the spleen and lymph nodes of non-tumor-bearing mice as early as day 3 (Figure 2A,B) after the initiation of

immunotherapy, and that they continue to expand through days 5 (Figure 2C,D) and 11 (Figure 2E,F).

Phenotypic analysis of CD8 T cells following immunotherapy determined that the majority of CD8 T cells following immunotherapy expressed CD44, indicating that they were of an effector or memory phenotype (Figure 3A-C). These effects were observed with both anti-CD40 and IL-2 immunotherapy (Figure 3B), as well as a IL-12 and IL-2 immunotherapy (Figure 3C). Because CD44 is upregulated during homeostatic proliferation as well as during effector and memory T cell differentiation, it was not clear if the CD8 populations that were proliferating after immunotherapy were the result of the expansion of pre-existing memory cells, or due to the conversion of naïve cells to an effector/memory phenotype.

To examine the expansion of memory cells versus the conversion of naïve cells to a memory phenotype after immunotherapy, we treated thymectomized C57BL/6 mice, or unaltered C57BL/6 littermates with immunotherapy and harvested spleens on day 11 and day 42 after the initiation of treatment for phenotypic analysis. The thymectomized mice allowed us to examine the effects of immunotherapy on naïve T cells in the absence of thymic output of new naïve T cells. We observed that the number of naïve CD8 T cells remained static 11 days after immunotherapy in both the thymectomized and unaltered mice (Supplemental Figure 2C). Interestingly, the numbers of naïve cells increased slightly at day 42 in the unaltered mice, a likely product of thymic output,

whereas the number of naïve T cells in the thymectomized mice remained unchanged (Supplemental Figure 2D). These data suggest that immunotherapy does not induce the expansion of naïve CD8 T cells. We also found that found that effector/memory CD8 T cells from unaltered and thymectomized mice are expanded 11 days after immunotherapy (Supplemental Figure 2A). Additionally, the effector/memory CD8 T cells from immunotherapy treated mice remain elevated 42 days after immunotherapy compared to control mice in both the unaltered and thymectomized mice (Supplemental Figure 2A,B). However, the numbers of effector/memory CD8 T cells are greatly contracted compared to their levels at day 11 (Supplemental Figure 2A,B). These data demonstrate that effector/memory CD8 T cells preferentially expand in response to immunotherapy, and remain expanded long after the cessation of therapy.

In keeping with the observations from the thymectomy experiments, BrDU analysis of CD8 T cells from immunotherapy treated C57BL/6 mice showed that CD44hi CD8 T cells are proliferating after immunotherapy (Figure 3D-G). These data support the evidence that the increased numbers of CD44hi CD8 T cells are due to the expansion of memory T cells, and not the conversion of naïve cells to an effector phenotype (Figure 3E). Remarkably, these experiments were carried out in naïve mice in the absence of tumor or antigenic stimulation, which demonstrates the cytokine sensitivity of effector/memory CD8 T cells to cytokine stimulation.

Memory CD8 T cells are activated after immunotherapy but do not bear surface markers consistent with recent TCR engagement

Once we had established that memory CD8 T cells were expanding after immunotherapy, we wanted to examine these cells for the expression of other activation molecules. Further analysis determined that in addition to expressing high levels of CD44, these cells also demonstrated increased expression of memory, activation and effector molecules such as CD122, granzyme B, IFN-gamma, Ly6C, and NKG2D (Figure 4). Surprisingly, these cells did not show appreciable increases in the expression of CD69, PD-1, or CD25 (Figure 4). The lack of PD-1 and CD25 upregulation following immunotherapy is particularly interesting, as this suggests that the activation of these cells has occurred in the absence of TCR ligation. In addition to their presence in the spleen (Figure 4) and lymph nodes (data not shown), it is important to note that $CD25^{low}$ $PD\text{-}1^{low}$ CD8 T cells have also been observed within the tumors of orthotopic renal cell carcinoma (Renca) bearing mice following immunotherapy (Data not shown) (Wilkins et al, manuscript in preparation). This is a tumor model in which cytokine immunotherapy with anti-CD40 and IL-2, as well as IL-12 and IL-2 has shown great efficacy in prolonging the life of tumor bearing mice, so the presence of these cells within tumors may be of significance to the therapeutic outcome of these therapies (143, 171, 174-177).

To further interrogate the expression of CD25 and PD-1 under conditions of cytokine treatment versus TCR ligation, we performed *in vitro* experiments

where splenocytes were cultured with either high doses of IL-2, or with anti-CD3 and anti-CD28 antibodies. We found in these studies that high doses of IL-2 did not lead to appreciable increases in the surface expression of PD-1 or CD25 on CD8 T cells (Figure 5). However, upon TCR ligation via anti-CD3 and so-stimulation via anti-CD28, CD8 T cells significantly upregulate the expression of PD-1 and CD25, thus confirming that these surface molecules are good indicators for recent TCR stimulation (Figure 5) (178-180).

Because CD25 is rapidly upregulated in response to TCR ligation, we questioned if the reason were not seeing CD25 expression on CD8 T cells following immunotherapy was due to the fact that we were looking too late after the initiation of immunotherapy (day 11). To exclude this possibility, we treated naïve C57BL/6 mice with the standard regimen of anti-CD40 and IL-2 and looked for CD25 expression on CD8 and CD4 T cells on day 3 (Figure 6A,B) and day 5 (Figure 6C,D) after the initiation of therapy. We did not observe a significant increase in the expression of CD25 on CD8 T cells at these timepoints; however, we did notice a marked increase in CD25 expression on CD4 T cells. We have published that this therapy results in a large expansion of regulatory T cells (Tregs) (169), so it is likely that the population of CD4 T cells expressing CD25 are Tregs.

Immunotherapy increases the lytic capabilities of CD8 T cells against antigen-irrelevant tumor targets

We next wanted to determine the functional capabilities of CD8 T cells following immunotherapy. To examine the TCR-independent lytic capabilities of CD8 T cells following immunotherapy we treated C57BL/6 mice with the standard regimen of anti-CD40 and IL-2 immunotherapy, harvested and enriched CD8 T cells, then performed redirected lysis assays against P815 targets. The redirected lysis assay allows for the assessment of the MHC-independent killing abilities of CD8 T cells through the crosslinking of their CD3-TCR complex with an anti-CD3 antibody bound to Fc receptor-bearing radiolabeled target cells (181). The lysis of target cells denotes the activation state and lytic capabilities of the CD8 T cells. We found that following immunotherapy, CD8 T cells are capable of mediating the antigen independent lysis of tumor targets (Figure 7A).

We also examined the lytic capabilities of CD8 T cells against allogeneic Renca tumor targets in a flow cytometry based assay. Renca tumor targets were labeled with CFSE and co-cultured with CD8 T cells isolated from mice that had received either control treatment or immunotherapy. We found that CD8 T cells isolated from immunotherapy treated mice were capable of decreasing the frequency of CFSE labeled tumor targets (Figure7B). These data demonstrate that CD8 T cells are effective killers after immunotherapy and do not require TCR recognition of tumor targets to mediate cytolytic function *in vitro*.

Summary

Collectively these data demonstrate that immunotherapy induces the expansion and activation of memory CD8 T cells. We also show that memory CD8 T cells do not express CD25 or PD-1 following immunotherapy. As we also demonstrate that TCR ligation leads to the upregulation of PD-1 and CD25 on CD8 T cells *in vitro*, these findings suggest that the CD8 T cell populations expanding after immunotherapy have not recently undergone TCR mediated activation. Despite the lack of apparent TCR stimulation, CD8 T cells are effective killers of tumors *in vivo*, and of TCR irrelevant tumor targets *in vitro* following immunotherapy. In light of these findings, we hypothesized that the anti-tumor effects generated by CD8 T cells following high dose cytokine immunotherapy are antigen independent, and not due to tumor recognition by antigen specific CD8 T cells. The next series of studies sought to identify the role of antigen specificity in the antitumor effects generated after anti-CD40 and IL-2 immunotherapy.

Part Two

CD8 T cell responses following anti-CD40 / IL-2 immunotherapy: The role of antigen specificity

Introduction

OT-1 mice: A model for examining antigen specific responses

Based on the data presented in part one of this dissertation, we had reason to believe that antigen recognition of the tumor by TCRs on CD8 T cells following immunotherapy was not required for the generation of antitumor effects. To examine this, we used OT-1 TCR transgenic mice in a variety of adoptive transfer and direct immunotherapy treatment scenarios. OT-1 mice provide an excellent model with which to examine the expansion and effector function of antigen specific responses. OT-1 mice are a strain of TCR transgenic mice that possess a TCR specific for chicken ovalbumin (OVA). These mice have been backcrossed onto a C57BL/6 background, and were initially developed as a tool to examine positive selection within the thymus (182); however, due to their reactivity to a well characterized xeno-antigen, their use has been greatly expanded. The TCR transgenes these mice express were derived from a CD8 T cell clone which expresses a TCR specific for OVA. The T cell clone 149.42 expresses the Valpha 2 and Vbeta 5 variable regions of the TCR and recognizes the SIINFEKL peptide of OVA presented within the context of the H2-K^b MHC I molecule (182-184). To generate mice expressing the complete TCR, cDNA from the entire alpha chain of the CD8 T cell clone 149.42 was cloned in a pES4

expression vector and co-injected into blastocysts along with the genomic beta chain construct (pK5913.CB18.31). The blastocysts were from F1 (B6 x bm1) mice. Two founder lines, 253-2 and 243-2, were identified, and the 243-2 line was backcrossed onto a B6 background and submitted to the Jackson Laboratory mouse repository for distribution (182-186).

OT-1 mice are slightly lymphopoenic and possess some additional unique phenotypic qualities. First, in the thymus there is a skewing of the CD4:CD8 T cell ratio in favor of CD8 T cells, all of which express high levels of the transgenic TCR. While there is a two-fold increase in the number of CD8 T cells in the spleen and lymph nodes of these animals, there have been reports that demonstrate as much as a 7-fold reduction in the number of CD4 T cells observed in 9 week old OT-1 mice (183, 187). Interestingly, during thymic positive selection, OT-1 T cells are positively selected by peptides other than the antigenic SIINFEKL OVA peptide to which they respond in the periphery, as it has been demonstrated by several groups that the stimulation of fetal thymic organ cultures from OT-1 mice with the SIINFEKL peptide results in the rapid deletion of double positive cells (183, 187). However, due to their high affinity for SIINFEKL peptide presented within the context of H2-K^b, CD8 T cells from these mice proliferate vigorously in response to stimulation in the periphery and display strong cytolytic functions against ova-bearing target cells.

The phenotypic and activation characteristics of OT-1 mice made them a good model for examining the effects of immunotherapy on antigen specific CD8

T cell responses. In the following studies, we employed a variety of adoptive transfer and direct treatment regimens which allowed us to examine and compare the proliferative, phenotypic, and cytolytic capabilities of antigen specific CD8 T cells in both the presence and absence of antigen.

Results

Adoptively transferred OT-1 memory CD8 T cells proliferate after anti-CD40 and IL-2 immunotherapy

It has been reported that approximately 90%-95% of the CD8 T cells in OT-1 mice on a C57BL/6 background express the transgenic TCR, and that these mice have a skewing of their CD4:CD8 T cell ratio, favoring the CD8 T cells (183, 187). To determine if our in-house bred OT-1 mice recapitulated these frequencies, we examined OT-1 and C57BL/6 littermates for their expression of the Valpha2 and Vbeta5.1/5.2 TCR subunits. Indeed, we found that approximately 95-98% of the CD8 T cells in OT-1 mice possessed dual expression of the Valpha 2 and Vbeta 5.1/5.2 TCR compared to only 2-5% observed in wildtype C57BL/6 mice (Figure 8A,B). Additionally, we observed that our in-house OT-1 mice possessed approximately 5-fold fewer CD4 T cells than CD8 T cells (Figure 8C).

Once we had established a method for identifying OT-1 T cells and validated the phenotype of our colony, we next wanted to determine if immunotherapy could expand antigen specific T cells in the absence of ovq antigen. To examine this, we performed adoptive transfer studies where we adoptively transferred 2.5 x 10^6 OT-1 lymph node cells into naive C57BL/6 hosts and then treated the recipients with control reagents, anti-CD40 and IL-2, or ova vaccination. This regimen is outlined in supplemental figure 3. The ova vaccinations consisted of the IP administration of ovalbumin emulsified in

incomplete freund's adjuvant. On day 11 after the initiation of therapy, spleens and lymph nodes were harvested and analyzed for the presence of CD8 T cells co-expressing the TCR antibodies for Valpha2, and Vbeta5.1/5.2. We found that immunotherapy results in the expansion of adoptively transferred OT-1 T cells in the spleens (Figure 9A) and lymph nodes (Figure 9B), even in the absence of ova vaccination (Figure 9). We next wanted to determine if the memory subsets of the adoptively transferred OT-1 T cells expanded in response to immunotherapy, as we has previously observed in wildtype C57BL/6 mice (Figure 3). Approximately 25-30% of transgene positive CD8 T cells in control treated mice express CD44 (Figure 10A), so we expected to see an expansion of these cells after immunotherapy. We found that the percentages and total numbers of adoptively transferred OT-1 CD8 T cells expressing CD44 increased following immunotherapy (Figure 10B,D,E). As expected, we also saw a slight increase in the frequency, and significant increases in the total numbers of CD44hi OT-1 CD8 T cells in the mice that received OVA vaccination (Figure 10C). We concluded that the increases in CD44hi OT-1 T cells following immunotherapy were likely due to the expansion of memory CD8 T cells, whereas the increases in CD44hi T cells after ova vaccination were due to the conversion of naïve CD8 T cells (CD44lo, CD62Lhi) to an effector phenotype (CD44hi, CD62Lhi) due to direct stimulation of the transgenic TCR with the OVA antigen. In keeping with the observation that immunotherapy is inducing the expansion of memory T

cells, we also found that the adoptively transferred OT-1 CD8 T cells that are BrDU positive, are also predominantly of a CD44hi phenotype (Figure 11).

Direct immunotherapy treatment of OT-1 mice induces the expansion of memory CD8 T cells, but not the upregulation of CD25

We next wanted to determine if the direct treatment of OT-1 mice recapitulated the effect of immunotherapy on adoptively transferred CD8 T cells. We treated OT-1 mice with the standard C57BL/6 dose of anti-CD40 and IL-2 and examined the memory expansion of transgene positive CD8 T cells following therapy. This treatment regimen is outlined in supplemental figure 4. We found that direct treatment with immunotherapy or ova vaccination increased the frequency of CD44hi transgene positive CD8 T cells (Figure 12B,C) compared to control. The treatment of OT-1 mice with OVA vaccination provided a unique opportunity to directly examine the upregulation of CD25 in response to TCR engagement. As we observed in C57BL/6 mice, immunotherapy treatment of OT-1 mice did not result in the upregulation of CD25 (Figure 13A,D) or PD-1 (Figure 13C,E) on transgene positive CD8 T cells. However, when we examined the blood of OT-1 mice that had been vaccinated with OVA, we saw an increase in the expression of CD25 by transgene positive CD8 T cells (Figure 13B,D). PD-1 levels were also elevated on splenic CD8 T cells following ova vaccination (Figure 13C,E).

We also performed *in vitro* studies to examine the role of TCR engagement on the upregulation of CD25 on OT-1 CD8 T cells. OT-1 splenocytes were cultured with either high doses of IL-2, or with anti-CD3 and anti-CD28 antibodies. We found in these studies that high doses of IL-2 did not lead to appreciable increases in the surface expression of PD-1 or CD25 on transgene positive CD8 T cells (Figure 14). However, upon TCR ligation via anti-CD3 and co-stimulation via anti-CD28, the transgene positive CD8 T cells significantly upregulate the expression of PD-1 (Figure 14E) and CD25 (Figure 14D). These data once again demonstrate that CD25 and PD-1 are valid markers for examining the presence of TCR engagement on populations of CD8 T cells (178-180).

In light of the increased frequency of memory T cells in OT-1 mice after direct treatment with immunotherapy, and due to the fact that we had observed increased proliferation and elevated numbers of OT-1 T cells following adoptive transfer, we expected to see an increase in the number of transgene positive cells in the OT-1 mice following immunotherapy. Surprisingly, this was not the case. We did not see increases in the numbers of transgene positive CD8 T cells following immunotherapy (Figure 15). Even more surprising, was the dramatic decrease in the number of transgene positive CD8 T cells we observed in the OT-1 mice that received OVA vaccination alone (Figure 15). Collectively these data demonstrate that OT-1 memory CD8 T cells expand in response to immunotherapy, however this expansion does not result in increased numbers of

transgene-bearing CD8 T cells. As we demonstrated in Figure 8, OT-1 mice have a deficit in CD4 T cells. It may be that there are too few CD4 T cells in OT-1 mice to provide the cytokine help needed to generate proliferative responses to OVA vaccination. Other possible explanations for the decreases in the numbers of CD8 T cells following the vaccination of OT-1 mice are presented in the discussion.

OT-1 CD8 T cells display effector functions and TCR independent tumor killing after direct treatment with immunotherapy

To further examine the role of antigen specificity in the anti-tumor effects generated after immunotherapy, we wanted to determine the functional capabilities of OT-1 CD8 T cells after immunotherapy. To examine the lytic function of OT-1 CD8 T cells following immunotherapy, we used EL4 as TCR irrelevant targets and the EL4 ova transfected cell line, EG.7, as a positive control for TCR-mediated killing. We observed that OVA vaccination resulted in the increased killing capacity of ova-expressing EG.7 targets cells, but not of the parental EL4 cell line. This was expected, as we had already demonstrated that ova vaccination induces the expansion of OT-1 CD8 T cells and the upregulation of CD25 (Figures 12 and 13), both factors indicating that the cells were activated. Remarkably, immunotherapy augmented the ability of OT-1 CD8 T cells to kill the parental EL-4 cell line (Figure 16A). This was in addition to augmenting the lysis of the ova-expressing EG.7 line (Figure 16A). These data

illustrate two important points; 1) TCR tg T cells are activated by cytokine and become antigen independent killers, and 2) Immunotherapy can augment antigen specific CD8 T cell responses in OT-1 mice despite the previously reported deleterious effects of immunotherapy on CD4 T cells, and the decreased CD4 cellularity in these mice. This was demonstrated by the vigorous killing of the ova-bearing EG.7 cell line by OT-1 CD8 T cells following immunotherapy (Figure 16A).

We also wanted to determine if OT-1 mice were capable of rejecting a tumor that did not express OVA *in vivo.* We proposed that if immunotherapy augmented the antigen independent killing capabilities of CD8 T cells, we would see anti-tumor effects in TCR transgenic mice where the majority of CD8 T cells possessed TCRs specific for a peptide not expressed by the tumor. For these studies, we subcutaneously inoculated OT-1 mice with $2x10^6$ 3LL cells in the right flank and initiated anti-CD40 and IL-2 immunotherapy 7 days later. We observed a significant increase in the survival of immunotherapy treated OT-1 mice compared to untreated controls (Figure 16B). These studies provide a proof of principle that the activation of effector memory cells by strong cytokine stimulation can result in antigen-independent antitumor effects.

Summary

OT-1 mice provided an excellent model with which to examine the effects of anti-CD40 and IL-2 immunotherapy on antigen specific CD8 T cell responses.

We found that the majority of CD8 T cells in OT-1 mice express the transgenic TCR (approx 95%), and that when treated with immunotherapy in either an adoptive transfer or direct treatment scenario, these cells adopted a memory phenotype. As expected, adoptively transferred OT-1 CD8 T cells proliferated and expanded vigorously in response to immunotherapy, however we were surprised to find that these proliferative responses were not recapitulated in the direct treatment model. While OVA vaccinated OT-1 CD8 T cells upregulated CD25, and there was an increase in the frequency of CD44hi T cells, the total numbers of CD8 T cells actually decreased after vaccination. Possible mechanisms behind this cell loss are addressed later in the discussion.

To determine if OT-1 T cells were capable of TCR-independent killing after immunotherapy, we examined the *ex vivo* killing of OVA expressing and TCR irrelevant tumors after immunotherapy, as well as the *in vivo* anti-tumor effects generated after immunotherapy. We found that OT-1 CD8 T cells are capable of mediating tumor lysis *in vitro* and anti-tumor effects *in vivo* after immunotherapy. Collectively, these data demonstrate that immunotherapy with anti-CD40 and IL-2 can induce TCR-independent activation and killing mechanisms in TCR transgenic T cells and support our hypothesis that the antitumor effects generated with this therapy are not antigen specific in nature. In light of these findings, we next wanted to determine a possible mechanism for tumor recognition by CD8 T cells following immunotherapy. We selected NKG2D

as a possible candidate for these interactions. These results are discussed in the next section.

Part Three

CD8 T cell responses following anti-CD40 / IL-2 immunotherapy: The role of NKG2D in tumor recognition

Introduction

NKG2D: The merging of innate and adaptive immune function

NKG2D is a stimulatory immunoreceptor expressed by NK cells, activated CD8 T cells, and to a lesser extent on gamma-delta T cells and CD4 T cells (188). NKG2D signaling plays a crucial role in the NK cell mediated antitiumor effects generated with IL-2 and IL-18 combination therapy (189), and it has been observed that NKG2D ligation on CD8 T cells can induce MHC-unrestricted cytotoxicity after cytoking stimulation *in vitro* (82). There are qualitative differences in NKG2D signaling between NK cells and CD8 T cells. While NKG2D signaling on NK cells can trigger immediate cytolytic activity, NKG2D ligation on CD8 T cells provides more of a co-stimulatory signal and acts to enhance proliferation and effector function. (80, 190, 191).

In humans, NKG2D ligation on CD8 T cells or NK cells triggers the DAP10 adaptor molecule to signal the lipid kinase cascade. However, NKG2D signaling is more complex in the mouse where there are two isoforms of NKG2D that have been identified (NKG2DS and NKG2DL), both of which associate with both DAP10 and DAP12 adaptor molecules. It was originally thought that DAP12 was only expressed by activated NK cells; however, subsequent studies found that

cytokine activated CD8 T cells upregulate the expression of DAP12. Karimi and colleagues found that silencing DAP 12 in human cytokine activated CD8 T cells reduced their cytolytic abilities (82, 192, 193). As DAP12 possesses an intracellular ITAM motif which can trigger protein tyrosine-kinase pathways, it stands to reason that loss of this pathway would have profound effects on the activity of CD8 T cells (82).

Traditionally, NKG2D ligands are not expressed on healthy tissues, but have been found to be upregulated on malignant tissues, tumor cell lines, virally infected cells, and upon engagement of the DNA-damage-response pathway (188, 190, 194-198). NKG2D ligands in humans include the stress-inducible nonclassical MHC molecules, MICA and MICB, as well ULBP1, ULBP2, ULBP4, and ULBP4. ULBP1 to 3 have been found to be expressed on malignant tissues (80, 198). In the mouse, the three families of NKG2D ligands include the Rae-1 family of molecules (α, β, γ, δ), H60, and MULT1. These ligands are also upregulated on malignant tissues and tumor lines (199).

Because of the selective expression of NKG2D ligands on stressed and malignant cells, and because the activation of NKG2D on immune effector cells can elicit cytotoxic responses from CD8 T cells and NK cells, it has been proposed that NKG2D signaling may play a role in the generation of a variety of immune responses. NKG2D interactions have been observed to have an impact on NK and CD8 T cell responses in the context of tumor immunity and immunoediting, autoimmunity, graft rejection, and viral immunity NKG2D (188,

196, 200, 201). In light of the ability of NKG2D activation to induce MHC-unrestricted CD8 T cell function, and the broad expression of NKG2D ligands on tumor cells, we considered NKG2D to be a likely mediator for the interactions between our immunotherapy expanded memory T cells and tumor cells. Both tumor lines in which anti-CD40 and IL-2 have been used to good effect express high levels of the NKG2D ligand Rae-1 (189, 199), among others, so we decided to perform *in vitro* and *in vivo* studies to examine the role of NKG2D signaling in the antitumor effects generated by anti-CD40 and IL-2 immunotherapy.

Results

Wildtype C57BL/6 and adoptively transferred OT-1 CD44hi CD8 T cells express NKG2D after immunotherapy or antigen stimulation

Once we had established that cytokine activated CD8 T cells were capable of non-specific killing after immunotherapy, the question still remained of how these cells were recognizing tumor targets *in vivo*. We turned to the lectin-like receptor, NKG2D, as a possible candidate. NKG2D is expressed on NK cells as well as activated CD8 T cells, upon which it acts as a co-stimulatory molecule. NKG2D ligation on cytokine activated CD8 T cells has been found to mediate anti-viral immunity, as well as anti-tumor effects (188, 191, 196, 200). Because a variety of NKG2D ligands are expressed on several tumor types, NKG2D is an attractive mechanism by which the immunotherapy expanded memory T cells may be recognizing tumor cells in the absence of TCR ligation.

We first wanted to determine if NKG2D was upregulated on CD8 T cells in C57BL/6 mice following immunotherapy. We found that NKG2D is upregulated exclusively on the CD44hi subset of CD8 T cells following immunotherapy (Figure 17A-C), and that both the frequencies and numbers of NKG2D expressing NKG2D+ CD8 T cells increase following immunotherapy (Figure 17D,E). NKG2D was also upregulated exclusively on the CD44hi subset of CD4 T cells after immunotherapy (Figure 17F,G and data not shown). Subsequent studies performed in mice that received adoptively transferred OT-1 CD8 T cells yielded similar results. We observed an increase in the frequency of NKG2D-expressing

CD44hi transgene positive CD8 T cells following immunotherapy (Figure 18B). Interestingly, we also observed a moderate increase in the frequency of NKG2D+ CD44hi transgene positive OT-1 CD8 T cells following OVA vaccination (Figure 18C,D); however, the total number of NKG2D positive cells was much greater in the immunotherapy treated mice (Figure 18B,E). These data support the notion that NKG2D acts in a co-stimulatory fashion on CD8 T cells after both cytokine and TCR mediated activation.

Direct treatment of OT-1 mice results in an increase in the number of NKG2D positive CD8 T cells

Because we had observed TCR-independent antitumor effects in OT-1 mice against TCR irrelevant tumors in OT-1 mice, we wanted to determine if NKG2D was upregulated following direct treatment. We observed the same phenomena after direct OT-1 treatment as seen after adoptive transfer and in wildtype mice. Immuntherapy or OVA vaccination resulted in an increase in the frequencies of NKG2D positive CD8 T cells (Figure 19A,B). As we observed in the adoptive transfer experiments, NKG2D expression increased after vaccination, however, the total number of NKG2D positive cells was higher in the immunotherapy treated group (Figure 19C). These differences are likely due in part to the method of stimulation (TCR versus cytokine), as well as to the vast decrease in CD8 T cell cellularity we observed in OT-1 mice following OVA vaccination (Figure 15).

NKG2D blockade diminishes antitumor effects *in vitro* and *in vivo*

Renca tumor cells express high levels of the NKG2D ligand, Rae-1 gamma, which makes them an attractive target for NKG2D mediated recognition *in vitro* and *in vivo* (Figure 20A). To examine a role for NKG2D in antitumor responses *in vitro*, we treated C57BL/6 mice with anti-CD40 and IL-2 and examined the ability of isolated CD8 T cells to lyse Renca tumor targets in the presence or absence of an NKG2D blocking antibody. We observed a two-fold decrease in the lysis of Renca cells when NKG2D was blocked *in vitro* (Figure 20B). To examine the role of NKG2D in antitumor responses *in vivo*, BALB/c mice were inoculated with subcutaneous Renca tumors and then treated with immunotherapy with or without the administration of an NKG2D blocking antibody. The immunotherapy treated mice displayed complete tumor regression, whereas, the tumors in the group that received NKG2D blockade and immunotherapy did not regress (Figure 20C,D). It is important to not that NKG2D blockade did not completely abrogate the anti-tumor responses after immunotherapy. These data suggest that the TCR independent tumor recognition which occurs after immunotherapy is NKG2D assisted, but that there are likely other mechanisms contributing to tumor recognition and killing.

Summary

In this final section, we submit NKG2D as a possible mechanism by which CD8 T cells recognize tumor cells following immunotherapy. We found that NKG2D is upregulated on CD8 T cells following immunotherapy in wildtype C57BL/6 mice, after OT-1 adoptive transfer, and after direct OT-1 treatment. We also observed an increase in NKG2D expression by OT-1 CD8 T cells following OVA vaccination, which suggests that NKG2D may have a function in the generation of antigen specific responses as well. Final proof of the role of NKG2D in anti-tumor effects following immunotherapy is provided with the observation that NKG2D blockade reduces the lytic capability of CD8 T cells *in vitro*, and that NKG2D diminishes the antitumor effects generated against NKG2D ligand-bearing tumors *in vivo*.

Discussion

Advantages of antigen-independent CD8 T cell activation

To date, most immunotherapy regimens have focused on the development of antigen specific responses by CD8 T cells against known tumor antigens. These methods are efficacious because they allow for the generation of targeted anti-tumor immunity and are less likely to cause damage to healthy tissues; however, these therapies are limited by our knowledge of tumor associated and tumor specific antigens. Other challenges to antigen specific therapies include the phenomena of immunoediting, where the antigenicity of a tumor is shaped or edited based on the immune response generated against it. This principle has been found to apply to tumor antigen targeted immunity in the development of antigen loss variants which downregulate the expression of the targeted antigen in response to immune recognition (23). While cytokine therapy increases the likelihood of toxicities due to the systemic administration, the generation of MHC-unrestricted killers may prove beneficial for the treatment of less antigenic tumors.

Using a model of systemic cytokine immunotherapy, we observed that effector memory CD8 T cells expand in secondary lymphoid tissues, as well as within tumor tissues. Remarkably, these cells do not express surface molecules that would denote recent TCR engagement, and are able to mediate the antigen-independent lysis of tumor targets, regardless of their TCR specificity. These

data suggest that under conditions that result in strong cytokine stimulation, tissue resident memory CD8 T cells are poised to function as innate effectors and contribute to tumor immunity, independent of their antigen specificity.

The expansion and activation of memory cells via cytokine rather than TCR stimulation may give these cells a survival advantage in the context of tumor immunity. PD-1 is a suppressive molecule that is upregulated on T cells following TCR ligation (168, 169, 202, 203) . One of the ligands for PD-1, B7-H1 (PDL-1) is upregulated on several tumors and is highly inducible by IFN-gamma (202, 203). B7-H1 is one method by which tumor cells are thought to control immune responses within their microenvironment. The ligation of PD-1 on the surface of tumor infiltrating T cells has been shown to induce anergy in those cells (204). The cytokine driven memory cells that we describe here are not activated via their TCR, and therefore do not upregulate PD-1, thus leaving them less susceptible to the suppressive environments created by tumor cells. This finding is significant in light of the high levels of IFN-g which are produced in response to these immunotherapies.

The paradoxical effects of immunotherapy and vaccination on TCR transgenic T cells: Are OT-1 CD8 T cells helpless?

OT-1 mice provide an attractive model with which to examine the effects of anti-CD40 and IL-2 immunotherapy on antigen specific T cells. Initial studies were performed which examined the expansion and function of OT-1 cells

following adoptive transfer. However, when direct treatments were performed into OT-1 mice, we observed a surprising lack of CD8 T cell expansion. This lack of expansion was surprising considering the fact that we had demonstrated that memory cells are the target population of this therapy, and that approximately 15%-25% of OT-1 CD8 T cells possess a memory phenotype in resting animals. The lack of expansion following anti-CD40 and IL-2 was also of interest because these cells were functionally active following therapy as evidenced by their expression of CD44 and NKG2D as well as their cytolytic function against OVA-bearing and antigen irrelevant tumors.

We have developed two hypotheses to address the lack of expansion of OT-1 CD8 T cells after immunotherapy, as well as the dramatic loss of CD8 T cells following OVA vaccination:

1. The "Helpless" Hypothesis: OT-1 mice have been found to possess fewer CD4 T cells than wildtype B6 mice. It may be that CD4 T cells are required for the expansion of CD8 T cells and to provide cytokine help, even in the presence of high levels of exogenous IL-2. While it has been established that CD4 T cells are not necessary for the antitumor effects generated with this immunotherapy, studies are currently underway to determine if CD4 T cells are necessary for the expansion of CD8 T cells following immunotherapy. Despite the decreased numbers of CD4 T cells in OT-1 mice, sufficient responses would still be expected after immunotherapy due to the fact that CD40 ligation can bypass the need for

CD4 T cell help in the generation of CD8 T cell responses. However, antigen specific responses after vaccination may require more CD4 help than is possible, given the decreased numbers in these mice.

2. The Precursor Frequency Hypothesis: This hypothesis likely explains the decreased number of CD8 T cells in the OT-1 mice following ova vaccination. It has been demonstrated that the expansion of TCR transgenic cells can be hindered by the transfer of too many of those cells in adoptive transfer studies (205-207). It is estimated that 1 in 100,000 cells will be specific for a particular antigen (129). In the case of transferring too many cells, or in the case of directly treating an OT-1 mouse, those frequencies are excessively higher. This puts these cells at a disadvantage due to excessive competition for antigen and cytokines. It may be that the precursor frequency of ova-specific T cells is so high, that even in the case of anti-CD40 and IL-2, they are unable to proliferate. This lack of proliferation is significant despite the observation that CD8 T cells from OT-1 mice treated directly with either immunotherapy or antigen vaccination are active and are capable of effector function. These data suggest that the activation threshold for cytolytic activity differs from that which is required to induce cell proliferation.

NKG2D, killing, and tumor recognition mechanisms

We propose that immunotherapy activated CD8 T cells possess multiple killing and recognition mechanisms that are beneficial to the generation of immune responses against a variety of tumor types. NKG2D has been found to play a role in the immunoediting of spontaneous tumor models where NKG2D ligand expression on spontaneous tumors was much higher in NKG2D knockout mice than in wildtype. NKG2D blockade did not result in the complete abrogation of anti-tumor effects *in vitro* or *in vivo*, therefore, there are likely other recognition and killing mechanisms that these cells use to identify tumor targets. Some of these may include LY49d, Fas-FasL interactions, and TRAIL receptor-TRAIL interactions. The presence of other recognition mechanisms is also displayed by the direct OT-1 *in vitro* killing data. The EL4 tumor line does not express any identifiable NKG2D ligands; therefore, other mechanisms were likely involved in the TCR-independent killing we observed *in vitro* after immunotherapy. It would be of particular interest to examine these post-immunotherapy cells for their expression of other NK cell activating and inhibitory receptors. The NK cell activating receptor, Ly49D signals through the same DAP12/ITAM as NKG2D and is expressed by T cells in patients suffering from chronic inflammatory disorders and autoimmunity (208-210). The triggering of transgene expressed Ly49D on cytokine activated CD8 T cells leads to T cell proliferation, IFN-g production, and cytolytic activity. These effects were triggered by the ligation of Ly49D with an MHC-I ligand in the absence of

TCR engagement (210). As such, Ly49D may play a supporting role in the antigen-independent recognition of tumor cells.

The differences between mouse and human NKG2D expression are significant. NKG2D has been found to be consitutively expressed on virtually all CD8 T cells in humans, whereas this expression is inducible on CD8 T cells in the mouse (211). In the human scenario, this would leave any NKG2D ligand-bearing tissue vulnerable to targeting by all CD8 T cells. A recent study by Cerboni and colleagues has proposed a mechanism by which NKG2D mediated killing is regulated. This study shows that antigen activated human CD4 and CD8 T cells upregulate the expression of the NKG2D ligands MICA and ULBP3, and that the expression of NKG2D ligands by CD4 T cells induces the downmodulation of NKG2D on CD8 T cells, thus effectively controlling the function of these cells (211). A similar method of NKG2D modulation has been observed in several clinical tumor scenarios, where tumors have been found to shed soluble NKG2D ligands which effectively bind NKG2D expressed on T cells and NK cells and decrease their function (211-217). It would be of interest to examine the expression of NKG2D ligands on CD4 and CD8 T cells following immunotherapy, as this may play a role in the diminution of the antigen-independent immune responses generated with this therapy.

Personal perspectives and future directions

Are antigen specific responses really needed to combat cancer? It is very likely that we are expanding both antigen specific and tumor antigen irrelevant T cell clones. Kory Alderson and I proposed several times to perform a timing experiment to determine if the early pre-immunotherapy exposure to tumor antigens during tumor engraftment affected the nature of the response generated ie, antigen specific or not. We proposed that the longer the period of antigenic exposure, the greater the opportunity there would be for the generation of antigen specific responses, and therefore, the greater the opportunity for these antigen specific T cells clones to expand in response to immunotherapy. This theory is supported by the observation that short-term vaccination with a xeno-antigen bearing B16ova tumor followed by 40/2 immunotherapy results in a loss of secondary responses. We propose that the CD4 T cell disregulation that occurs as a result of immunotherapy had a greater impact in the B16ova model than in the orthotopic renca model because immunotherapy was initiated within 4 days of tumor injections. This is in contrast to the orthotopic renca model, where mice were exposed to antigen for 11 days before the initiation of therapy, thus allowing for the effective priming of antigen specific T cells before the initiation of immunotherapy and any deleterious effects on CD4 T cells could occur. These data are supported by the secondary anti-tumor responses we observed in the orthotopic Renca model, but not in the B16ova model after immunotherapy. Long term studies examining the

effects of this therapy on spontaneously arising tumors would be of interest and may give greater insight into the role of tumor antigen exposure in the types of primary and memory CD8 T cell responses generated with immunotherapy. These studies would also be more clinically relevant, as human tumors develop over long periods of time and in the presence of immune effectors that may or may not recognize tumor antigens.

In a grander view of the implications of antigen independent immune activation, cytokine expanded CD8 memory T cells have an advantage over antigen specific responses because they don't express high levels of the regulatory molecules that tumors can use to derail an immune response (ie PD-1). Additionally, because they are able to kill independently of any one particular antigen, they are less likely to affect the development of antigen loss variants. On the other hand, one can not ignore the very real toxicities and possibilities for autoimmunity that may arise from the antigen-independent polyclonal activation of memory T cells. Anti-CD40 and IL-2 immunotherapy is associated with severe toxicities. Our group and others have shown that systemic administration of anti-CD40 and IL-2 can result in dose limiting toxicities which include splenomegaly, the induction of pro-inflammatory cytokines such as TNFα, IFNγ, and IL-12, and the development of intestinal lesions (173, 218-220). The intestinal effects of CD40 stimulation are such that an agonist CD40 mAb was recently used to simulate a model of wasting disease and colitis in immune deficient mice (221).

While the acute toxicities observed after immunotherapy are severe, they generally regress quickly upon cessation of treatment. We have unpublished data which shows that the NKG2D^{+} subset of CD8 T cells disappear within 8 days of cytokine withdrawal, thus suggesting that this subset of cytokine expanded memory T cells rapidly contract in the absence of continued cytokine support. This contraction may be the product of the preferential expression of the intermediate affinity IL-2 receptor, CD122, and not the high affinity IL-2 receptor, CD25. Additionally, the fact that many of the CD8 T cells possess an effector memory phenotype after immunotherapy may mark them for decreased longevity. Effector memory cells are noted for their immediate effector functions upon re-stimulation; however, they are terminally differentiated and do not undergo successive rounds of proliferation as would be expected of central memory T cells. Further study into the longevity and contraction of these cells is warranted in light of the toxicities observed with high dose cytokine immunotherapy.

The phenomena of bystander proliferation is an example of cytokine sensitivity, in which memory T cells of a particular specificity can proliferate and gain effector function by responding to the cytokines produced by an immune response that is generated to another antigen. In the present study, we demonstrate that memory CD8 T cells expand in response to strong cytokine immunotherapy. We also show that memory CD8 T cells are able to mediate

anti-tumor effects after cytokine immunotherapy in an antigen and TCR-independent manner, and that tumor recognition is NKG2D assisted.

We have unpublished data which demonstrates that memory CD8 T cells upregulate NKG2D in the lungs of influenza infected mice, and that these cells are capable of killing in an antigen independent fashion. These are tissue-resident memory CD8 T cells and are not recruited from the draining mediastinal lymph nodes, distant lymph nodes, or spleen as we only see an expansion of these CD8 T cells in the lungs of infected mice. These data, combined with the studies presented in this dissertation make a case that memory CD8 T cells may play a dual role in the periphery. Their primary role is to lie in wait for a reencounter with their cognate antigen, but they can also act as a pool of "innate" effectors which respond to high cytokine milieus such as those generated in the case of cytokine immunotherapy, or during viral infections. Follow-up experiments are currently underway to further examine the role of pathogen-induced inflammation on the expansion of tissue-resident memory T cells in a systemic *Listeria monocytogenes* model.

The exact killing mechanisms utilized by the expanded memory CD8 T cells have yet to be determined. We found that CD8 T cells upregulate the expression of granzyme B following immunotherapy, and as perforin and granzyme release are the common mediators in short-term killing assays, these mechanisms likely played a role in our *in vitro* killing studies. Early studies from our lab also demonstrated that IFN-gamma and Fas Ligand are necessary for the

antitumor responses generated against Renca (171). It may be that the killing pathways utilized are determined by the nature of CD8 T cell response. NKG2D signaling on CIK CD8 T cells has been found to exclusively trigger perforin-dependent tumor killing, so this may be the dominant killing mechanism which occurs in the absence of TCR ligation, whereas TCR triggering induces killing through the Fas-FasL pathway.

In summary, it is likely that both antigen specific and antigen independent responses are generated during anti-CD40 and IL-2 immunotherapy, and that the killing mechanisms involved are likely determined by the nature of the response ie: NKG2D/perforin-granzyme, or TCR/ Fas-FasL. Due to its co-stimulatory role in the event of TCR signaling, it is possible that NKG2D activation plays a role in both antigen dependent and antigen independent responses. Further studies into the role of antigen and tumor timing before the administration of immunotherapy, and differences in killing mechanisms would be of great interest.

Figure 1

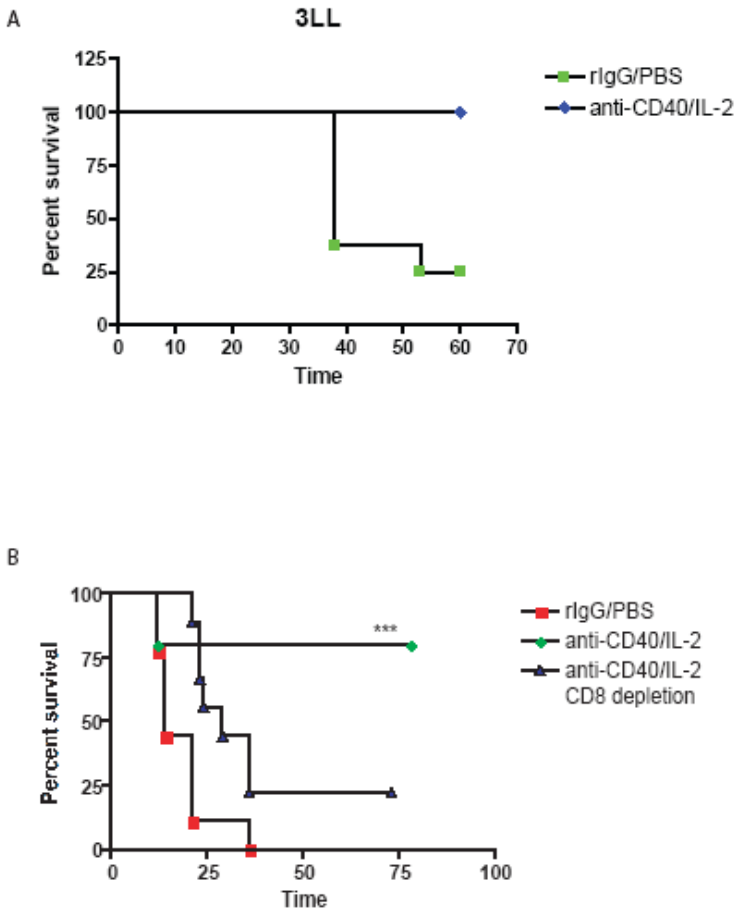

A
3LL
Percent survival
Time
rIgG/PBS
anti-CD40/IL-2
B
***
Percent survival
Time
rIgG/PBS
anti-CD40/IL-2
anti-CD40/IL-2
CD8 depletion

Figure 1. Anti-CD40 and IL-2 immunotherapy prolongs the survival of tumor bearing mice in a CD8 T cell dependent manner. A-B) C57BL/6 mice were injected S.C. with $2x10^6$ 3LL tumor cells on Day 0. On day 11 post tumor injection, mice were treated with the standard regimen of anti-CD40 and IL-2 or control reagents, and were monitored for survival. (B) In some groups, CD8 T cells were depleted by the administration of a CD8 T cell depleting antibody on the first day of immunotherapy treatment. Data are representative of at least two independent experiments. Data were plotted using the Kaplan-Meier technique and statistics were generated by Log-rank test.

Figure 2

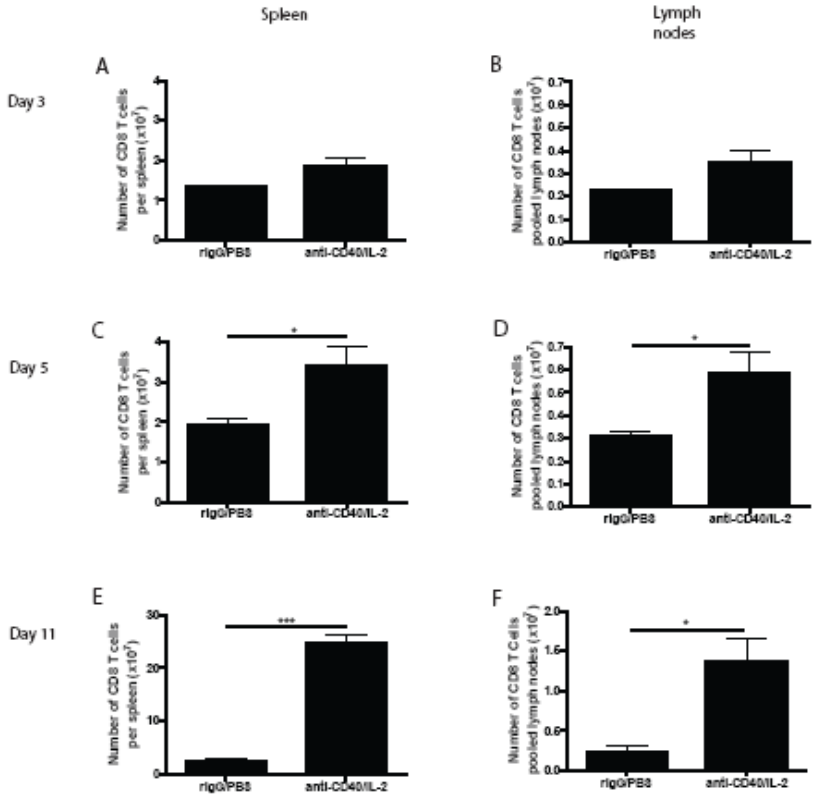

Spleen
Lymph nodes
Day 3
Day 5
Day 11
A
B
C
D
E
F
Number of CD8 T cells per spleen (x10⁷)
Number of CD8 T cells pooled lymph nodes (x10⁷)
rIgG/PBS
anti-CD40/IL-2
*
***

Figure 2. Immunotherapy with anti-CD40 and IL-2 induces the expansion of CD8 T cells in the absence of tumor or exogenous antigen. Naïve C57BL/6 mice were treated with the standard immunotherapy regimen and A,C,E) spleens and B,D,F) axillary, brachial, and inguinal lymph nodes were harvested on days A,B) 3, C,D) 5, and E,F) 11. The frequency of CD8 T cells was determined by flow cytometry and total numbers were generated by applying the percent positive against the total cell number as determined with a coulter counter. Data are representative of at least 3 independent experiments. Statistics were generated by Student's *t*- test with Welch's correction.

Figure 3

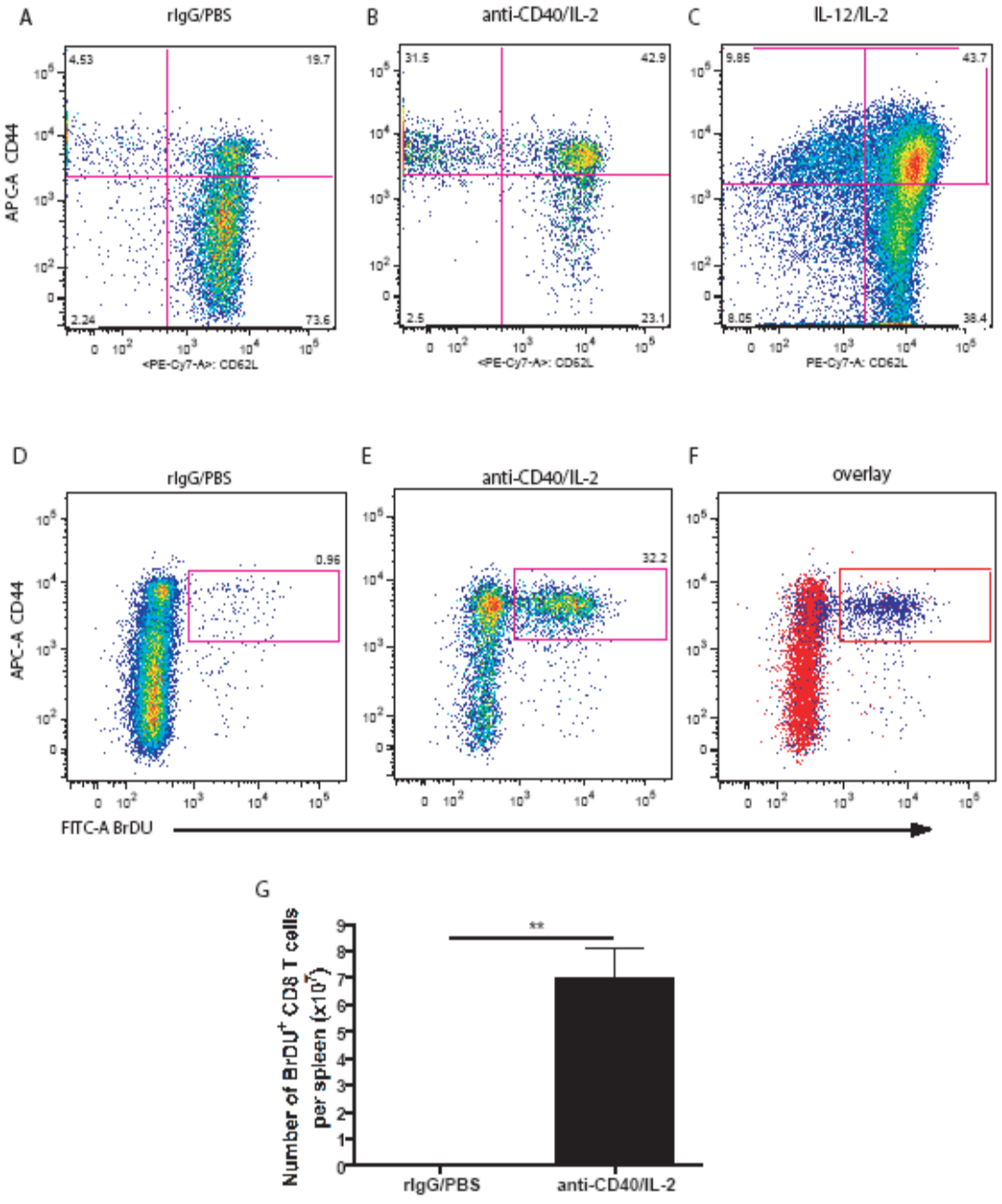

A
rIgG/PBS
4.53
19.7
2.24
73.6
APC-A CD44
<PE-Cy7-A>: CD62L
B
anti-CD40/IL-2
31.5
42.9
2.5
23.1
<PE-Cy7-A>: CD62L
C
IL-12/IL-2
9.85
43.7
8.05
38.4
PE-Cy7-A: CD62L
D
rIgG/PBS
0.96
APC-A CD44
FITC-A BrDU
E
anti-CD40/IL-2
32.2
F
overlay
G
Number of BrDU+ CD8 T cells per spleen (x10^7)
**
rIgG/PBS
anti-CD40/IL-2

Figure 3. Memory CD8 T cells expand and proliferate after immunotherapy. C57BL/6 mice received the standard regimen of anti-CD40 and IL-2 (B,E), IL-12 and IL-2 (E,F), or rat IgG and PBS control treatments (A,D). Mice were sacrificed on day 5 (C) or day 11 (A-B) and spleens cells were labeled for CD8, CD44, and CD62L and analyzed by flow cytometry. Data were gated on CD8 T cells and are shown as CD44 plotted against CD62L (A-C). (D-F) Mice were injected with 1mg BrDU IP on day 10, and harvested on day 11. Splenocytes were labeled for CD8, CD44, and BrDU incorporation and analyzed by flow cytometry. Data were gated on CD8 T cells and dot plots show CD44 populations plotted against BrDU. F) BrDU incorporation is shown as an overlay of control versus immunotherapy treatment. G) Total number of BrDU positive CD8 T cells per spleen. Statistics were generated by Student's t-test. Data are representative of at least three independent experiments.

Figure 4

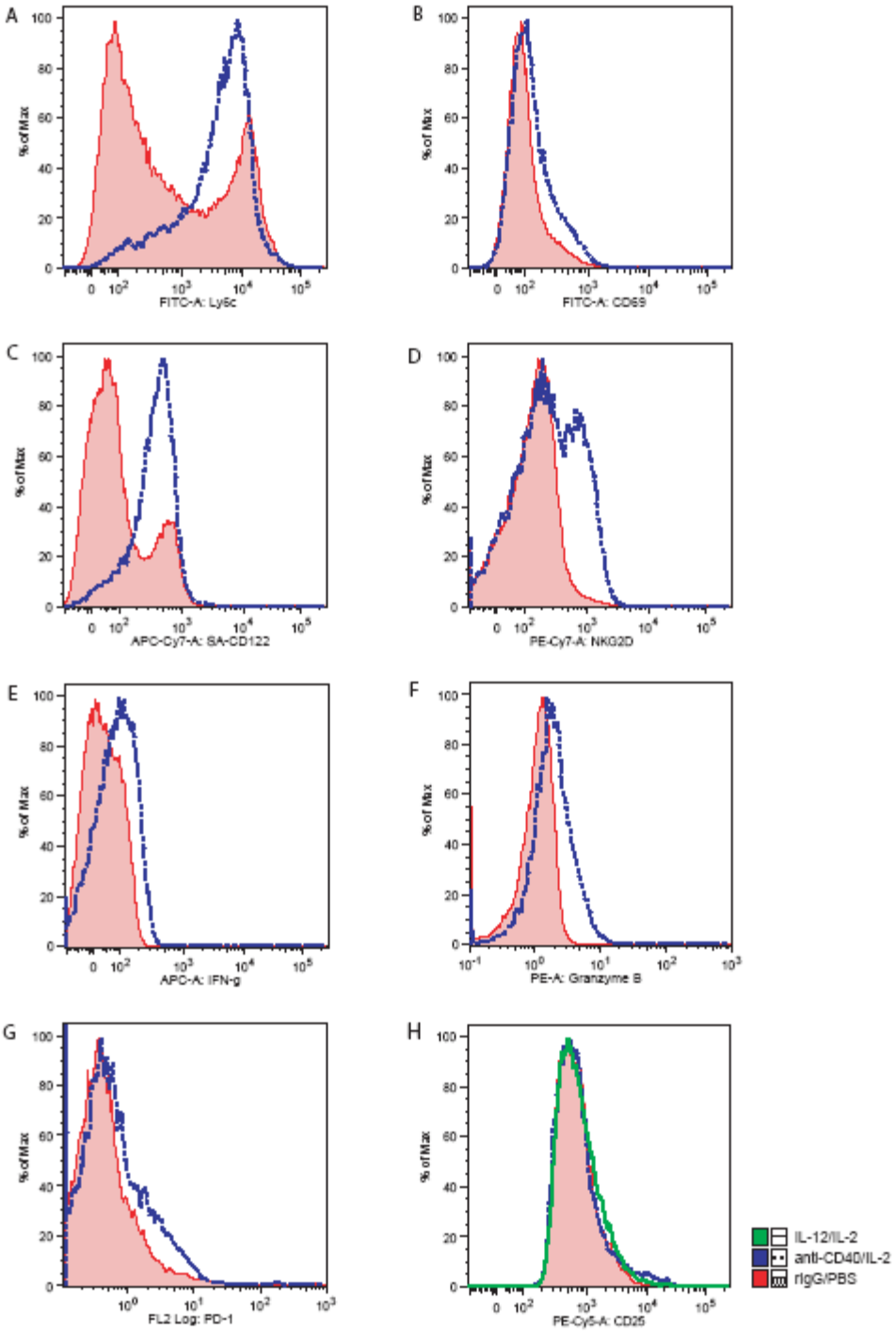

A
B
C
D
E
F
G
H
% of Max
FITC-A: Ly6c
FITC-A: CD69
APC-Cy7-A: SA-CD122
PE-Cy7-A: NKG2D
APC-A: IFN-g
PE-A: Granzyme B
FL2 Log: PD-1
PE-Cy5-A: CD25
IL-12/IL-2
anti-CD40/IL-2
rIgG/PBS

Figure 4. Phenotype of CD8 T cells following the standard regimen of anti-CD40 and IL-2 immunotherapy. C57BL/6 mice were treated with the standard regimen of anti-CD40 and IL-2. Spleens were harvested on day 11 post initiation of immunotherapy and CD8 T cells were analyzed for their expression of surface and activation molecules: A) LY6c, B) CD69, C) CD122, D) NKG2D, E) IFN-gamma, F) Granzyme B, G) PD-1 H) CD25. (H) Another group of mice also received treatment with IL-12 and IL-2.

Figure 5

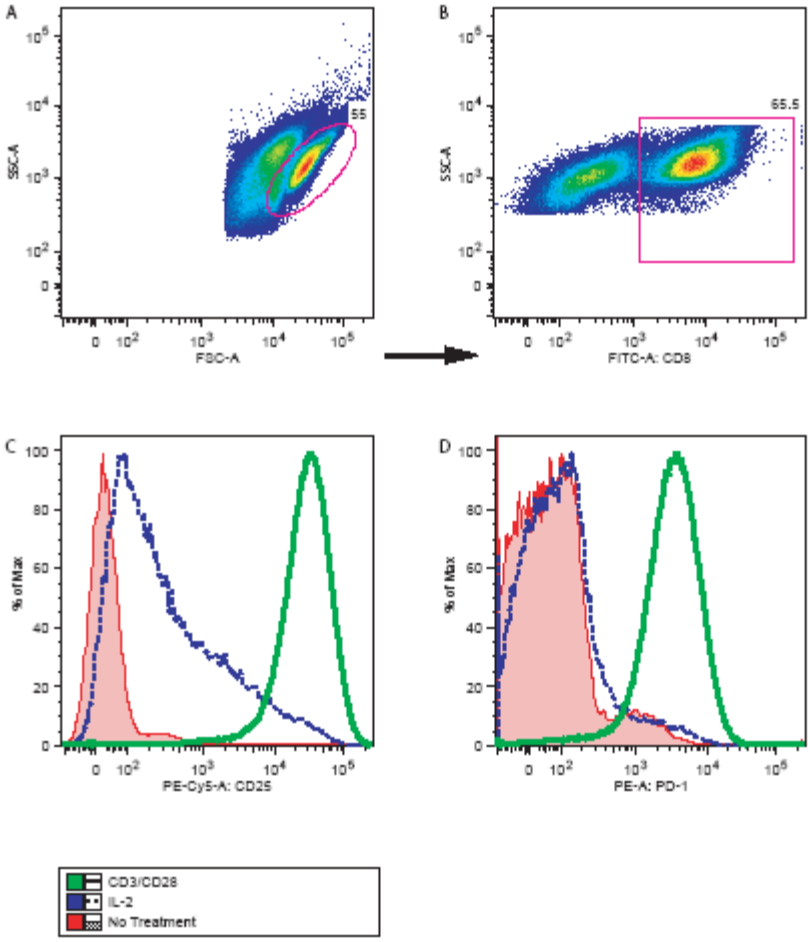

A
SSC-A
FSC-A
55
B
SSC-A
FITC-A: CD8
65.5
C
% of Max
PE-Cy5-A: CD25
D
% of Max
PE-A: PD-1
CD3/CD28
IL-2
No Treatment

Figure 5. TCR ligation with anti-CD3 and anti-CD28 upregulates the expression of CD25 and PD-1 on CD8 T cells *in vitro.* Spleens were harvested from naïve C57BL/6 mice, dissociated into single cell suspensions, and cultured with 1ug/ml anti-CD3 antibody and 5ug/ml anti-CD28 antibody (solid lines), 20,000 IU recombinant human IL-2 (dashed lines), or were left untreated (shaded) for 72 hours. Cultures were then washed and CD8 T cells were labeled for their expression of CD25 and PD-1.

A) Forward scatter/ Side Scatter plot and gating scheme

B) CD8 T cell gating scheme

C) CD25 expression on CD8 T cells

D) PD-1 expression on CD8 T cells

Figure 6

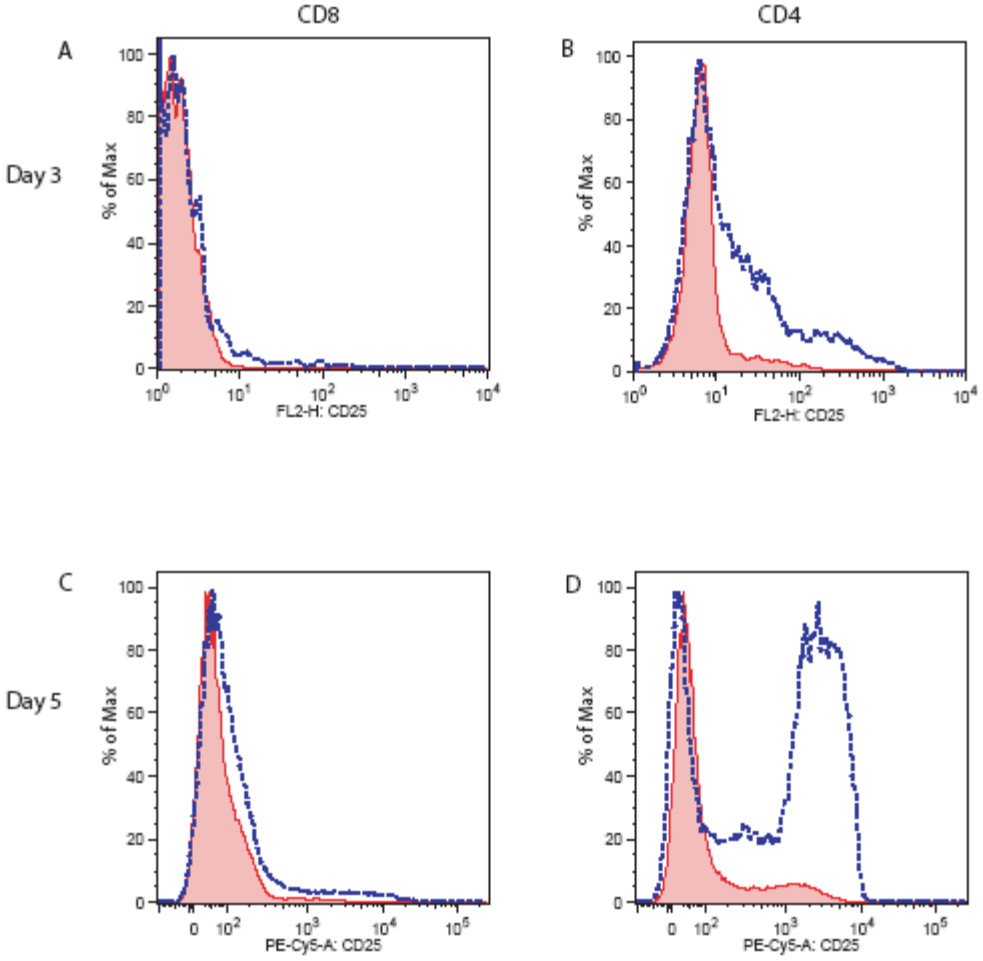


Figure 6. CD25 is expressed on CD4 T cells but not CD8 T cells early after immunotherapy. C57BL/6 mice were treated with the standard regimen of anti-CD40 and IL-2 (blue dashed histograms) or (red shaded histograms) control treatments. Spleens were harvested on days 3 and 5 post initiation of immunotherapy and CD4 and CD8 T cells were analyzed for their expression of CD25. Data were gated on CD4 (B,D) or CD8 (A,C) T cells and CD25 expression is expressed as histograms.

Figure 7

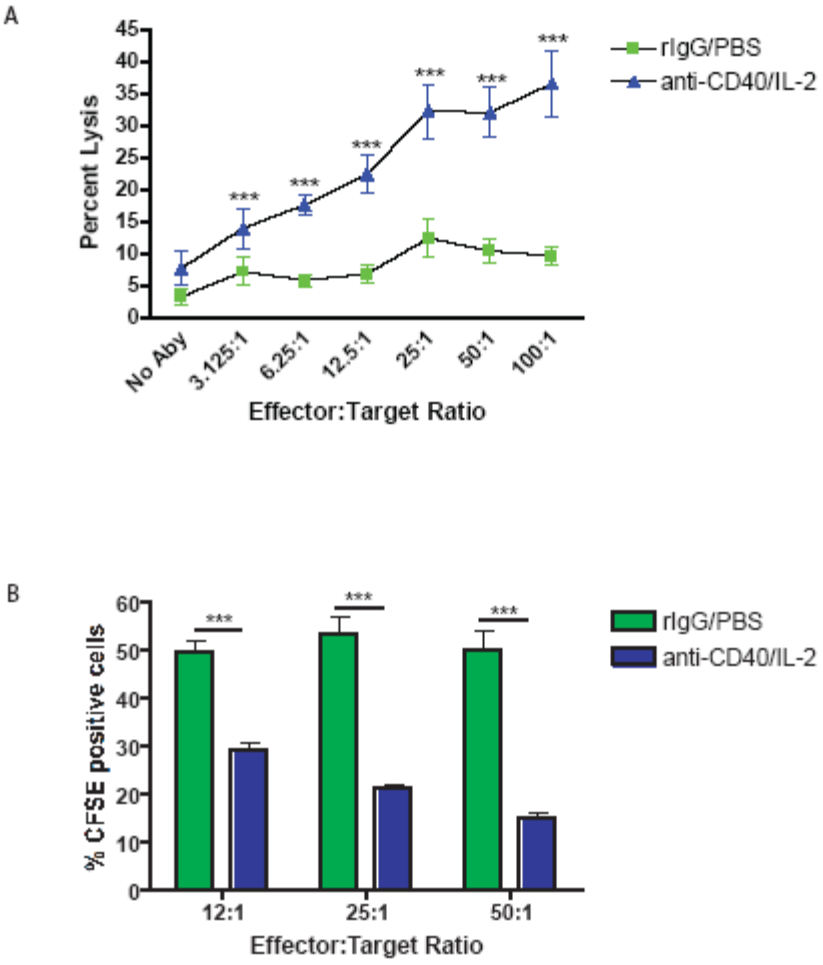
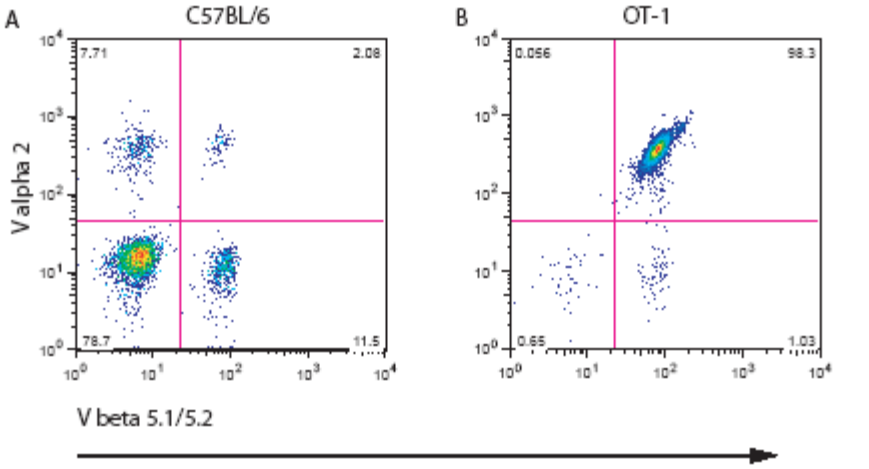

A
Percent Lysis
45
40
35
30
25
20
15
10
5
0
***
***
***
***
***
***
No Aby
3.125:1
6.25:1
12.5:1
25:1
50:1
100:1
Effector:Target Ratio
rIgG/PBS
anti-CD40/IL-2
B
% CFSE positive cells
60
50
40
30
20
10
0
***
***
***
12:1
25:1
50:1
Effector:Target Ratio
rIgG/PBS
anti-CD40/IL-2

Figure 7. Splenocytes harvested from immunotherapy treated mice display increased antigen independent cyotoxicity . (A) C57BL/6 mice were treated with anti-CD40 and IL-2 and harvested on day 11. Spleens were dissociated into single cell suspensions and CD8 T cells were isolated with MACS columns and incubated with anti-CD3 bound, chromium-51 labeled P815 tumor target cells for 18 hours. Supernatants were removed, mixed 1:1 with scintillation fluid and analyzed by scintillation counter. Statistics were generated by two way ANOVA with Bonferoni post test. (B) C57BL/6 mice were treated with anti-CD40 and IL-2 and harvested on day 11. Spleens were dissociated into single cell suspensions and CD8 T cells were isolated with MACS columns. CD8 T cells were then cultured for 18 hours in the presence of CFSE labeled Renca tumor targets. CFSE labeled cells were identified by flow cytometry, and the percentage of remaining Renca cells are reported here as a percentage of CFSE positive cells. Statistics were generated by Mann-Whitney test.

Figure 8


A
C57BL/6
B
OT-1
7.71
2.08
78.7
11.5
0.056
98.3
0.65
1.03
V alpha 2
V beta 5.1/5.2

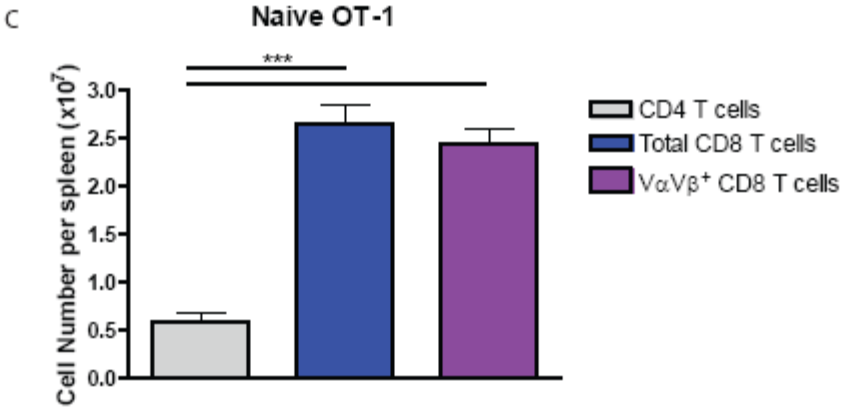

C
Naive OT-1
***
Cell Number per spleen (x10^7)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CD4 T cells
Total CD8 T cells
VαVβ+ CD8 T cells

Figure 8. Gating Scheme and cellularities for OT-1 mice Blood was collected from the tail vein of an (B) OT-1 mouse and a (A) transgene negative C57BL/6 littermate and CD8 T cells were analyzed for the expression of the V alpha 2 and V beta 5.1/5.2 T cell receptor subunits by flow cytometry. C) Spleens were collected from rIgG/PBS- treated control mice and labeled for CD4, CD8, and the expression of the V alpha 2 and V beta 5.1/5.2 T cell receptor subunits by flow cytometry. The frequency of CD8 T cells was determined by flow cytometry and total numbers were generated by multiplying the percent positive against the total cell number as determined with a coulter counter. Data are representative of at least 3 independent experiments. Statistics were generated by one-way ANOVA with the Tukey post-test.

Figure 9

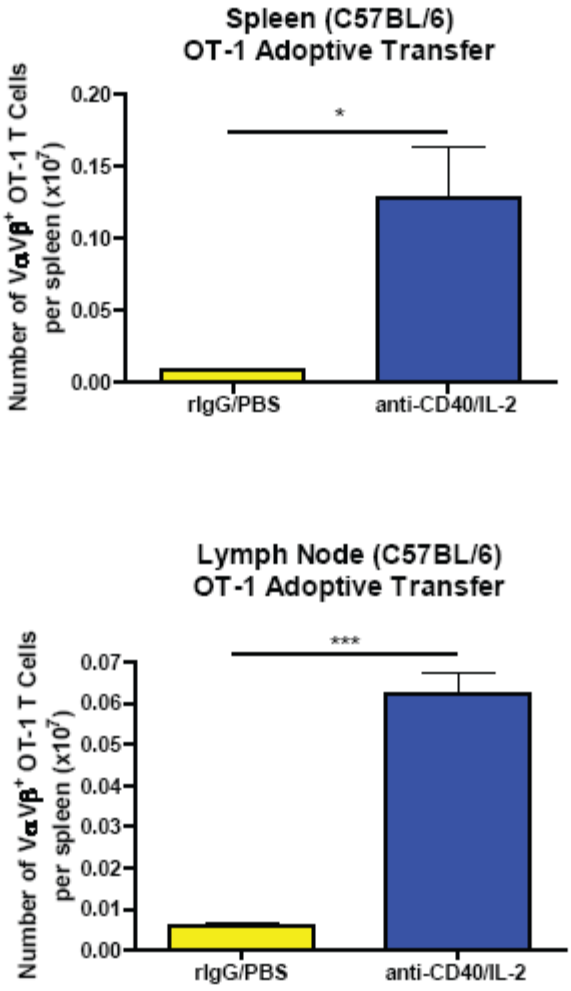
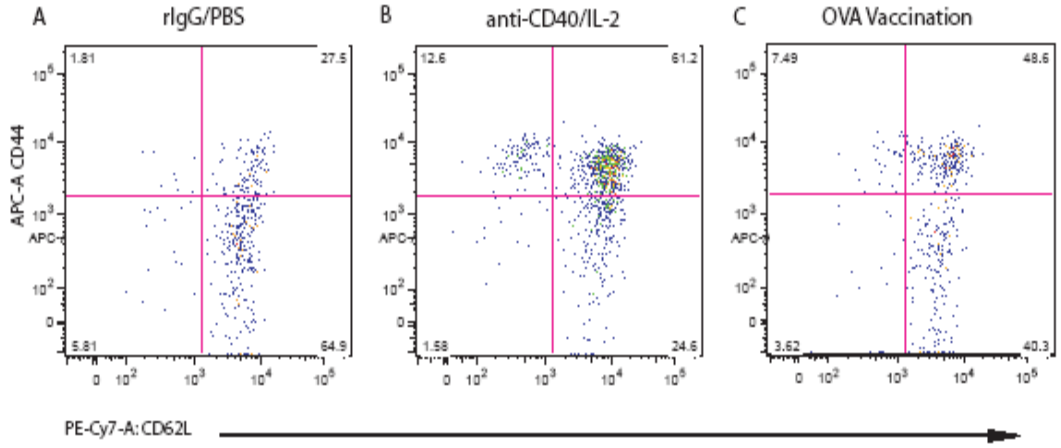
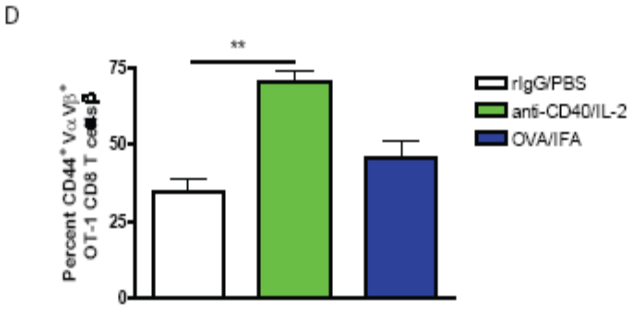

Spleen (C57BL/6)
OT-1 Adoptive Transfer
Number of Vα Vβ+ OT-1 T Cells per spleen (x10^7)
0.20
0.15
0.10
0.05
0.00
*
rIgG/PBS
anti-CD40/IL-2
Lymph Node (C57BL/6)
OT-1 Adoptive Transfer
Number of Vα Vβ+ OT-1 T Cells per spleen (x10^7)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
***
rIgG/PBS
anti-CD40/IL-2

Figure 9. Immunotherapy with anti-CD40 and IL-2 results in the expansion of OT-1 TCR Tg CD8+ T cells in the absence of antigen. C57BL/6 received an adoptive transfer of 2.5 x 10^6 OT-1 lymph node cells IV on day -4. On day 0, mice received treatment with anti-CD40 and IL-2 or control reagents. Spleens (A) and axillary, brachial, and inguinal Lymph nodes (B) were harvested 11 days after the initiation of treatments. Statistics were generated by Student's *t*-test with Welch's correction and one-way ANOVA with the tukey post-test. Data are representative of at least three independent experiments.

Figure 10


A
rIgG/PBS
B
anti-CD40/IL-2
C
OVA Vaccination
APC-A CD44
PE-Cy7-A: CD62L
1.81
27.5
5.81
64.9
12.6
61.2
1.58
24.6
7.49
48.6
3.62
40.3


D
**
rIgG/PBS
anti-CD40/IL-2
OVA/IFA

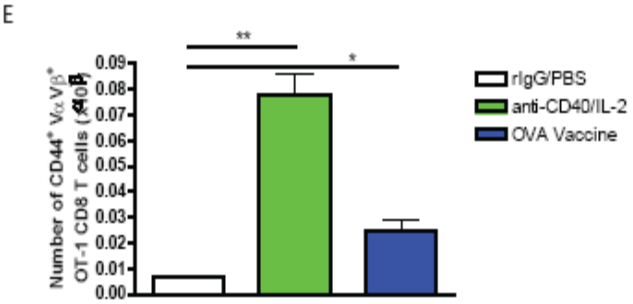

E
**
*
rIgG/PBS
anti-CD40/IL-2
OVA Vaccine

Figure 10. Immunotherapy increases frequencies and numbers of CD44^{hi} OT-1 CD8 T adoptively transferred cells. C57BL/6 mice received an adoptive transfer of 2.5 x 10^6 OT-1 lymph node cells IV on day -4. On day 0, mice received a vaccination with an emulsion of 500ug ovalbumin (OVA) in 200ul incomplete Freund's adjuvant (IFA) IP (C), anti-CD40 and IL-2 (B), or control reagents (A). Spleens were harvested 11 days after the initiation of treatments, and splenocytes were labeled for CD8, Valpha 2 TCR, Vbeta 5.1/5.2 TCR, CD62L, and CD44 expression and were analyzed by flow cytometry. Data were gated on CD8 positive T cells, followed by gating on cells expressing both the Valpha 2 and Vbeta 5.1/5.2 TCRs (data not shown). Data are shown as CD44 populations plotted against CD62L. Frequencies (D) and total numbers (E) of CD44hi OT-1 CD8 T cells increase after immunotherapy. Data are representative of at least 3 independent experiments. Statistics were generated by one-way ANOVA with the tukey post-test.

Figure 11

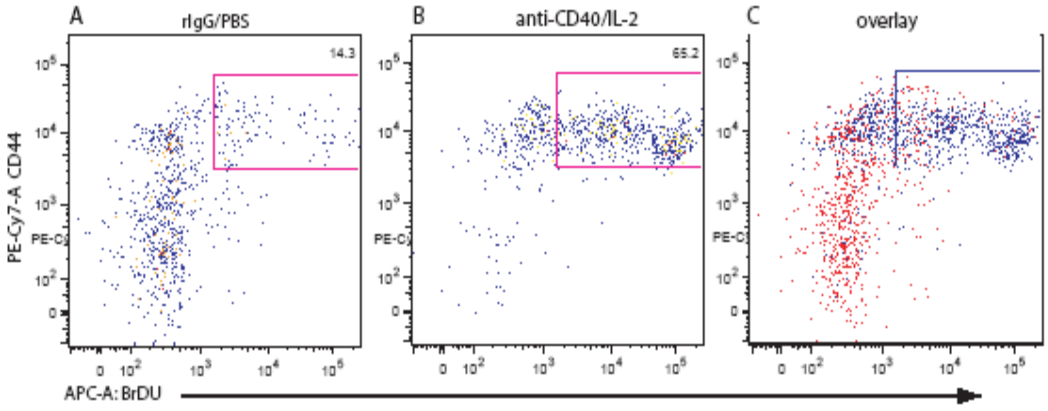
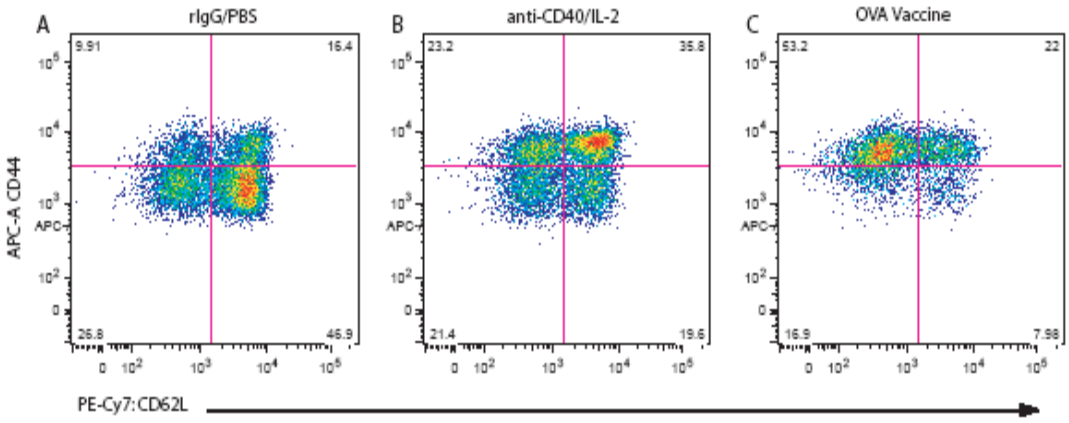

A
rIgG/PBS
14.3
B
anti-CD40/IL-2
65.2
C
overlay
PE-Cy7-A CD44
APC-A: BrDU

Figure 11. Adoptively transferred CD44hi CD8 T cells proliferate in response to immunotherapy in the absence of antigen. C57BL/6 mice received an adoptive transfer of 2.5 x 10^6 OT-1 lymph node cells IV on day -4 and began treatment with the standard (B) anti-CD40 and IL-2 immunotherapy or (A) control regimen on day 0. Mice were injected with 1mg BrDU IP on day 10, and harvested on day 11. Splenocytes were labeled for CD8, Valpha 2 TCR, Vbeta 5.1/5.2 TCR, and CD44 expression and BrDU incorporation and were analyzed by flow cytometry. Data were gated on CD8 positive T cells, followed by gating on cells expressing both the Valpha 2 and beta 5.1/5.2 TCRs (data not shown). Data are shown as CD44 populations plotted against BrDU. (C) BrDU incorporation is shown as an overlay of control versus immunotherapy treatment.

Figure 12


A
rIgG/PBS
B
anti-CD40/IL-2
C
OVA Vaccine
APC-A CD44
PE-Cy7:CD62L
9.91
16.4
26.8
46.9
23.2
35.8
21.4
19.6
53.2
22
16.9
7.98

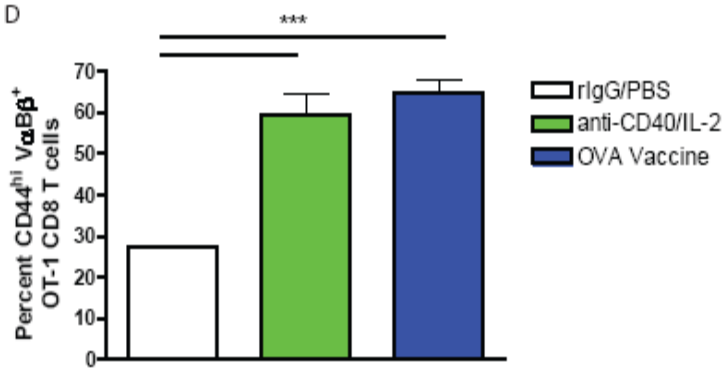

D
***
Percent CD44hi VαBβ+ OT-1 CD8 T cells
rIgG/PBS
anti-CD40/IL-2
OVA Vaccine

Figure 12. Memory CD8 T cells from OT-1 mice expand after direct treatment with immunotherapy or vaccination. On day 0, OT-1 mice received a vaccination with an emulsion of 500ug ovalbumin (OVA) in 200ul incomplete Freund's adjuvant (IFA) IP (C), anti-CD40 and IL-2 (B), or control reagents (A). Spleens were harvested 11 days after the initiation of treatments, and splenocytes were labeled for CD8, Valpha 2 TCR, Vbeta 5.1/5.2 TCR, CD62L, and CD44 expression and were analyzed by flow cytometry. Data were gated on CD8 positive T cells, followed by gating on cells expressing both the Valpha 2 and Vbeta 5.1/5.2 TCRs (data not shown). Data are shown as CD44 populations plotted against CD62L (A-C). Frequencies of CD44hi OT-1 CD8 T cells increase after immunotherapy (D). Data are representative of at least 3 independent experiments. Statistics were generated by one-way ANOVA with the tukey post-test.

Figure 13

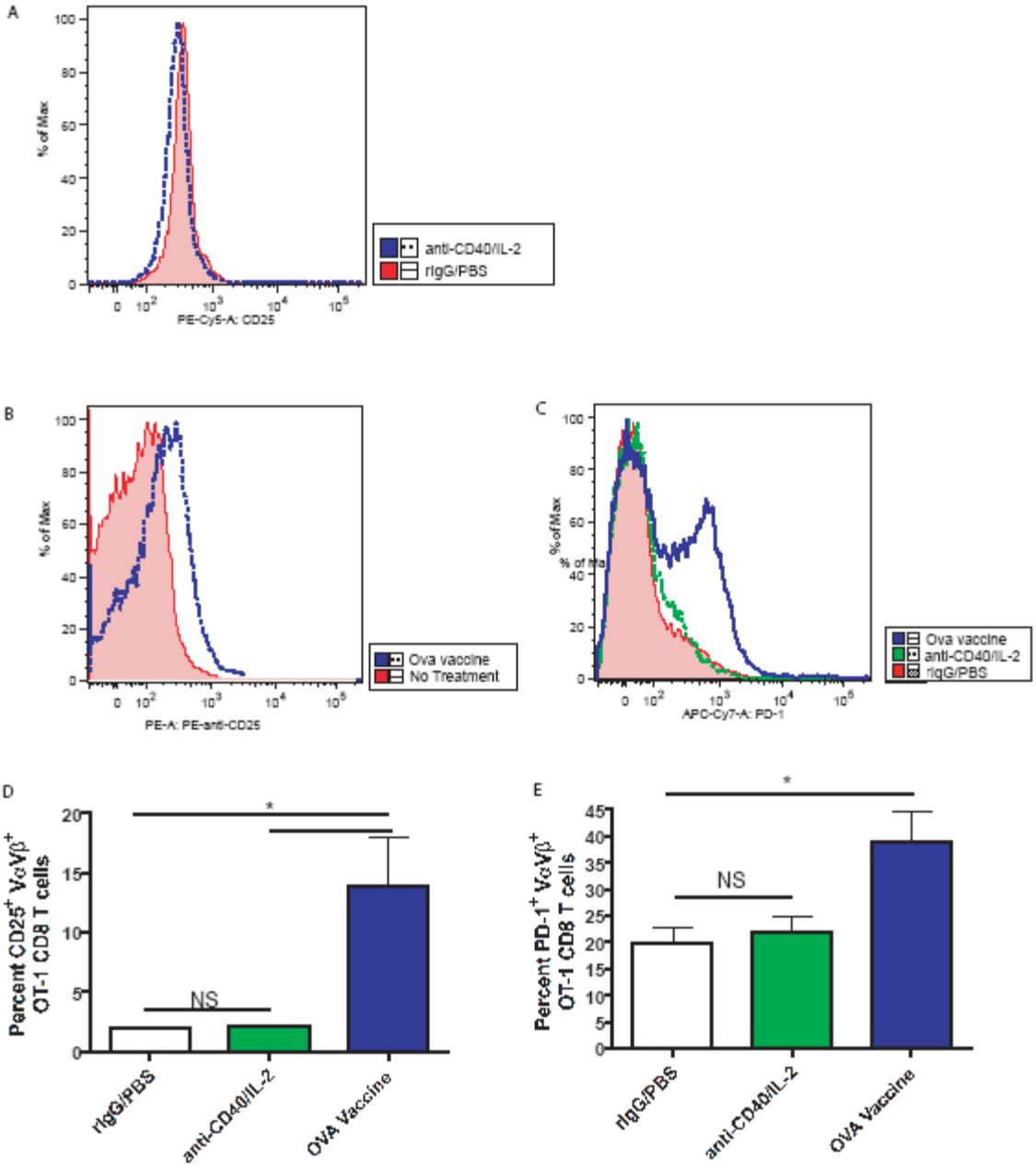

A
% of Max
PE-Cy5-A: CD25
anti-CD40/IL-2
rIgG/PBS
B
% of Max
PE-A: PE-anti-CD25
Ova vaccine
No Treatment
C
% of Max
APC-Cy7-A: PD-1
Ova vaccine
anti-CD40/IL-2
rIgG/PBS
D
Percent CD25+ VαVβ+ OT-1 CD8 T cells
NS
*
rIgG/PBS
anti-CD40/IL-2
OVA Vaccine
E
Percent PD-1+ VαVβ+ OT-1 CD8 T cells
NS
*
rIgG/PBS
anti-CD40/IL-2
OVA Vaccine

Figure 13. OT-1 CD8 T cells upregulate CD25 and PD-1 in response to OVA vaccination but not immunotherapy *in vivo*. (A) OT-1 mice received the standard regimen of anti-CD40 and IL-2 or rat IgG and PBS control treatments. Mice were sacrificed on day 11 and spleens cells were labeled for CD8, Valpha 2, Vbeta 5.1/5.2, and CD25 and were analyzed by flow cytometry. (B) OT-1 mice were vaccinated with an emulsion of 500ug OVA in 200ul incomplete Freund's adjuvant (IFA) IP and blood was collected from vaccinated mice and untreated control mice three days later and labeled for CD8, Valpha 2, Vbeta 5.1/5.2, and CD25. (C) OT-1 mice received a vaccination with an emulsion of 500ug OVA in 200ul incomplete Freund's adjuvant (IFA) IP, or anti-CD40 and IL-2, or control reagents. Spleens were harvested 11 days after the initiation of treatments, and splenocytes were labeled for CD8, Valpha 2 TCR, Vbeta 5.1/5.2 TCR, and PD-1. Data were gated on CD8 positive T cells, followed by gating on cells expressing both the Valpha 2 and Vbeta 5.1/5.2 TCRs (data not shown). The frequency of $CD25^+$ (D) and PD-1^+ (E) TCR transgenic CD8 T cells in OT-1 mice following immunotherapy or OVA vaccination. Data are representative of at least two independent experiments. Statistics were generated by one-way ANOVA with the Tukey post-test.

Figure 14

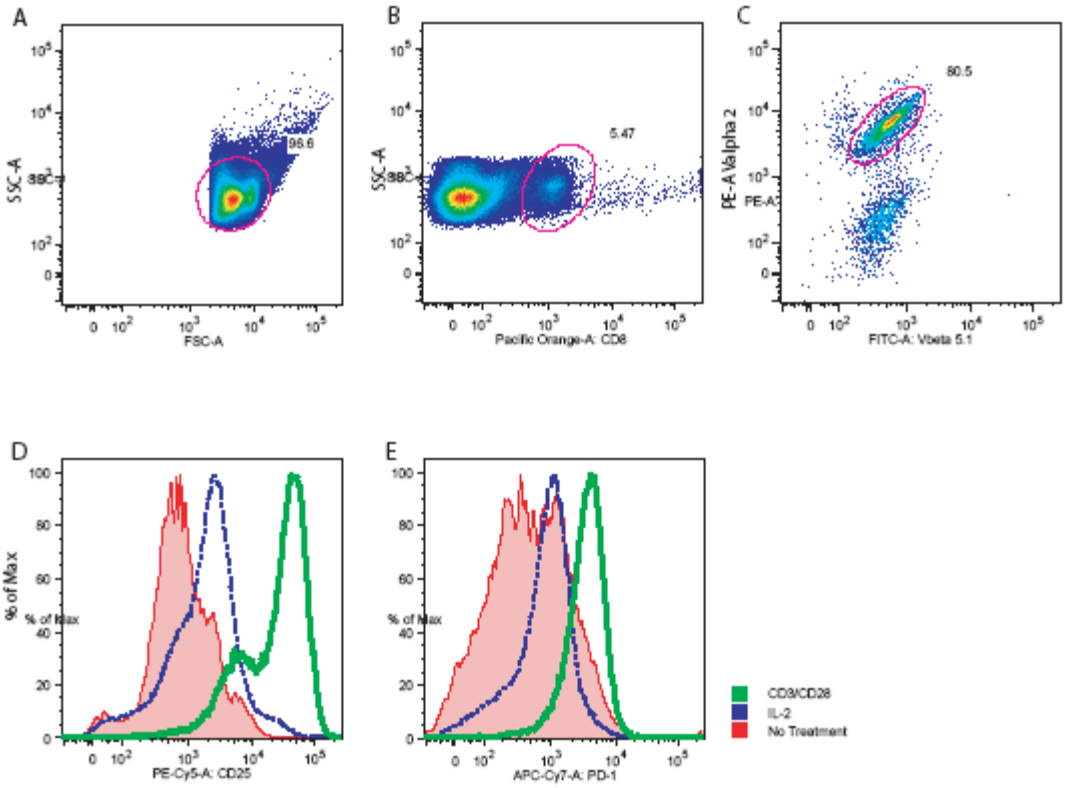

A
SSC-A
FSC-A
96.6
B
SSC-A
Pacific Orange-A: CD8
5.47
C
PE-A Valpha 2
PE-A
FITC-A: Vbeta 5.1
80.5
D
% of Max
PE-Cy5-A: CD25
E
% of Max
APC-Cy7-A: PD-1
CD3/CD28
IL-2
No Treatment

Figure 14. OT-1 CD8 T cells upregulate CD25 in response to OVA vaccination but not immunotherapy *in vitro*. Spleens were harvested from naïve OT-1 mice, dissociated into single cell suspensions, and cultured with 1ug/ml anti-CD3 antibody and 5ug/ml anti-CD28 antibody (solid lines), 20,000 IU recombinant human IL-2 (dashed lines), or were left untreated (shaded) for 72 hours. Cultures were then washed and CD8 T cells were labeled for their expression of CD25 and PD-1.

A) Forward scatter/ Side Scatter plot and gating scheme

B) CD8 T cell gating scheme

C) Valpha 2 and Vbeta 5.1/5.2 gating scheme

D) CD25 expression on CD8 T cells

E) PD-1 expression on CD8 T cells

Figure 15

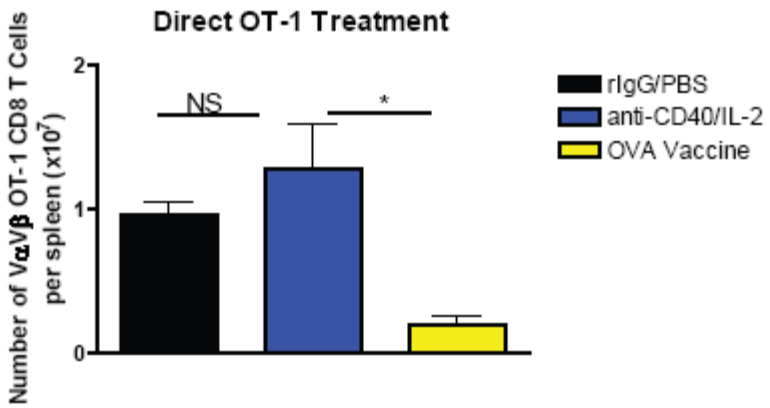

Direct OT-1 Treatment
Number of VαVβ OT-1 CD8 T Cells per spleen (x10^7)
2
1
0
NS
*
rIgG/PBS
anti-CD40/IL-2
OVA Vaccine

Figure 15. CD8 T cells do not expand when OT-1 mice are directly treated with immunotherapy or OVA vaccination. On day 22, some mice received a vaccination with an emulsion of 500ug ovalbumin (OVA) in 200ul incomplete Freund's adjuvant (IFA) IP. On day 0, mice received a boost with 500ug OVA, anti-CD40 and IL-2, or control reagents. Spleens were harvested 11 days after the initiation of treatments, and splenocytes were labeled for CD8, Valpha 2 TCR, and Vbeta 5.1/5.2 TCR and were analyzed by flow cytometry. Data were gated on CD8 positive T cells, followed by gating on cells expressing both the Valpha 2 and Vbeta 5.1/5.2 TCRs (data not shown). Data are shown as the total number of Valpha 2 and Vbeta 5.1/5.2 positive CD8 T cells per spleen. Data are representative of at least 3 independent experiments. Statistics were generated by one-way ANOVA with the tukey post-test.

Figure 16

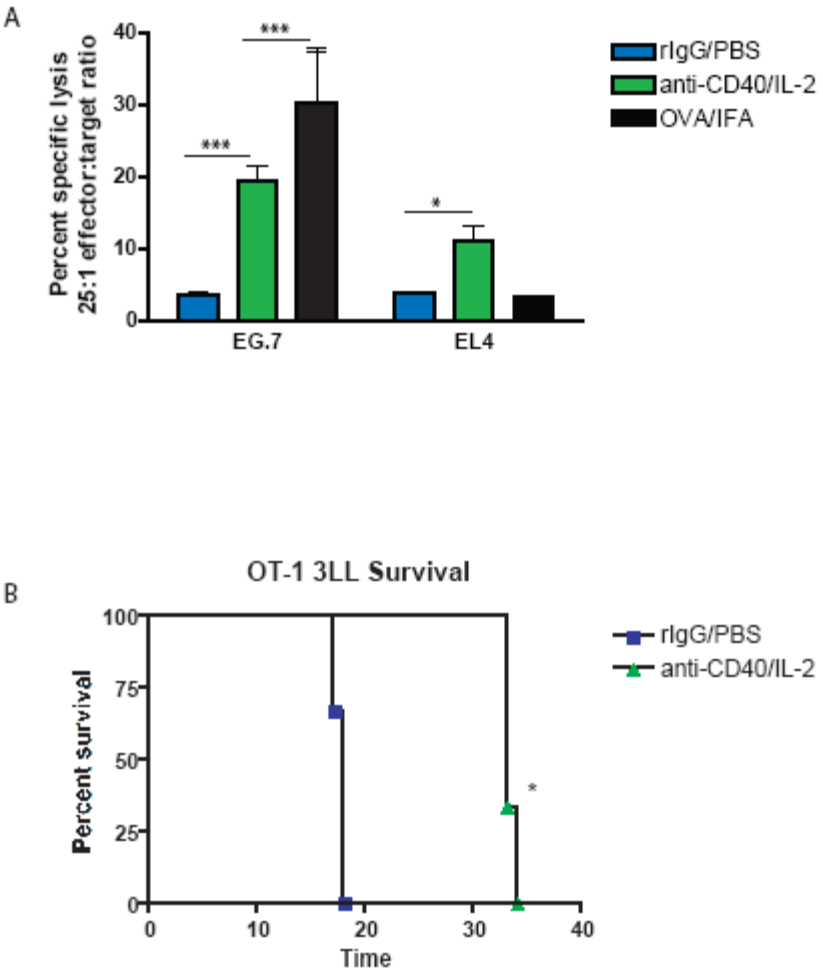

A
Percent specific lysis
25:1 effector:target ratio
40
30
20
10
0
***
***
*
EG.7
EL4
rIgG/PBS
anti-CD40/IL-2
OVA/IFA
B
OT-1 3LL Survival
Percent survival
100
75
50
25
0
0
10
20
30
40
Time
*
rIgG/PBS
anti-CD40/IL-2

Figure 16. OT-1 mice reject TCR irrelevant tumors after immunotherapy *in vivo* and *in vitro*. (A) OT-1 mice were treated with anti-CD40 and IL-2, control regimen, or were immunized with 100ug OVA emulsified in IFA and harvested on day 11. Spleens were dissociated into a single cell suspension and incubated with chromium-51 labeled EG.7 (ova-EL4) or EL4 tumor target cells for 4 hours. Supernatants were removed and analyzed on a gamma counter. Data are presented as percent lysis at a 50:1 effector:target ratio. Statistics were generated by one way ANOVA with Tukey post test. (B) OT-1 mice were injected subcutaneously with $2x10^6$ 3LL tumor cells on Day 0. On day 7 post tumor injection, mice were treated with the BALB/c regimen of anti-CD40, and a reduced dose of $3x10^5$ IU rhIL-2, or control reagents and were monitored for survival. Data were plotted using the Kaplan-Meier technique and statistics were generated by Log-rank test.

Figure 17

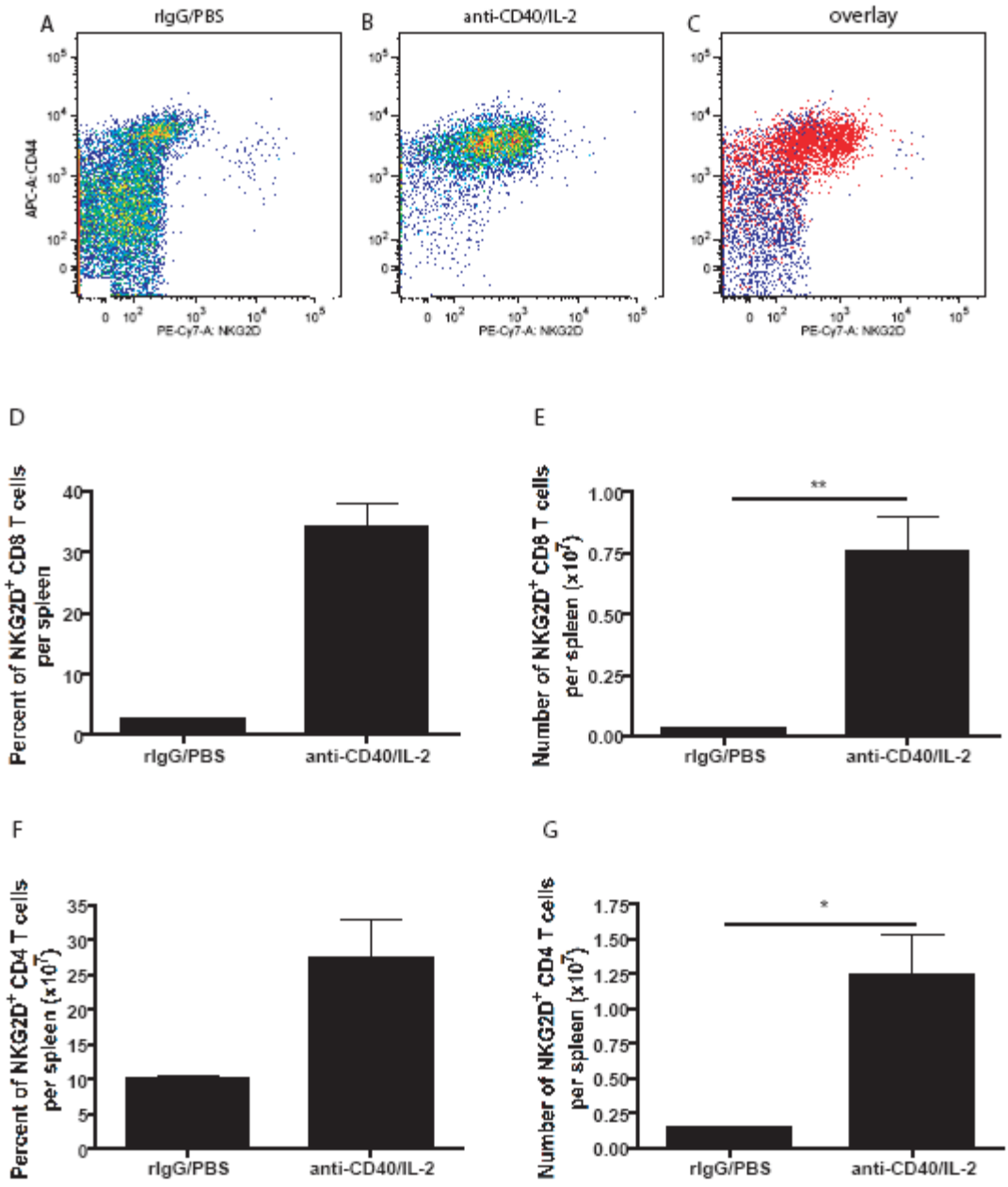
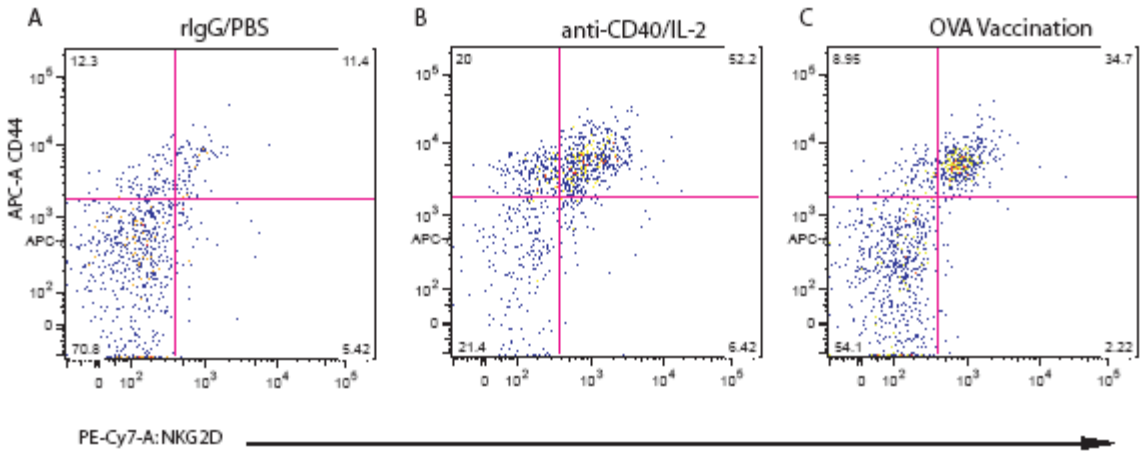
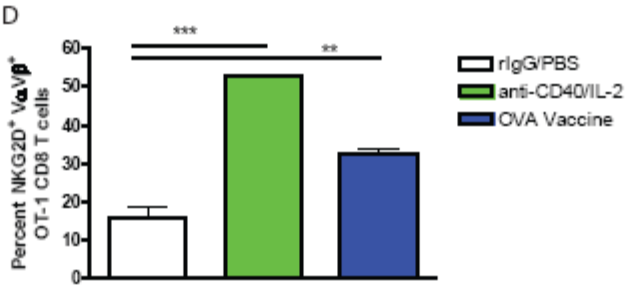

A
rIgG/PBS
B
anti-CD40/IL-2
C
overlay
APC-A:CD44
PE-Cy7-A: NKG2D
D
Percent of NKG2D+ CD8 T cells per spleen
rIgG/PBS
anti-CD40/IL-2
E
Number of NKG2D+ CD8 T cells per spleen (x10^7)
**
F
Percent of NKG2D+ CD4 T cells per spleen (x10^7)
G
Number of NKG2D+ CD4 T cells per spleen (x10^7)
*

Figure 17. Immunotherapy increases the frequency and number of NKG2D+ CD8 T cells. C57BL/6 mice received the standard regimen of (B) anti-CD40 and IL-2 or (A) rat IgG and PBS control treatments. (A-C) Mice were sacrificed on day 11 and spleens cells were labeled for CD8, CD44, and NKG2D and analyzed by flow cytometry. Data were gated on CD8 T cells and are shown as CD44 plotted against NKG2D. (C) NKG2D expression is shown as an overlay of control versus immunotherapy treatment. (D) Frequencies and (E) total number of NKG2D+ CD8 T cells per spleen following control and immunotherapy treatment. Statistics were generated by Mann-Whitney test (D) or Student's t-test (E).

Figure 18


A
rIgG/PBS
B
anti-CD40/IL-2
C
OVA Vaccination
APC-A CD44
PE-Cy7-A: NKG2D
12.3
11.4
70.8
5.42
20
52.2
21.4
6.42
8.95
34.7
54.1
2.22


D
Percent NKG2D+ VαVβ+ OT-1 CD8 T cells
***
**
rIgG/PBS
anti-CD40/IL-2
OVA Vaccine

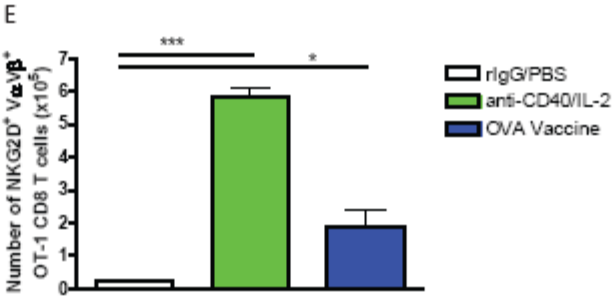

E
Number of NKG2D+ VαVβ+ OT-1 CD8 T cells (x10^5)
***
*
rIgG/PBS
anti-CD40/IL-2
OVA Vaccine

Figure 18. Immunotherapy and OVA vaccination results in increased frequencies and numbers of adoptively transferred NKG2D$^+$ OT-1 CD8 T cells. C57BL/6 received an adoptive transfer of 2.5 x 10^6 OT-1 lymph node cells IV on day -4. On day 0, mice received (C) a vaccination with an emulsion of 500ug ovalbumin (OVA) in 200ul incomplete Freund's adjuvant (IFA) IP, (B) anti-CD40 and IL-2, or (A) control reagents. Spleens were harvested 11 days after the initiation of treatments, and plenocytes were labeled for CD8, Valpha 2 TCR, Vbeta 5.1/5.2 TCR, NKG2D, and CD44 expression and were analyzed by flow cytometry. Data were gated on CD8 positive T cells, followed by gating on cells expressing both the Valpha 2 and Vbeta 5.1/5.2 TCRs (data not shown). Data are shown as CD44 populations plotted against NKG2D. (D) Frequencies and (E) total numbers of NKG2D$^+$ VαVβ$^+$ CD8 T cells following adoptive transfer and immunotherapy with or without OVA vaccination. Data are representative of at least 3 independent experiments. Statistics were generated by one-way ANOVA with the tukey post-test.

Figure 19

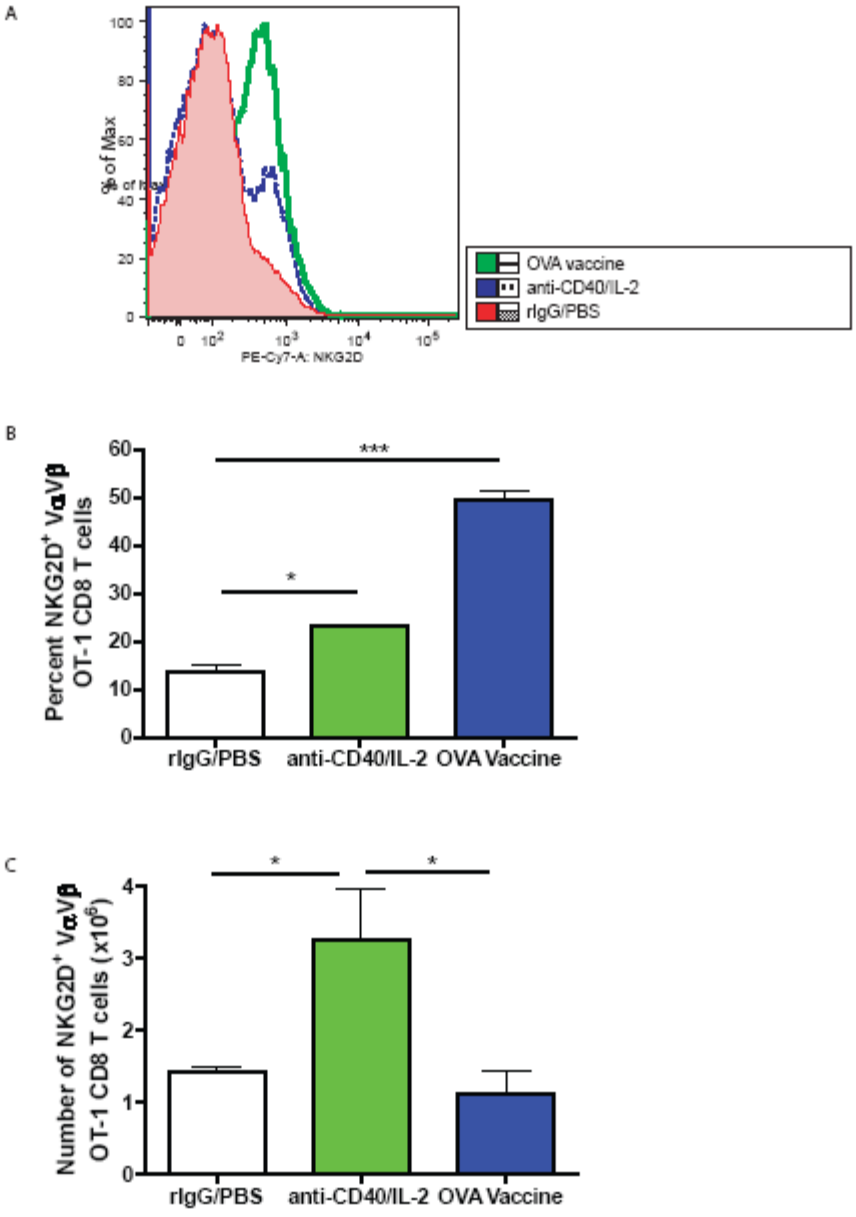

A
% of Max
PE-Cy7-A: NKG2D
OVA vaccine
anti-CD40/IL-2
rIgG/PBS
B
Percent NKG2D+ VαVβ OT-1 CD8 T cells
***
*
rIgG/PBS
anti-CD40/IL-2
OVA Vaccine
C
Number of NKG2D+ VαVβ OT-1 CD8 T cells (x10^6)
*
*
rIgG/PBS
anti-CD40/IL-2
OVA Vaccine

Figure 19. The number and frequency of NKG2D+ TCR Tg CD8 T cells increases in OT-1 mice after direct immunotherapy treatment. OT-1 mice were treated with anti-CD40 and IL-2, control regimen, or were immunized with 100ug OVA emulsified in IFA and harvested on day 11. (A) Spleen cells were labeled for CD8, Valpha 2, Vbeta 5.1/5.2, and NKG2D and were analyzed by flow cytometry (Shaded: control, Dashed blue: immunotherapy, Solid green: OVA vaccine). The (B) frequencies and (C) total numbers of NKG2D+ TCR Tg T cells in OT-1 mice following direct control, OVA vaccination, or immunotherapy treatment. Data are representative of at least 2 independent experiments. Statistics were generated by one-way ANOVA with the tukey post-test.

Figure 20

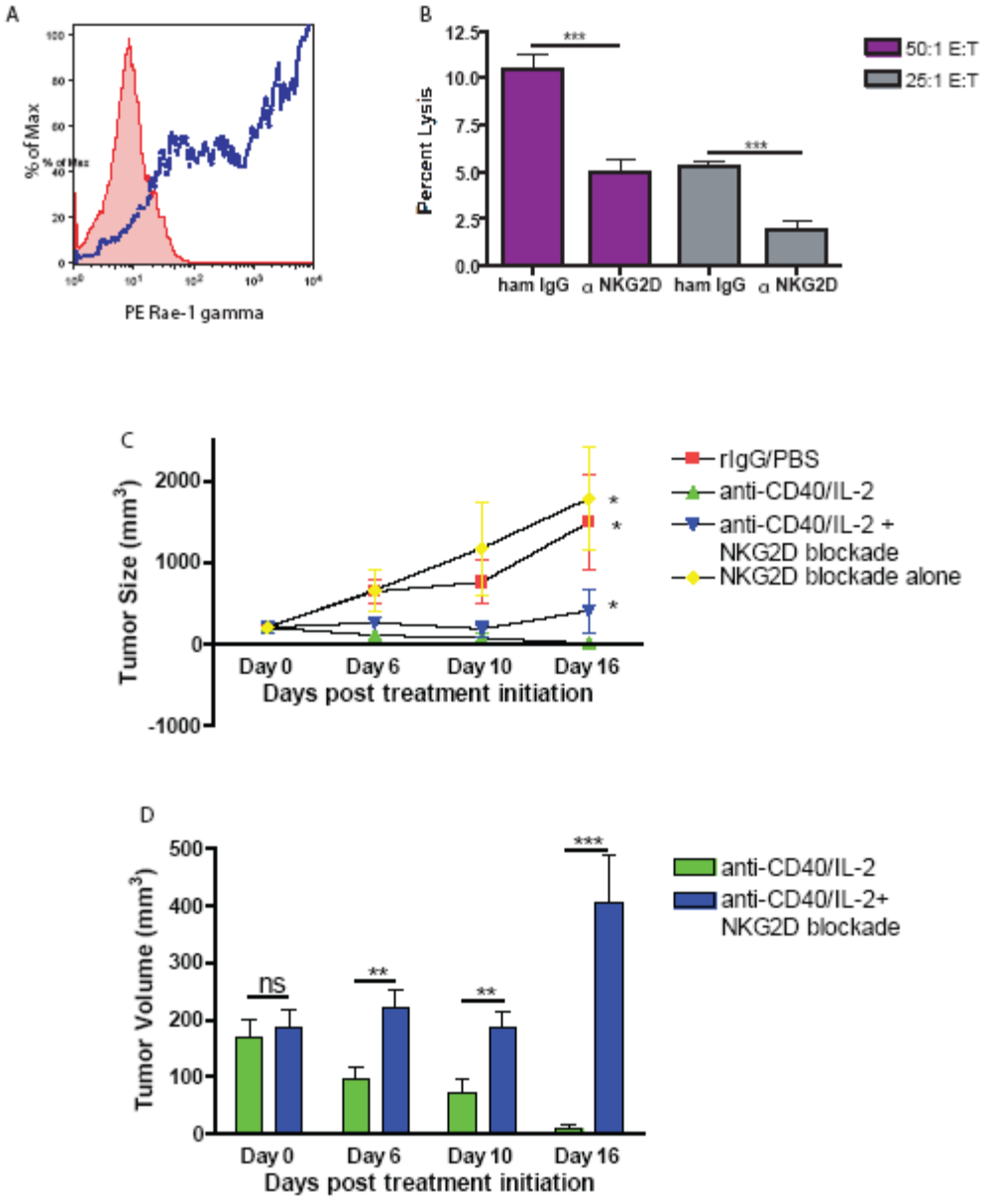

A
% of Max
PE Rae-1 gamma
B
Percent Lysis
12.5
10.0
7.5
5.0
2.5
0.0
***
***
ham IgG
α NKG2D
ham IgG
α NKG2D
50:1 E:T
25:1 E:T
C
Tumor Size (mm3)
2000
1000
0
-1000
Day 0
Day 6
Day 10
Day 16
Days post treatment initiation
*
*
*
rIgG/PBS
anti-CD40/IL-2
anti-CD40/IL-2 + NKG2D blockade
NKG2D blockade alone
D
Tumor Volume (mm3)
500
400
300
200
100
0
ns
**
**
***
Day 0
Day 6
Day 10
Day 16
Days post treatment initiation
anti-CD40/IL-2
anti-CD40/IL-2+ NKG2D blockade

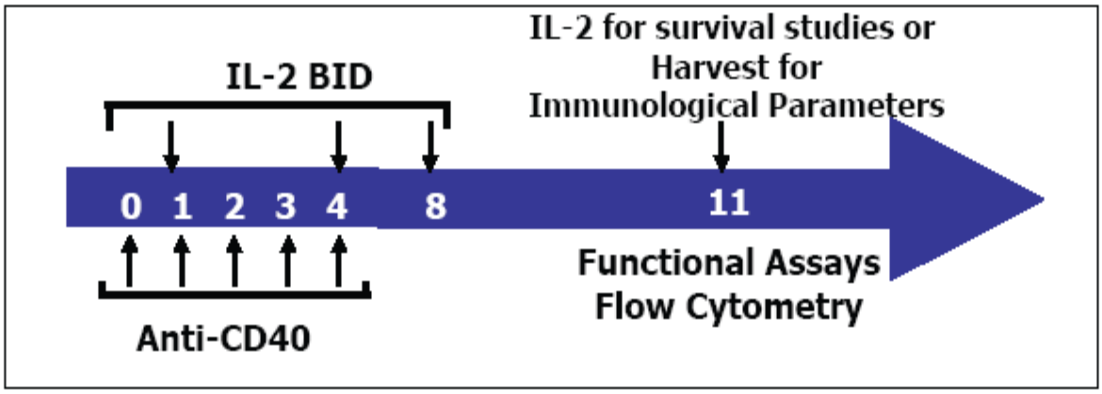
Figure 20. CD8 T cells mediate anti-tumor effects *in vitro* and *in vivo* in an NKG2D assisted manner after immunotherapy. (A) Renca tumor cells were cultured in RF10C media, dissociated with collagenase, and labeled with an antibody against Rae-1γ. (B) C57BL/6 mice were treated with anti-CD40 and IL-2 and harvested on day 11. Spleens were dissociated into single cell suspensions and CD8 T cells were isolated with MACS columns. CD8 T cells were then cultured for 4 hours in the presence of chromium-51 labeled Renca tumor targets with 20μg/mL anti-NKG2D blocking antibody or a hamster IgG control. Supernatants were removed, mixed 1:1 with scintillation fluid and analyzed on a scintillation counter. Statistics were generated by Student's *t* test with Welch's correction. (C-D) BALB/c mice were inoculated with $2x10^6$ Renca tumor cells subcutaneously. Anti-CD40 and IL-2 immunotherapy was initiated 7 days later with or without the administration of an NKG2D blocking antibody. Tumor volumes were measured and mice were monitored for survival. Statistics were generated by Student's *t* -test with Welch's correction (C) and two-way ANOVA with the Bonerfoni post-test. Data are representative of at least two independent experiments.

Table 1. Human Tumor Antigens

Category	Example Antigen	Cancer Histology
1. Oncofetal	CEA	Colorectal carcinoma
2. Oncoviral	HPV E6, E7	Cervical carcinoma
3. Overexpressed/ Accumulated	Her2/neu Telomerase	Multi Multi
4. Cancer-testis	MAGE family	Multi
5. Lineage Restricted	Melan-A/MART-1 Tyrosinase Prostate-specific antigen	Melanoma Melanoma Prostate
6. Mutated	BCRA1/2 BCR-Abl MART-2 P53 Ras	Breast, ovarian carcinoma CML Melanoma Multi Multi
7. Posttranslationally altered	MUC1	Ductal carcinoma, RCC
8. Idiotypic	Ig, TCR	B, T leukemia, lymphoma, myeloma

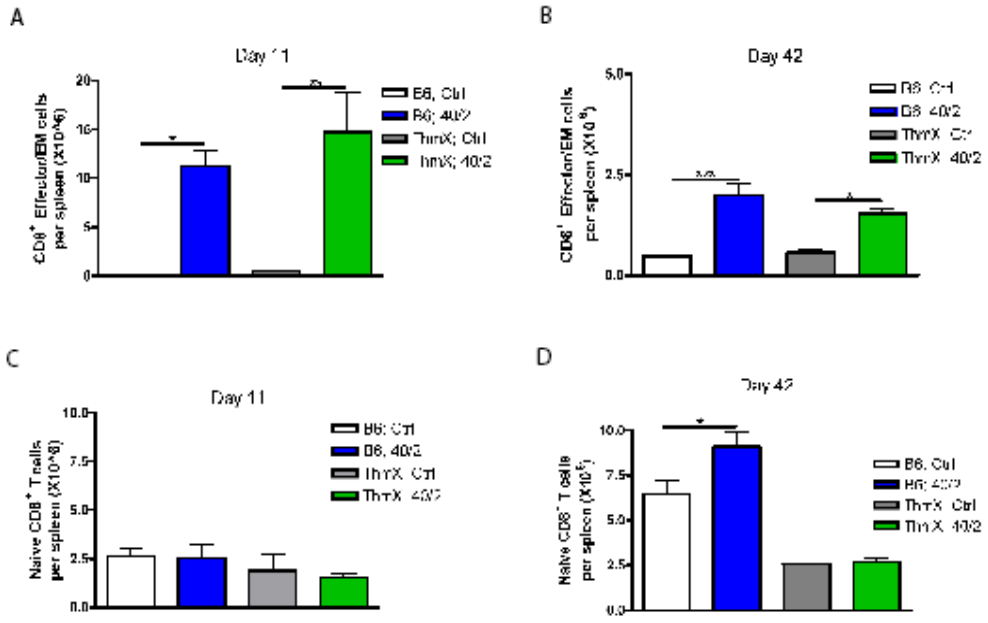
Adapted from *Principles of Cancer Biology* by L.J. Kliensmith (223) and *The Biology of Cancer* by R.A. Weinberg (224).

Supplemental Figure 1



Supplemental Figure 1. Schema of the standard anti-CD40 and IL-2 immunotherapy regimen. Mice received 5 consecutive days of treatment with anti-CD40. IL-2 was administered IP twice a day on days 2, 5, 8, and 11. The dosing of anti-CD40 for C57BL/6 mice consisted of 80ug/0.2ml administered intraperitoneally (IP). BALB/c mice received a reduced dose of anti-CD40 consisting of 65ug/0.2ml IP. The doses of IL-2 consisted of 10^6 IU for C57BL/6 mice and $5x10^5$ IU of recombinant human IL-2 administered IP. Control mice received equivalent doses of rat gamma globulin in 0.2ml administered IP instead of anti-CD40, and 0.2ml of sterile PBS in place of IL-2.

Supplemental Figure 2

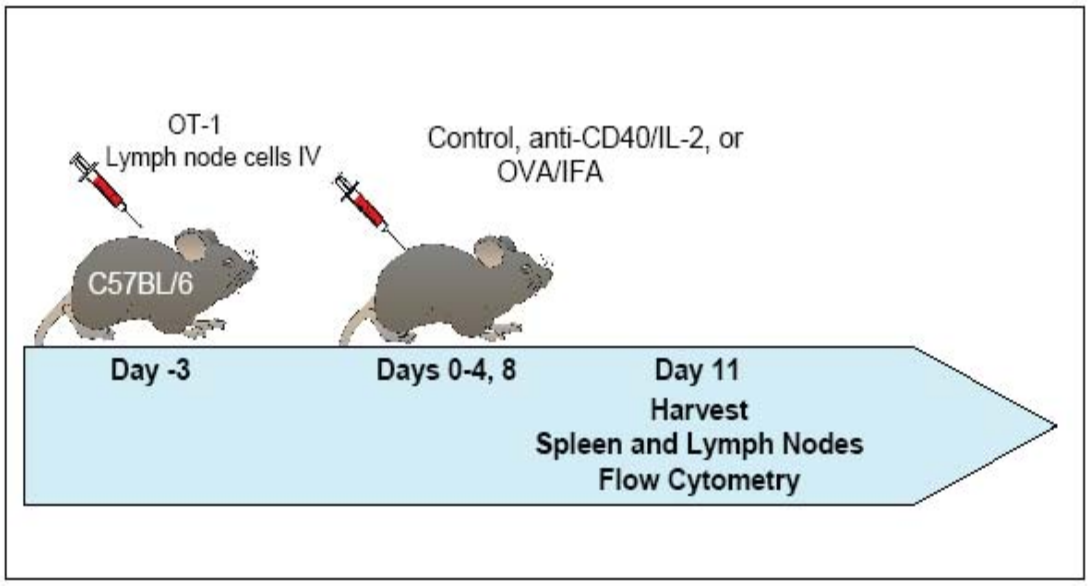


Supplemental Figure 2. Effector/Memory CD8 T cells expand in response to immunotherapy, but naïve CD8 T cells do not.

Surgically thymectomized C57BL/6 mice or their unaltered littermates were treated with the standard anti-CD40 and IL-2 immunotherapy regimen and harvested 11 (A,C,) and 42 (B,D) days later. The numbers of CD8 T cells with a naïve T cell phenotype (CD62Lhi CD44lo) or memory phenotype (CD44hi, CD62L hi/lo) were determined by flow cytometry. A) The number of effector/memory CD8 T cells per spleen on day 11. B) The number of effector/memory CD8 T cells per spleen on day 42. C) The number of naïve CD8 T cells per spleen on day 11. D) The number of naïve CD8 T cells per spleen on day 42.

(*Adapted from Alderson et al. Manuscript in preparation*)

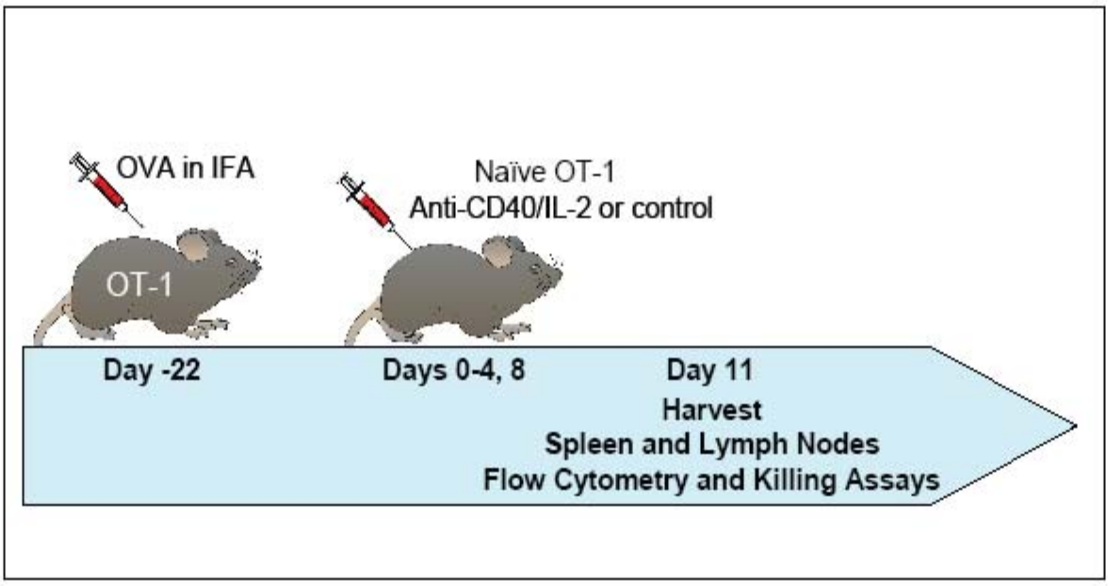
Supplemental Figure 3



Supplemental Figure 3. Schema for OT-1 adoptive transfer experiments

C57BL/6 mice received an adoptive transfer of 2.5 x 10^6 OT-1 lymph node cells IV on day -4 and began treatment with the standard anti-CD40 and IL-2 immunotherapy or control regimen on day 0. Spleens were harvested on day 11 following immunotherapy and analyzed for phenotype and proliferation by BrDU incorporation.

Supplemental Figure 4



Supplemental Figure 4. Schema for the direct treatment of OT-1 mice. OT-1 mice received a vaccination with an emulsion of 500ug ovalbumin (OVA) in 200ul incomplete Freund's adjuvant (IFA) IP 22 days before the initiation of immunotherapy. On day 0, mice received the standard C57BL/6 regimen of anti-CD40 and IL-2, control treatments, a boost with 500ug of OVA IV, or an OVA boost combined with anti-CD40 and IL-2. Spleens were harvested on day 11 following immunotherapy and analyzed for phenotype and function by flow cytometry and killing assays.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). Male and Female OT-1 (C57BL/6-Tg(TcraTcrb)1100Mjb/J) breeder pairs were purchased from the Jackson Laboratories (Bar Harbor, ME). Wiltype B6 females were bred to hemizygous OT-1 males and offspring were tested for transgene expression by flow cytometry for dual expression of the Valpha 2 and Vbeta 5 TCRs. TCR positive (experimental) and TCR negative (control) offspring with birthdates within two weeks of each other were consider age matched and were used together for experiments. For some studies, male and female OT-1 mice and age matched C57BL/6 controls were purchased from Jackson Laboratories for direct use. ThmX mice received a surgical thymectomy procedure by Charles River Laboratories at 6-8 weeks of age. Mice were housed under specific pathogen free conditions at the University of Nevada, Reno, Laboratory Animal Research Facility, and were between 12 and 24 weeks of age at the initiation of each experiment. All mouse experiments were performed with the consent and approval of the University of Nevada, Reno, Institutional Animal Care and Use Committee.

Cell Lines and Reagents

Renca is a spontaneously originating renal adenocarcinoma line derived from BALB/c mice. Renca were maintained in vitro in RF10 complete media (RF10C), which consists of RPMI 1640 (Cambrex, Walkersville, MD) supplemented with 10% Fetal bovine serum (Gemini Bio-Products, Sacramento, CA), 2mM L-glutamine (Cambrex, Walkersville, MD), 1mM sodium pyruvate, 1X non-essential amino acids, 10mM Hepes buffer (Invitrogen, Grand Island, NY), 50ug/mL penicillin/streptomycin (Mediatech, Herndon, VA), and 5 x 10^{-5}M 2-mercaptoethanol (Invitrogen, Grand Island, NY). Renca was dissociated from flasks using Trypsin EDTA 1X (Mediatech, Herndon, VA). 3LL is a Lewis Lung carcinoma of C57BL/6 origin. Culturing conditions for 3LL were identical to those used for Renca except DPBS without calcium and magnesium was used for dissociation instead of trypsin (Mediatech, Herndon, VA). P815, EL-4, and EG.7 cell lines were also maintained in RF10 complete media. EG.7 was supplemented with 0.1% G418 sulfate solution (Invitrogen, Carlsbad, CA).

The anti-mouse CD40 antibody, clone FGK115 was generously donated by Bruce Blazar M.D. (University of Minnesota). The subclone FGK115-B3 was kindly isolated by the laboratory of Thomas Kozel Ph.D (University of Nevada, Reno) and antibody was generated via ascites production in our laboratory, as previously described (222). Total protein content was determined by spectrophotometry. Antibody content was determined by rat IgG ELISA. Endotoxin content was tested by Limulus Amoebocyte Lysate (LAL) assay (QCL-

1000) (Lonza, Basel, Switzerland). The endotoxin levels between antibody lots ranged between 0.51 EU/mg of antibody and 10 EU/mg. Rat gamma globulin was utilized as an antibody control for all assays and was purchased from Jackson Immunoresearch (West Grove, PA). Recombinant human Interleukin-2 (IL-2; TECIN. Teceleukin) was provided by the National Cancer Institute (Frederick, MD). Recombinant murine IL-12 was purchased from Peprotech (Rocky Hill, NJ). BrDU was purchased from BD Pharmingen (San Diego, CA) as part of a FITC BrDU labeling kit.

Purified anti-mouse-CD3 (clone 145-2C11) and anti-mouse CD28 (clone 37.51) antibodies were purchased from ebioscience (San Diego, CA) and used *in vitro* at concentrations of 1ug/ml and 5ug/ml, resectively. The non-depleting NKG2D blocking antibody (Clone CX5) was a generous gift from Dr. Lewis Lanier of the University of California, San Francisco. The CD8 depletion antibody (clone YTS169.4) was purchased as whole ascites from Taconic labs (Hudson, NY)

Flow Cytometry

Single cell suspensions of spleen and lymph node tissues were gently dissociated with 25G needles. Cells were filtered through nylon mesh and washed two times in staining buffer before antibody labeling. Staining buffer consisted of DPBS (Mediatech, Herndon, VA) with 1% FBS (Gemini Bio-products, Sacramento, CA) and 1% penicillin/streptomycin (Mediatech, Herndon, VA). Cells were labeled with Fc Block and antibodies for 20 minutes, and then washed two times with

staining buffer before data acquisition on a three color FACScan flow cytometer using Cell Quest software (Becton Dickinson, San Jose, CA), a 5-color FC 500/MPL (Beckman Coulter, Fullerton CA), or on a custom configured LSRII using the FACDiva software (Becton Dickinson, San Jose, CA)**.** All data was analyzed using FlowJo software (Treestar, Ashland, OR). The laser and filter configurations for the custom LSRII flow cytometer are presented in addendums 2-5. A list of antibodies, vendors, clones, and dilutions are included in addendum 1. FITC BrDU staining kits were purchased from BD Pharmingen (San Diego, CA) and used according to manufacture's instructions. For intracellular cytokine staining, cells were stimulated for 4 hours in RF10 complete media supplemented with 400ng/ml phorbol 12-myristate 13-acetat (PMA) (Fluka-Sigma, St. Louis, MO), 1uM ionomycin calcium salt (Sigma, St. Louis, MO), and 1ug/ml Golgi plug (BD Pharmingen, San Diego, CA) before surface antibody labeling. IntraPrep Reagent kits (Beckman Coulter, Brea, CA) were used for the fixation and permabilization of cells, according to manufacturer's instructions.

Cell Preparations

Single cell suspensions of spleen and lymph node tissues were gently dissociated with 25G needles. Cells were filtered through nylon mesh and washed two times in staining buffer and were resuspended in RF10C media or staining buffer. Red blood cells were lysed with zap-oglobin II lytic reagent (Beckman Coulter, Brea, CA) and total tissue cell counts were determined on a Coulter Z1 particle counter

(Coulter Electronics, Arlington, TX). Blood was collected in BD Micro-fine tubes containing EDTA (Bedford, MA). Red blood cells were lysed in blood samples with FACSLyse (BD Biosciences, San Jose, CA).

Redirected lysis assay

Spleens were harvested from C57BL/6 mice 5 days after the initiation of immunotherapy. CD8 T cells were enriched with MACS column according to manufacturer's instructions (Miltenyi, Auburn, CA) and were serial diluted in 96 well plates in RF10C media. P815 mastocytoma cells were labeled with 100uCi Cr-51 (NEZ030S, Perkin Elmer, Waltham, MA) per 10^6 cells for 1 hour, washed twice with 50ml DPBS and resuspended in RF10C. P815 tumor cells were then incubated for 30 minutes with 10ug/ml anti-CD3 antibody (clone 2C11, ebioscience, San Diego, CA). 10,000 P815 target cells were added per well and assays were incubated at 37C for 4 hours. Plates were centrifuged at 1000 RPM for 5 minutes and supernatants were removed and mixed 1:1 with scintillation fluid and analyzed on a Wallac scintillation counter (Wallac, Turku, Finland). Specific release was calculated as:

$$\text{Percent lysis} = \frac{\text{Experimental - Spontaneous}}{\text{Total - Spontaneous}} \times 100\%$$

CFSE cytotoxicity assay

Renca was dissociated from flasks using Trypsin EDTA 1X (Mediatech, Herndon, VA), washed twice in DPBS, and resuspended in 5uM Vybrant CFDA SE Cell tracter reagent (Invitrogen, Eugene, OR) and incubated for 10 minutes at 37C. Cells were washed once, resuspended in 20ml DPBS and allowed to purge for an additional 10 minutes at room temperature. 50,000 Cells were then added to 5ml tubes and co-incubated with various concentrations of splenocytes harvested from immunotherapy and control treated mice. Tubes were incubated overnight and CFSE positive cells were analyzed by flow cytometry.

OVA Vaccinantion

Ovalbumin (Sigma, St. Louis, MO) was diluted at a concentration of 5mg/ml in DPBS (Mediatech, Herndon, VA). Appropriate concentrations of Ovalbumin were subjected to water into oil emulsification with a 1:1 ratio of ovalbumin to incomplete Freund's adjuvant (Sigma, St. Louis, MO). Emulsification was performed with glass syringes and 47.6mm micro-emulsifying needles (Fisher Scientific, Pittsburgh, PA). Emulsifications were immediately injected IP into recipient mice.

Treatment Protocols

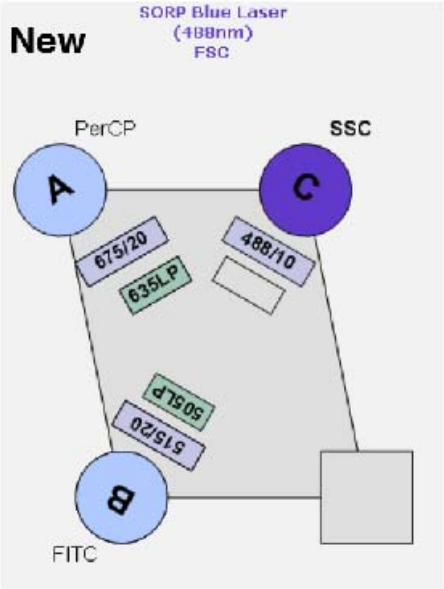
C57BL/6 mice were treated with 80ug anti-CD40 antibody for 5 consecutive days. $1x10^6$ IU recombinant human IL-2 was administered BID on days 2, 5, 8, and 11.

BALB/c mice received the same timed regimen, except the doses of anti-CD40 were lowered to 65ug, and 500,000 IU IL-2 was administered. Administration of IL-2 and CD40 was separated by a minimum of 4 hours, and a maximum of 20 hours to prevent toxicities. In experiments where IL-12 was used as an alternative to anti-CD40, 0.5ug recombinant murine IL-12 was administered during 5 consecutive days. $1x10^6$ IU of IL-2 were administered in the IL-12 studies at the same time as given in the anti-CD40/IL-2 regimen. In experiments involving BrDU staining, 1mg/0.2ml of BrDU was injected IP one day before harvest. For in vivo depletion studies, 50ug/0.2ml anti-NKG2D (clone CX5) antibody was injected intratumorally concurrent with anti-CD40 and IL-2 therapy (days 1-5, 8, and 11). For CD8 T cell depletions, one IP injection of 300ug/0.2ml was given on the first day of immunotherapy treatment.

Statistics. Statistical analysis was performed using Prism software (Graphpad Software Inc.) Analysis of percentages with two test groups was performed with the Mann-Whitney analysis. For analysis of three or more groups, the non-parametric ANOVA test was utilized with the Bonferroni or Tukey post-tests. Analysis of cell numbers between two test groups was performed using Student's *t*-test. Welch's correction was applied to Student's *t*-test data sets with significant differences in variance. Survival data were analyzed by Log Rank Test. Data were tested for normality and variance, a p-value of <0.05 was considered significant. * <0.05, ** <0.01, *** <0.001.

Flow Cytometry antibodies, and LSRII laser and filter configurations

Reagent	Supplier	Isotype	Clone	Dilution (10ul/tube)
FITC-anti-mouse TCR Vbeta 5.1/5.2	BD PharMingen	M IgG1 K	MR9-4	20
PE-anti-mouse TCR Valpha 2	eBioscience	R IgG2a	B20.1	16
AF700-anti-mouse CD4	BioLegend	R IgG2b K	GK1.5	80
Pacific Orange-anti-mouse CD8a	Invitrogen	R IgG2b	5H10	4
FITC-anti-mouse CD8a	eBioscience	R IgG2a k	53-6.7	40
anti-mouse CD16/32	eBioscience	R-IgG2b k	93	20
PE-anti-mouse CD25	eBioscience	R IgG1 L	PC61.5	32
PE-Cy5-anti-mouse CD25	eBioscience	R IgG1 L	PC61.5	16
FITC-anti-mouse CD27	eBioscience	H IgG	LG.7F9	40
APC-anti-mouse/human CD44	eBioscience	R IgG2b k	IM7	64
PC7-anti-mouse/human CD44	eBioscience	R IgG2b k	IM7	32
Pacific Blue-anti-mouse CD45	BioLegend	R-IgG2b k	30-F11	40
PC7-anti-mouse-CD62L	eBioscience	R IgG2a k	MEL-14	128
FITC-anti-mouse CD69	eBioscience	H IgG	H1.2F3	20
Biotin-anti-mouse CD122	eBioscience	R IgG2a K	5H4	40
PE-anti-mouse PD-1	eBioscience	Ar H IgG	J43	32
Biotin-anti-mouse PD-1	eBioscience	H IgG	J43	20
FITC-anti-mouse Ly-6C	PharMingen	R IgM K	AL-21	32
PE-Isotype control for a-GZMB	Invitrogen	M IgG1		2
PE-anti-Granzyme B	Invitrogen	M IgG1	GB12	2
AF647-Rat-IgG1 k (Ctrl for IFNg)	eBioscience	R-IgG1 k	eBRG1	16
AF647-anti-mouse IFNg	eBioscience	R IgG1 k	XMG1.2	16
PC7-anti-mouse NKG2D	eBioscience	R IgG1 K	CX5	8
PE-anti-mouse NKG2D	eBioscience	R IgG1 K	CX5	8
APC-Cy7-Streptavidin	eBioscience			16
IntraPrep Reagent kit 150 tests	Beckman-Coulter			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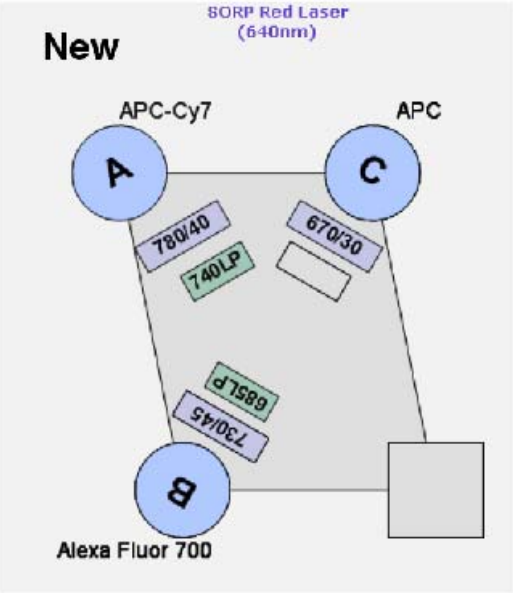
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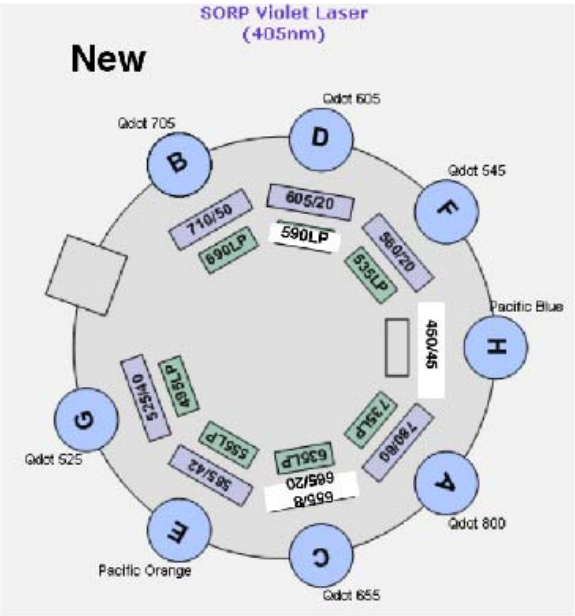
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(405nm)
New
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D
Qdot 705
B
Qdot 545
F
605/20
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690LP
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560/20
535LP
Pacific Blue
450/45
H
A
Qdot 800
780/60
735LP
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655/8
665/20
585LP
585/42
C
Qdot 655
Pacific Orange
E
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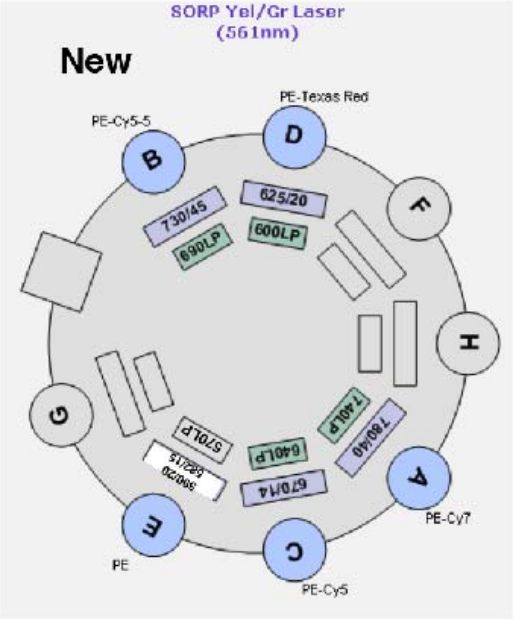
525/40
G
Qdot 525


SORP Yel/Gr Laser
(561nm)
New
PE-Texas Red
PE-Cy5-5
B
D
F
H
A
C
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G
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600LP
740LP
780/40
670/14
640LP
570LP
582/15
PE-Cy7
PE
PE-Cy5

References

1. Smyth, M. J., G. P. Dunn, and R. D. Schreiber. 2006. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Advances in immunology* 90:1-50.
2. Dunn, G. P., A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber. 2002. Cancer immunoediting: from immunosurveillance to tumor escape. *Nature immunology* 3:991-998.
3. Dunn, G. P., L. J. Old, and R. D. Schreiber. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 21:137-148.
4. Burnet, F. M. 1971. Implications of immunological surveillance for cancer therapy. *Israel journal of medical sciences* 7:9-16.
5. Burnet, F. M. 1970. The concept of immunological surveillance. *Progress in experimental tumor research. Fortschritte der experimentellen Tumorforschung* 13:1-27.
6. Burnet, F. M. 1971. Immunological surveillance in neoplasia. *Transplantation reviews* 7:3-25.
7. Burnet, F. M. 1973. Implications of cancer immunity. *Australian and New Zealand journal of medicine* 3:71-77.
8. Thomas, L. 1982. On immunosurveillance in human cancer. *The Yale journal of biology and medicine* 55:329-333.
9. Dunn, G. P., L. J. Old, and R. D. Schreiber. 2004. The three Es of cancer immunoediting. *Annual review of immunology* 22:329-360.
10. Stutman, O. 1974. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science (New York, N.Y* 183:534-536.
11. Shankaran, V., H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old, and R. D. Schreiber. 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 410:1107-1111.
12. Kaplan, D. H., V. Shankaran, A. S. Dighe, E. Stockert, M. Aguet, L. J. Old, and R. D. Schreiber. 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proceedings of the National Academy of Sciences of the United States of America* 95:7556-7561.
13. Street, S. E., E. Cretney, and M. J. Smyth. 2001. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. *Blood* 97:192-197.
14. Smyth, M. J., K. Y. Thia, S. E. Street, E. Cretney, J. A. Trapani, M. Taniguchi, T. Kawano, S. B. Pelikan, N. Y. Crowe, and D. I. Godfrey. 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. *The Journal of experimental medicine* 191:661-668.

15. Dunn, G. P., C. M. Koebel, and R. D. Schreiber. 2006. Interferons, immunity and cancer immunoediting. *Nature reviews* 6:836-848.
16. Boshoff, C., and R. Weiss. 2002. AIDS-related malignancies. *Nat Rev Cancer* 2:373-382.
17. Birkeland, S. A., H. H. Storm, L. U. Lamm, L. Barlow, I. Blohme, B. Forsberg, B. Eklund, O. Fjeldborg, M. Friedberg, L. Frodin, and et al. 1995. Cancer risk after renal transplantation in the Nordic countries, 1964-1986. *International journal of cancer* 60:183-189.
18. Galon, J., A. Costes, F. Sanchez-Cabo, A. Kirilovsky, B. Mlecnik, C. Lagorce-Pages, M. Tosolini, M. Camus, A. Berger, P. Wind, F. Zinzindohoue, P. Bruneval, P. H. Cugnenc, Z. Trajanoski, W. H. Fridman, and F. Pages. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science (New York, N.Y* 313:1960-1964.
19. MacKie, R. M., R. Reid, and B. Junor. 2003. Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. *The New England journal of medicine* 348:567-568.
20. Khong, H. T., Q. J. Wang, and S. A. Rosenberg. 2004. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. *J Immunother* 27:184-190.
21. Jager, E., M. Ringhoffer, M. Altmannsberger, M. Arand, J. Karbach, D. Jager, F. Oesch, and A. Knuth. 1997. Immunoselection in vivo: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma. *International journal of cancer* 71:142-147.
22. Yee, C., J. A. Thompson, D. Byrd, S. R. Riddell, P. Roche, E. Celis, and P. D. Greenberg. 2002. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proceedings of the National Academy of Sciences of the United States of America* 99:16168-16173.
23. Koebel, C. M., W. Vermi, J. B. Swann, N. Zerafa, S. J. Rodig, L. J. Old, M. J. Smyth, and R. D. Schreiber. 2007. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450:903-907.
24. Sokoloff, M. H., S. Daneshmand, and C. W. Ryan. 2005. Current clinical trials in renal cell carcinoma. *Urol Oncol* 23:289-292.
25. Sokoloff, M. H., J. B. deKernion, R. A. Figlin, and A. Belldegrun. 1996. Current management of renal cell carcinoma. *CA: a cancer journal for clinicians* 46:284-302.
26. Haas, G. P., B. G. Redman, V. K. Rao, E. Dybal, J. E. Pontes, and G. G. Hillman. 1993. Immunotherapy for metastatic renal cell cancer: effect on the primary tumor. *J Immunother Emphasis Tumor Immunol* 13:130-135.

27. Haas, G. P., G. G. Hillman, B. G. Redman, and J. E. Pontes. 1993. Immunotherapy of renal cell carcinoma. *CA: a cancer journal for clinicians* 43:177-187.
28. Parmiani, G., A. De Filippo, L. Novellino, and C. Castelli. 2007. Unique human tumor antigens: immunobiology and use in clinical trials. *J Immunol* 178:1975-1979.
29. Farkas, A. M., and O. J. Finn. Vaccines based on abnormal self-antigens as tumor-associated antigens: Immune regulation. *Seminars in immunology*.
30. Govan, V. A. 2008. A novel vaccine for cervical cancer: quadrivalent human papillomavirus (types 6, 11, 16 and 18) recombinant vaccine (Gardasil). *Therapeutics and clinical risk management* 4:65-70.
31. Muderspach, L., S. Wilczynski, L. Roman, L. Bade, J. Felix, L. A. Small, W. M. Kast, G. Fascio, V. Marty, and J. Weber. 2000. A phase I trial of a human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV 16 positive. *Clin Cancer Res* 6:3406-3416.
32. Cha, E., and L. Fong. Therapeutic vaccines for prostate cancer. *Current opinion in molecular therapeutics* 12:77-85.
33. Heslop, H. E., C. Y. Ng, C. Li, C. A. Smith, S. K. Loftin, R. A. Krance, M. K. Brenner, and C. M. Rooney. 1996. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nature medicine* 2:551-555.
34. Riddell, S. R., and P. D. Greenberg. 1995. Principles for adoptive T cell therapy of human viral diseases. *Annual review of immunology* 13:545-586.
35. Greenberg, P., J. Goodrich, and S. Riddell. 1991. Adoptive immunotherapy of human cytomegalovirus infection: potential role in protection from disease progression. *Transplantation proceedings* 23:97-101.
36. Perez-Diez, A., N. T. Joncker, K. Choi, W. F. Chan, C. C. Anderson, O. Lantz, and P. Matzinger. 2007. CD4 cells can be more efficient at tumor rejection than CD8 cells. *Blood* 109:5346-5354.
37. Quezada, S. A., T. R. Simpson, K. S. Peggs, T. Merghoub, J. Vider, X. Fan, R. Blasberg, H. Yagita, P. Muranski, P. A. Antony, N. P. Restifo, and J. P. Allison. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *The Journal of experimental medicine* 207:637-650.
38. Xie, Y., A. Akpinarli, C. Maris, E. L. Hipkiss, M. Lane, E. K. Kwon, P. Muranski, N. P. Restifo, and P. A. Antony. Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. *The Journal of experimental medicine* 207:651-667.
39. Riddell, S. R., and P. D. Greenberg. 1990. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. *Journal of immunological methods* 128:189-201.

40. June, C. H. 2007. Adoptive T cell therapy for cancer in the clinic. *The Journal of clinical investigation* 117:1466-1476.
41. June, C. H. 2007. Principles of adoptive T cell cancer therapy. *The Journal of clinical investigation* 117:1204-1212.
42. Schumacher, T. N., and N. P. Restifo. 2009. Adoptive T cell therapy of cancer. *Current opinion in immunology* 21:187-189.
43. Antony, P. A., C. A. Piccirillo, A. Akpinarli, S. E. Finkelstein, P. J. Speiss, D. R. Surman, D. C. Palmer, C. C. Chan, C. A. Klebanoff, W. W. Overwijk, S. A. Rosenberg, and N. P. Restifo. 2005. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol* 174:2591-2601.
44. Antony, P. A., C. M. Paulos, M. Ahmadzadeh, A. Akpinarli, D. C. Palmer, N. Sato, A. Kaiser, C. S. Hinrichs, C. A. Klebanoff, Y. Tagaya, and N. P. Restifo. 2006. Interleukin-2-dependent mechanisms of tolerance and immunity in vivo. *J Immunol* 176:5255-5266.
45. Einsele, H., E. Roosnek, N. Rufer, C. Sinzger, S. Riegler, J. Loffler, U. Grigoleit, A. Moris, H. G. Rammensee, L. Kanz, A. Kleihauer, F. Frank, G. Jahn, and H. Hebart. 2002. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood* 99:3916-3922.
46. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421:852-856.
47. Janssen, E. M., N. M. Droin, E. E. Lemmens, M. J. Pinkoski, S. J. Bensinger, B. D. Ehst, T. S. Griffith, D. R. Green, and S. P. Schoenberger. 2005. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature* 434:88-93.
48. Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393:480-483.
49. Klebanoff, C. A., S. E. Finkelstein, D. R. Surman, M. K. Lichtman, L. Gattinoni, M. R. Theoret, N. Grewal, P. J. Spiess, P. A. Antony, D. C. Palmer, Y. Tagaya, S. A. Rosenberg, T. A. Waldmann, and N. P. Restifo. 2004. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. *Proceedings of the National Academy of Sciences of the United States of America* 101:1969-1974.
50. Kalia, V., S. Sarkar, T. S. Gourley, B. T. Rouse, and R. Ahmed. 2006. Differentiation of memory B and T cells. *Current opinion in immunology* 18:255-264.
51. MacLeod, M., M. J. Kwakkenbos, A. Crawford, S. Brown, B. Stockinger, K. Schepers, T. Schumacher, and D. Gray. 2006. CD4 memory T cells survive and proliferate but fail to differentiate in the absence of CD40. *The Journal of experimental medicine* 203:897-906.

52. Hataye, J., J. J. Moon, A. Khoruts, C. Reilly, and M. K. Jenkins. 2006. Naive and memory CD4+ T cell survival controlled by clonal abundance. *Science (New York, N.Y* 312:114-116.
53. Klebanoff, C. A., L. Gattinoni, P. Torabi-Parizi, K. Kerstann, A. R. Cardones, S. E. Finkelstein, D. C. Palmer, P. A. Antony, S. T. Hwang, S. A. Rosenberg, T. A. Waldmann, and N. P. Restifo. 2005. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proceedings of the National Academy of Sciences of the United States of America* 102:9571-9576.
54. Berger, C., M. C. Jensen, P. M. Lansdorp, M. Gough, C. Elliott, and S. R. Riddell. 2008. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *The Journal of clinical investigation* 118:294-305.
55. Hinrichs, C. S., Z. A. Borman, L. Cassard, L. Gattinoni, R. Spolski, Z. Yu, L. Sanchez-Perez, P. Muranski, S. J. Kern, C. Logun, D. C. Palmer, Y. Ji, R. N. Reger, W. J. Leonard, R. L. Danner, S. A. Rosenberg, and N. P. Restifo. 2009. Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proceedings of the National Academy of Sciences of the United States of America* 106:17469-17474.
56. Dudley, M. E., J. C. Yang, R. Sherry, M. S. Hughes, R. Royal, U. Kammula, P. F. Robbins, J. Huang, D. E. Citrin, S. F. Leitman, J. Wunderlich, N. P. Restifo, A. Thomasian, S. G. Downey, F. O. Smith, J. Klapper, K. Morton, C. Laurencot, D. E. White, and S. A. Rosenberg. 2008. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 26:5233-5239.
57. Wrzesinski, C., C. M. Paulos, A. Kaiser, P. Muranski, D. C. Palmer, L. Gattinoni, Z. Yu, S. A. Rosenberg, and N. P. Restifo. Increased intensity lymphodepletion enhances tumor treatment efficacy of adoptively transferred tumor-specific T cells. *J Immunother* 33:1-7.
58. Gattinoni, L., S. E. Finkelstein, C. A. Klebanoff, P. A. Antony, D. C. Palmer, P. J. Spiess, L. N. Hwang, Z. Yu, C. Wrzesinski, D. M. Heimann, C. D. Surh, S. A. Rosenberg, and N. P. Restifo. 2005. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. *The Journal of experimental medicine* 202:907-912.
59. Klebanoff, C. A., H. T. Khong, P. A. Antony, D. C. Palmer, and N. P. Restifo. 2005. Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. *Trends in immunology* 26:111-117.
60. Paulos, C. M., C. Wrzesinski, A. Kaiser, C. S. Hinrichs, M. Chieppa, L. Cassard, D. C. Palmer, A. Boni, P. Muranski, Z. Yu, L. Gattinoni, P. A.

Antony, S. A. Rosenberg, and N. P. Restifo. 2007. Microbial translocation augments the function of adoptively transferred self/tumor-specific CD8+ T cells via TLR4 signaling. *The Journal of clinical investigation* 117:2197-2204.

61. Berry, L. J., M. Moeller, and P. K. Darcy. 2009. Adoptive immunotherapy for cancer: the next generation of gene-engineered immune cells. *Tissue antigens* 74:277-289.
62. Yee, C., S. R. Riddell, and P. D. Greenberg. 1997. Prospects for adoptive T cell therapy. *Current opinion in immunology* 9:702-708.
63. Mier, J. W., F. R. Aronson, R. P. Numerof, G. Vachino, and M. B. Atkins. 1988. Toxicity of immunotherapy with interleukin-2 and lymphokine-activated killer cells. *Pathology and immunopathology research* 7:459-476.
64. Dudley, M. E., J. R. Wunderlich, P. F. Robbins, J. C. Yang, P. Hwu, D. J. Schwartzentruber, S. L. Topalian, R. Sherry, N. P. Restifo, A. M. Hubicki, M. R. Robinson, M. Raffeld, P. Duray, C. A. Seipp, L. Rogers-Freezer, K. E. Morton, S. A. Mavroukakis, D. E. White, and S. A. Rosenberg. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science (New York, N.Y* 298:850-854.
65. Yee, C., J. A. Thompson, P. Roche, D. R. Byrd, P. P. Lee, M. Piepkorn, K. Kenyon, M. M. Davis, S. R. Riddell, and P. D. Greenberg. 2000. Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of t cell-mediated vitiligo. *The Journal of experimental medicine* 192:1637-1644.
66. Atkins, M. B., J. W. Mier, D. R. Parkinson, J. A. Gould, E. M. Berkman, and M. M. Kaplan. 1988. Hypothyroidism after treatment with interleukin-2 and lymphokine-activated killer cells. *The New England journal of medicine* 318:1557-1563.
67. Kernan, N. A., N. H. Collins, L. Juliano, T. Cartagena, B. Dupont, and R. J. O'Reilly. 1986. Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. *Blood* 68:770-773.
68. Sullivan, K. M., R. Storb, C. D. Buckner, A. Fefer, L. Fisher, P. L. Weiden, R. P. Witherspoon, F. R. Appelbaum, M. Banaji, J. Hansen, and et al. 1989. Graft-versus-host disease as adoptive immunotherapy in patients with advanced hematologic neoplasms. *The New England journal of medicine* 320:828-834.
69. Morgan, R. A., J. C. Yang, M. Kitano, M. E. Dudley, C. M. Laurencot, and S. A. Rosenberg. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 18:843-851.
70. Rosenberg, S. A., J. C. Yang, S. L. Topalian, D. J. Schwartzentruber, J. S. Weber, D. R. Parkinson, C. A. Seipp, J. H. Einhorn, and D. E. White. 1994.

Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *Jama* 271:907-913.

71. Siegel, J. P., and R. K. Puri. 1991. Interleukin-2 toxicity. *J Clin Oncol* 9:694-704.
72. Baker, J., M. R. Verneris, M. Ito, J. A. Shizuru, and R. S. Negrin. 2001. Expansion of cytolytic CD8(+) natural killer T cells with limited capacity for graft-versus-host disease induction due to interferon gamma production. *Blood* 97:2923-2931.
73. Verneris, M. R., M. Kornacker, V. Mailander, and R. S. Negrin. 2000. Resistance of ex vivo expanded CD3+CD56+ T cells to Fas-mediated apoptosis. *Cancer Immunol Immunother* 49:335-345.
74. Hoyle, C., C. D. Bangs, P. Chang, O. Kamel, B. Mehta, and R. S. Negrin. 1998. Expansion of Philadelphia chromosome-negative CD3(+)CD56(+) cytotoxic cells from chronic myeloid leukemia patients: in vitro and in vivo efficacy in severe combined immunodeficiency disease mice. *Blood* 92:3318-3327.
75. Schmidt-Wolf, I. G., R. S. Negrin, H. P. Kiem, K. G. Blume, and I. L. Weissman. 1991. Use of a SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. *The Journal of experimental medicine* 174:139-149.
76. Shi, M., B. Zhang, Z. R. Tang, Z. Y. Lei, H. F. Wang, Y. Y. Feng, Z. P. Fan, D. P. Xu, and F. S. Wang. 2004. Autologous cytokine-induced killer cell therapy in clinical trial phase I is safe in patients with primary hepatocellular carcinoma. *World J Gastroenterol* 10:1146-1151.
77. Leemhuis, T., S. Wells, C. Scheffold, M. Edinger, and R. S. Negrin. 2005. A phase I trial of autologous cytokine-induced killer cells for the treatment of relapsed Hodgkin disease and non-Hodgkin lymphoma. *Biol Blood Marrow Transplant* 11:181-187.
78. Ortaldo, J. R., R. T. Winkler-Pickett, H. Yagita, and H. A. Young. 1991. Comparative studies of CD3- and CD3+ CD56+ cells: examination of morphology, functions, T cell receptor rearrangement, and pore-forming protein expression. *Cellular immunology* 136:486-495.
79. Ortaldo, J. R. 1991. Human cytotoxic effector cells: definition and analysis of activity. *Allergologia et immunopathologia* 19:145-156.
80. Verneris, M. R., M. Karami, J. Baker, A. Jayaswal, and R. S. Negrin. 2004. Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. *Blood* 103:3065-3072.
81. Lu, P. H., and R. S. Negrin. 1994. A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. *J Immunol* 153:1687-1696.
82. Karimi, M., T. M. Cao, J. A. Baker, M. R. Verneris, L. Soares, and R. S. Negrin. 2005. Silencing human NKG2D, DAP10, and DAP12 reduces

cytotoxicity of activated CD8+ T cells and NK cells. *J Immunol* 175:7819-7828.

83. Doherty, P. C., and R. M. Zinkernagel. 1975. A biological role for the major histocompatibility antigens. *Lancet* 1:1406-1409.
84. Zinkernagel, R. M., M. B. Dunlop, and P. C. Doherty. 1975. Cytotoxic T cell activity is strain-specific in outbred mice infected with lymphocytic choriomeningitis virus. *J Immunol* 115:1613-1616.
85. Zinkernagel, R. M., and P. C. Doherty. 1975. H-2 compatability requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T-cell specificities are associated with structures coded for in H-2K or H-2D. *The Journal of experimental medicine* 141:1427-1436.
86. Doherty, P. C., and R. M. Zinkernagel. 1975. H-2 compatibility is required for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. *The Journal of experimental medicine* 141:502-507.
87. Zinkernagel, R. M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Advances in immunology* 27:51-177.
88. Shedlock, D. J., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science (New York, N.Y* 300:337-339.
89. Sun, J. C., M. A. Williams, and M. J. Bevan. 2004. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nature immunology* 5:927-933.
90. Bevan, M. J. 2004. Helping the CD8(+) T-cell response. *Nature reviews* 4:595-602.
91. Sprent, J., and C. D. Surh. 2002. T cell memory. *Annual review of immunology* 20:551-579.
92. Hamann, D., P. A. Baars, M. H. Rep, B. Hooibrink, S. R. Kerkhof-Garde, M. R. Klein, and R. A. van Lier. 1997. Phenotypic and functional separation of memory and effector human CD8+ T cells. *The Journal of experimental medicine* 186:1407-1418.
93. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.
94. Marzo, A. L., H. Yagita, and L. Lefrancois. 2007. Cutting edge: migration to nonlymphoid tissues results in functional conversion of central to effector memory CD8 T cells. *J Immunol* 179:36-40.
95. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annual review of immunology* 22:745-763.

96. Lanzavecchia, A., and F. Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science (New York, N.Y* 290:92-97.
97. Pihlgren, M., P. M. Dubois, M. Tomkowiak, T. Sjogren, and J. Marvel. 1996. Resting memory CD8+ T cells are hyperreactive to antigenic challenge in vitro. *The Journal of experimental medicine* 184:2141-2151.
98. Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science (New York, N.Y* 291:2413-2417.
99. Marzo, A. L., V. Vezys, K. D. Klonowski, S. J. Lee, G. Muralimohan, M. Moore, D. F. Tough, and L. Lefrancois. 2004. Fully functional memory CD8 T cells in the absence of CD4 T cells. *J Immunol* 173:969-975.
100. Chang, J. T., V. R. Palanivel, I. Kinjyo, F. Schambach, A. M. Intlekofer, A. Banerjee, S. A. Longworth, K. E. Vinup, P. Mrass, J. Oliaro, N. Killeen, J. S. Orange, S. M. Russell, W. Weninger, and S. L. Reiner. 2007. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science (New York, N.Y* 315:1687-1691.
101. Feau, S., and S. P. Schoenberger. 2009. Immunology. Ex uno plura. *Science (New York, N.Y* 323:466-467.
102. Bannard, O., M. Kraman, and D. T. Fearon. 2009. Secondary replicative function of CD8+ T cells that had developed an effector phenotype. *Science (New York, N.Y* 323:505-509.
103. Sun, J. C., and M. J. Bevan. 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science (New York, N.Y* 300:339-342.
104. Williams, M. A., A. J. Tyznik, and M. J. Bevan. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 441:890-893.
105. Masopust, D., V. Vezys, E. J. Usherwood, L. S. Cauley, S. Olson, A. L. Marzo, R. L. Ward, D. L. Woodland, and L. Lefrancois. 2004. Activated primary and memory CD8 T cells migrate to nonlymphoid tissues regardless of site of activation or tissue of origin. *J Immunol* 172:4875-4882.
106. Lefrancois, L. 2006. Development, trafficking, and function of memory T-cell subsets. *Immunological reviews* 211:93-103.
107. Lefrancois, L., and D. Masopust. 2002. T cell immunity in lymphoid and non-lymphoid tissues. *Current opinion in immunology* 14:503-508.
108. Masopust, D., J. Jiang, H. Shen, and L. Lefrancois. 2001. Direct analysis of the dynamics of the intestinal mucosa CD8 T cell response to systemic virus infection. *J Immunol* 166:2348-2356.
109. Wakim, L. M., J. Waithman, N. van Rooijen, W. R. Heath, and F. R. Carbone. 2008. Dendritic cell-induced memory T cell activation in nonlymphoid tissues. *Science (New York, N.Y* 319:198-202.

110. Moyron-Quiroz, J. E., J. Rangel-Moreno, L. Hartson, K. Kusser, M. P. Tighe, K. D. Klonowski, L. Lefrancois, L. S. Cauley, A. G. Harmsen, F. E. Lund, and T. D. Randall. 2006. Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs. *Immunity* 25:643-654.
111. Gebhardt, T., and F. R. Carbone. 2009. Immunology: A helpers' guide to infection. *Nature*.
112. Goldrath, A. W., P. V. Sivakumar, M. Glaccum, M. K. Kennedy, M. J. Bevan, C. Benoist, D. Mathis, and E. A. Butz. 2002. Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. *The Journal of experimental medicine* 195:1515-1522.
113. Hamilton, S. E., M. C. Wolkers, S. P. Schoenberger, and S. C. Jameson. 2006. The generation of protective memory-like CD8+ T cells during homeostatic proliferation requires CD4+ T cells. *Nature immunology* 7:475-481.
114. Lin, S. J., A. T. Chen, and R. M. Welsh. 2008. An immune system derived from homeostatic proliferation generates normal CD8 T cell memory but altered repertoires and diminished heterologous immune responses. *Blood*.
115. Goldrath, A. W. 2002. Maintaining the status quo: T-cell homeostasis. *Microbes and infection / Institut Pasteur* 4:539-545.
116. Goldrath, A. W., C. J. Luckey, R. Park, C. Benoist, and D. Mathis. 2004. The molecular program induced in T cells undergoing homeostatic proliferation. *Proceedings of the National Academy of Sciences of the United States of America* 101:16885-16890.
117. Goldrath, A. W., L. Y. Bogatzki, and M. J. Bevan. 2000. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *The Journal of experimental medicine* 192:557-564.
118. Haluszczak, C., A. D. Akue, S. E. Hamilton, L. D. Johnson, L. Pujanauski, L. Teodorovic, S. C. Jameson, and R. M. Kedl. 2009. The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *The Journal of experimental medicine* 206:435-448.
119. Cho, J. H., O. Boyman, H. O. Kim, B. Hahm, M. P. Rubinstein, C. Ramsey, D. M. Kim, C. D. Surh, and J. Sprent. 2007. An intense form of homeostatic proliferation of naive CD8+ cells driven by IL-2. *The Journal of experimental medicine* 204:1787-1801.
120. Murali-Krishna, K., and R. Ahmed. 2000. Cutting edge: naive T cells masquerading as memory cells. *J Immunol* 165:1733-1737.
121. Tanchot, C., F. A. Lemonnier, B. Perarnau, A. A. Freitas, and B. Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science (New York, N.Y* 276:2057-2062.

122. Khoruts, A., and J. M. Fraser. 2005. A causal link between lymphopenia and autoimmunity. *Immunology letters* 98:23-31.
123. Cheung, K. P., E. Yang, and A. W. Goldrath. 2009. Memory-like CD8+ T cells generated during homeostatic proliferation defer to antigen-experienced memory cells. *J Immunol* 183:3364-3372.
124. Dummer, W., A. G. Niethammer, R. Baccala, B. R. Lawson, N. Wagner, R. A. Reisfeld, and A. N. Theofilopoulos. 2002. T cell homeostatic proliferation elicits effective antitumor autoimmunity. *The Journal of clinical investigation* 110:185-192.
125. Tough, D. F., and J. Sprent. 1998. Bystander stimulation of T cells in vivo by cytokines. *Veterinary immunology and immunopathology* 63:123-129.
126. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8:177-187.
127. Murali-Krishna, K., J. D. Altman, M. Suresh, D. Sourdive, A. Zajac, and R. Ahmed. 1998. In vivo dynamics of anti-viral CD8 T cell responses to different epitopes. An evaluation of bystander activation in primary and secondary responses to viral infection. *Advances in experimental medicine and biology* 452:123-142.
128. Sprent, J., X. Zhang, S. Sun, and D. Tough. 1999. T-cell turnover in vivo and the role of cytokines. *Immunology letters* 65:21-25.
129. Tough, D. F., P. Borrow, and J. Sprent. 1996. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science (New York, N.Y* 272:1947-1950.
130. Tough, D. F., and J. Sprent. 1996. Viruses and T cell turnover: evidence for bystander proliferation. *Immunological reviews* 150:129-142.
131. Judge, A. D., X. Zhang, H. Fujii, C. D. Surh, and J. Sprent. 2002. Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells. *The Journal of experimental medicine* 196:935-946.
132. Belz, G. T., and P. C. Doherty. 2001. Virus-specific and bystander CD8+ T-cell proliferation in the acute and persistent phases of a gammaherpesvirus infection. *Journal of virology* 75:4435-4438.
133. Tripp, R. A., S. Hou, A. McMickle, J. Houston, and P. C. Doherty. 1995. Recruitment and proliferation of CD8+ T cells in respiratory virus infections. *J Immunol* 154:6013-6021.
134. Dhanji, S., S. J. Teh, D. Oble, J. J. Priatel, and H. S. Teh. 2004. Self-reactive memory-phenotype CD8 T cells exhibit both MHC-restricted and non-MHC-restricted cytotoxicity: a role for the T-cell receptor and natural killer cell receptors. *Blood* 104:2116-2123.
135. Ehl, S., J. Hombach, P. Aichele, H. Hengartner, and R. M. Zinkernagel. 1997. Bystander activation of cytotoxic T cells: studies on the mechanism

and evaluation of in vivo significance in a transgenic mouse model. *The Journal of experimental medicine* 185:1241-1251.
136. Welsh, R. M., J. M. McNally, M. A. Brehm, and L. K. Selin. 2000. Consequences of cross-reactive and bystander CTL responses during viral infections. *Virology* 270:4-8.
137. Zarozinski, C. C., and R. M. Welsh. 1997. Minimal bystander activation of CD8 T cells during the virus-induced polyclonal T cell response. *The Journal of experimental medicine* 185:1629-1639.
138. Hillman, G. G., P. Slos, Y. Wang, J. L. Wright, A. Layer, M. De Meyer, M. Yudelev, M. Che, and J. D. Forman. 2004. Tumor irradiation followed by intratumoral cytokine gene therapy for murine renal adenocarcinoma. *Cancer gene therapy* 11:61-72.
139. Younes, E., G. P. Haas, B. Dezso, E. Ali, R. L. Maughan, M. A. Kukuruga, E. Montecillo, J. E. Pontes, and G. G. Hillman. 1995. Local tumor irradiation augments the response to IL-2 therapy in a murine renal adenocarcinoma. *Cellular immunology* 165:243-251.
140. Malek, T. R. 2008. The biology of interleukin-2. *Annual review of immunology* 26:453-479.
141. Smith, K. A. 1989. The interleukin 2 receptor. *Annual review of cell biology* 5:397-425.
142. Rogers, W. O., C. T. Weaver, L. A. Kraus, J. Li, L. Li, and R. P. Bucy. 1997. Visualization of antigen-specific T cell activation and cytokine expression in vivo. *J Immunol* 158:649-657.
143. Weiss, J. M., J. J. Subleski, J. M. Wigginton, and R. H. Wiltrout. 2007. Immunotherapy of cancer by IL-12-based cytokine combinations. *Expert opinion on biological therapy* 7:1705-1721.
144. Kubin, M., M. Kamoun, and G. Trinchieri. 1994. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *The Journal of experimental medicine* 180:211-222.
145. Coughlin, C. M., K. E. Salhany, M. Wysocka, E. Aruga, H. Kurzawa, A. E. Chang, C. A. Hunter, J. C. Fox, G. Trinchieri, and W. M. Lee. 1998. Interleukin-12 and interleukin-18 synergistically induce murine tumor regression which involves inhibition of angiogenesis. *The Journal of clinical investigation* 101:1441-1452.
146. Zhang, M., Z. Yao, S. Dubois, W. Ju, J. R. Muller, and T. A. Waldmann. 2009. Interleukin-15 combined with an anti-CD40 antibody provides enhanced therapeutic efficacy for murine models of colon cancer. *Proceedings of the National Academy of Sciences of the United States of America* 106:7513-7518.
147. Lodolce, J. P., P. R. Burkett, R. M. Koka, D. L. Boone, and A. Ma. 2002. Regulation of lymphoid homeostasis by interleukin-15. *Cytokine & growth factor reviews* 13:429-439.

148. Ma, A., R. Koka, and P. Burkett. 2006. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annual review of immunology* 24:657-679.
149. Oh, S., J. A. Berzofsky, D. S. Burke, T. A. Waldmann, and L. P. Perera. 2003. Coadministration of HIV vaccine vectors with vaccinia viruses expressing IL-15 but not IL-2 induces long-lasting cellular immunity. *Proceedings of the National Academy of Sciences of the United States of America* 100:3392-3397.
150. Chapoval, A. I., J. A. Fuller, S. G. Kremlev, S. J. Kamdar, and R. Evans. 1998. Combination chemotherapy and IL-15 administration induce permanent tumor regression in a mouse lung tumor model: NK and T cell-mediated effects antagonized by B cells. *J Immunol* 161:6977-6984.
151. Epardaud, M., K. G. Elpek, M. P. Rubinstein, A. R. Yonekura, A. Bellemare-Pelletier, R. Bronson, J. A. Hamerman, A. W. Goldrath, and S. J. Turley. 2008. Interleukin-15/interleukin-15R alpha complexes promote destruction of established tumors by reviving tumor-resident CD8+ T cells. *Cancer research* 68:2972-2983.
152. Berger, C., M. Berger, R. C. Hackman, M. Gough, C. Elliott, M. C. Jensen, and S. R. Riddell. 2009. Safety and immunologic effects of IL-15 administration in nonhuman primates. *Blood* 114:2417-2426.
153. Gagnon, J., S. Ramanathan, C. Leblanc, A. Cloutier, P. P. McDonald, and S. Ilangumaran. 2008. IL-6, in synergy with IL-7 or IL-15, stimulates TCR-independent proliferation and functional differentiation of CD8+ T lymphocytes. *J Immunol* 180:7958-7968.
154. Carson, W. E., J. E. Dierksheide, S. Jabbour, M. Anghelina, P. Bouchard, G. Ku, H. Yu, H. Baumann, M. H. Shah, M. A. Cooper, J. Durbin, and M. A. Caligiuri. 2000. Coadministration of interleukin-18 and interleukin-12 induces a fatal inflammatory response in mice: critical role of natural killer cell interferon-gamma production and STAT-mediated signal transduction. *Blood* 96:1465-1473.
155. Assier, E., V. Jullien, J. Lefort, J. L. Moreau, J. P. Di Santo, B. B. Vargaftig, J. R. Lapa e Silva, and J. Theze. 2004. NK cells and polymorphonuclear neutrophils are both critical for IL-2-induced pulmonary vascular leak syndrome. *J Immunol* 172:7661-7668.
156. van Kooten, C., and J. Banchereau. 2000. CD40-CD40 ligand. *Journal of leukocyte biology* 67:2-17.
157. Grewal, I. S., and R. A. Flavell. 1998. CD40 and CD154 in cell-mediated immunity. *Annual review of immunology* 16:111-135.
158. Lum, H. D., I. N. Buhtoiarov, B. E. Schmidt, G. Berke, D. M. Paulnock, P. M. Sondel, and A. L. Rakhmilevich. 2006. In vivo CD40 ligation can induce T-cell-independent antitumor effects that involve macrophages. *Journal of leukocyte biology* 79:1181-1192.

159. Vogel, L. A., and R. J. Noelle. 1998. CD40 and its crucial role as a member of the TNFR family. *Seminars in immunology* 10:435-442.
160. Tian, L., R. J. Noelle, and D. A. Lawrence. 1995. Activated T cells enhance nitric oxide production by murine splenic macrophages through gp39 and LFA-1. *European journal of immunology* 25:306-309.
161. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity* 1:167-178.
162. Eliopoulos, A. G., and L. S. Young. 2004. The role of the CD40 pathway in the pathogenesis and treatment of cancer. *Curr Opin Pharmacol* 4:360-367.
163. Callard, R. E., R. J. Armitage, W. C. Fanslow, and M. K. Spriggs. 1993. CD40 ligand and its role in X-linked hyper-IgM syndrome. *Immunology today* 14:559-564.
164. Kozel, T. R., W. J. Murphy, S. Brandt, B. R. Blazar, J. A. Lovchik, P. Thorkildson, A. Percival, and C. R. Lyons. 2004. mAbs to Bacillus anthracis capsular antigen for immunoprotection in anthrax and detection of antigenemia. *Proceedings of the National Academy of Sciences of the United States of America* 101:5042-5047.
165. Altenburg, A., S. E. Baldus, H. Smola, H. Pfister, and S. Hess. 1999. CD40 ligand-CD40 interaction induces chemokines in cervical carcinoma cells in synergism with IFN-gamma. *J Immunol* 162:4140-4147.
166. Hess, S., and H. Engelmann. 1996. A novel function of CD40: induction of cell death in transformed cells. *The Journal of experimental medicine* 183:159-167.
167. Fyfe, G., R. I. Fisher, S. A. Rosenberg, M. Sznol, D. R. Parkinson, and A. C. Louie. 1995. Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. *J Clin Oncol* 13:688-696.
168. Berner, V., H. Liu, Q. Zhou, K. L. Alderson, K. Sun, J. M. Weiss, T. C. Back, D. L. Longo, B. R. Blazar, R. H. Wiltrout, L. A. Welniak, D. Redelman, and W. J. Murphy. 2007. IFN-gamma mediates CD4+ T-cell loss and impairs secondary antitumor responses after successful initial immunotherapy. *Nature medicine* 13:354-360.
169. Alderson, K. L., Q. Zhou, V. Berner, D. E. Wilkins, J. M. Weiss, B. R. Blazar, L. A. Welniak, R. H. Wiltrout, D. Redelman, and W. J. Murphy. 2008. Regulatory and conventional CD4+ T cells show differential effects correlating with PD-1 and B7-H1 expression after immunotherapy. *J Immunol* 180:2981-2988.
170. Weiss, J. M., T. C. Back, A. J. Scarzello, J. J. Subleski, V. L. Hall, J. K. Stauffer, X. Chen, D. Micic, K. Alderson, W. J. Murphy, and R. H. Wiltrout. 2009. Successful immunotherapy with IL-2/anti-CD40 induces the

chemokine-mediated mitigation of an immunosuppressive tumor microenvironment. *Proceedings of the National Academy of Sciences of the United States of America* 106:19455-19460.

171. Murphy, W. J., L. Welniak, T. Back, J. Hixon, J. Subleski, N. Seki, J. M. Wigginton, S. E. Wilson, B. R. Blazar, A. M. Malyguine, T. J. Sayers, and R. H. Wiltrout. 2003. Synergistic anti-tumor responses after administration of agonistic antibodies to CD40 and IL-2: coordination of dendritic and CD8+ cell responses. *J Immunol* 170:2727-2733.
172. Schuurhuis, D. H., S. Laban, R. E. Toes, P. Ricciardi-Castagnoli, M. J. Kleijmeer, E. I. van der Voort, D. Rea, R. Offringa, H. J. Geuze, C. J. Melief, and F. Ossendorp. 2000. Immature dendritic cells acquire CD8(+) cytotoxic T lymphocyte priming capacity upon activation by T helper cell-independent or -dependent stimuli. *The Journal of experimental medicine* 192:145-150.
173. Hamzah, J., D. Nelson, G. Moldenhauer, B. Arnold, G. J. Hammerling, and R. Ganss. 2008. Vascular targeting of anti-CD40 antibodies and IL-2 into autochthonous tumors enhances immunotherapy in mice. *The Journal of clinical investigation* 118:1691-1699.
174. Wigginton, J. M., K. L. Komschlies, T. C. Back, J. L. Franco, M. J. Brunda, and R. H. Wiltrout. 1996. Administration of interleukin 12 with pulse interleukin 2 and the rapid and complete eradication of murine renal carcinoma. *Journal of the National Cancer Institute* 88:38-43.
175. Wigginton, J. M., K. L. Komschlies, J. E. Green, G. W. Cox, C. L. Jorcyk, T. C. Back, J. L. Franco, M. J. Brunda, and R. H. Wiltrout. 1996. Evaluation of the antitumor activity of the interleukin-12/pulse interleukin-2 combination. *Annals of the New York Academy of Sciences* 795:434-439.
176. Wigginton, J. M., E. Gruys, L. Geiselhart, J. Subleski, K. L. Komschlies, J. W. Park, T. A. Wiltrout, K. Nagashima, T. C. Back, and R. H. Wiltrout. 2001. IFN-gamma and Fas/FasL are required for the antitumor and antiangiogenic effects of IL-12/pulse IL-2 therapy. *The Journal of clinical investigation* 108:51-62.
177. Brunda, M. J., L. Luistro, L. Rumennik, R. B. Wright, J. M. Wigginton, R. H. Wiltrout, J. A. Hendrzak, and A. V. Palleroni. 1996. Interleukin-12: murine models of a potent antitumor agent. *Annals of the New York Academy of Sciences* 795:266-274.
178. Pimentel-Muinos, F. X., M. A. Munoz-Fernandez, and M. Fresno. 1994. Control of T lymphocyte activation and IL-2 receptor expression by endogenously secreted lymphokines. *J Immunol* 152:5714-5722.
179. Pimentel-Muinos, F. X., J. Mazana, and M. Fresno. 1994. Regulation of interleukin-2 receptor alpha chain expression and nuclear factor.kappa B activation by protein kinase C in T lymphocytes. Autocrine role of tumor necrosis factor alpha. *The Journal of biological chemistry* 269:24424-24429.

180. Carter, L., L. A. Fouser, J. Jussif, L. Fitz, B. Deng, C. R. Wood, M. Collins, T. Honjo, G. J. Freeman, and B. M. Carreno. 2002. PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. *European journal of immunology* 32:634-643.
181. Seth, A., L. Gote, M. Nagarkatti, and P. S. Nagarkatti. 1991. T-cell-receptor-independent activation of cytolytic activity of cytotoxic T lymphocytes mediated through CD44 and gp90MEL-14. *Proceedings of the National Academy of Sciences of the United States of America* 88:7877-7881.
182. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76:17-27.
183. Clarke, S. R., M. Barnden, C. Kurts, F. R. Carbone, J. F. Miller, and W. R. Heath. 2000. Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. *Immunology and cell biology* 78:110-117.
184. Kelly, J. M., S. J. Sterry, S. Cose, S. J. Turner, J. Fecondo, S. Rodda, P. J. Fink, and F. R. Carbone. 1993. Identification of conserved T cell receptor CDR3 residues contacting known exposed peptide side chains from a major histocompatibility complex class I-bound determinant. *European journal of immunology* 23:3318-3326.
185. Carbone, F. R., S. J. Sterry, J. Butler, S. Rodda, and M. W. Moore. 1992. T cell receptor alpha-chain pairing determines the specificity of residue 262 within the Kb-restricted, ovalbumin257-264 determinant. *International immunology* 4:861-867.
186. Kaye, J., N. J. Vasquez, and S. M. Hedrick. 1992. Involvement of the same region of the T cell antigen receptor in thymic selection and foreign peptide recognition. *J Immunol* 148:3342-3353.
187. Hogquist, K. A., A. J. Tomlinson, W. C. Kieper, M. A. McGargill, M. C. Hart, S. Naylor, and S. C. Jameson. 1997. Identification of a naturally occurring ligand for thymic positive selection. *Immunity* 6:389-399.
188. Guerra, N., Y. X. Tan, N. T. Joncker, A. Choy, F. Gallardo, N. Xiong, S. Knoblaugh, D. Cado, N. M. Greenberg, and D. H. Raulet. 2008. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* 28:571-580.
189. Smyth, M. J., J. Swann, J. M. Kelly, E. Cretney, W. M. Yokoyama, A. Diefenbach, T. J. Sayers, and Y. Hayakawa. 2004. NKG2D recognition and perforin effector function mediate effective cytokine immunotherapy of cancer. *The Journal of experimental medicine* 200:1325-1335.
190. Groh, V., R. Rhinehart, J. Randolph-Habecker, M. S. Topp, S. R. Riddell, and T. Spies. 2001. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nature immunology* 2:255-260.

191. Walsh, K. B., M. B. Lodoen, R. A. Edwards, L. L. Lanier, and T. E. Lane. 2008. Evidence for differential roles for NKG2D receptor signaling in innate host defense against coronavirus-induced neurological and liver disease. *Journal of virology* 82:3021-3030.
192. Dhanji, S., and H. S. Teh. 2003. IL-2-activated CD8+CD44high cells express both adaptive and innate immune system receptors and demonstrate specificity for syngeneic tumor cells. *J Immunol* 171:3442-3450.
193. Diefenbach, A., and D. H. Raulet. 2002. The innate immune response to tumors and its role in the induction of T-cell immunity. *Immunological reviews* 188:9-21.
194. Diefenbach, A., J. K. Hsia, M. Y. Hsiung, and D. H. Raulet. 2003. A novel ligand for the NKG2D receptor activates NK cells and macrophages and induces tumor immunity. *European journal of immunology* 33:381-391.
195. Diefenbach, A., E. R. Jensen, A. M. Jamieson, and D. H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 413:165-171.
196. Pinto, A. K., A. M. Jamieson, D. H. Raulet, and A. B. Hill. 2007. The role of NKG2D signaling in inhibition of cytotoxic T-lymphocyte lysis by the Murine cytomegalovirus immunoevasin m152/gp40. *Journal of virology* 81:12564-12571.
197. Gasser, S., S. Orsulic, E. J. Brown, and D. H. Raulet. 2005. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 436:1186-1190.
198. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science (New York, N.Y* 285:727-729.
199. Abdool, K., E. Cretney, A. D. Brooks, J. M. Kelly, J. Swann, A. Shanker, E. W. Bere, Jr., W. M. Yokoyama, J. R. Ortaldo, M. J. Smyth, and T. J. Sayers. 2006. NK cells use NKG2D to recognize a mouse renal cancer (Renca), yet require intercellular adhesion molecule-1 expression on the tumor cells for optimal perforin-dependent effector function. *J Immunol* 177:2575-2583.
200. Kim, J., C. K. Chang, T. Hayden, F. C. Liu, J. Benjamin, J. A. Hamerman, L. L. Lanier, and S. M. Kang. 2007. The activating immunoreceptor NKG2D and its ligands are involved in allograft transplant rejection. *J Immunol* 179:6416-6420.
201. Walsh, K. B., M. B. Lodoen, L. L. Lanier, and T. E. Lane. 2006. NKG2D signaling and host defense after mouse hepatitis virus infection of the central nervous system. *Advances in experimental medicine and biology* 581:369-372.
202. Keir, M. E., L. M. Francisco, and A. H. Sharpe. 2007. PD-1 and its ligands in T-cell immunity. *Current opinion in immunology* 19:309-314.

203. Dong, H., S. E. Strome, D. R. Salomao, H. Tamura, F. Hirano, D. B. Flies, P. C. Roche, J. Lu, G. Zhu, K. Tamada, V. A. Lennon, E. Celis, and L. Chen. 2002. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nature medicine* 8:793-800.
204. Ahmadzadeh, M., L. A. Johnson, B. Heemskerk, J. R. Wunderlich, M. E. Dudley, D. E. White, and S. A. Rosenberg. 2009. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* 114:1537-1544.
205. Mintern, J. D., G. M. Davey, G. T. Belz, F. R. Carbone, and W. R. Heath. 2002. Cutting edge: precursor frequency affects the helper dependence of cytotoxic T cells. *J Immunol* 168:977-980.
206. Badovinac, V. P., J. S. Haring, and J. T. Harty. 2007. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity* 26:827-841.
207. Rizzuto, G. A., T. Merghoub, D. Hirschhorn-Cymerman, C. Liu, A. M. Lesokhin, D. Sahawneh, H. Zhong, K. S. Panageas, M. A. Perales, G. Altan-Bonnet, J. D. Wolchok, and A. N. Houghton. 2009. Self-antigen-specific CD8+ T cell precursor frequency determines the quality of the antitumor immune response. *The Journal of experimental medicine* 206:849-866.
208. Meresse, B., S. A. Curran, C. Ciszewski, G. Orbelyan, M. Setty, G. Bhagat, L. Lee, M. Tretiakova, C. Semrad, E. Kistner, R. J. Winchester, V. Braud, L. L. Lanier, D. E. Geraghty, P. H. Green, S. Guandalini, and B. Jabri. 2006. Reprogramming of CTLs into natural killer-like cells in celiac disease. *The Journal of experimental medicine* 203:1343-1355.
209. Namekawa, T., M. R. Snyder, J. H. Yen, B. E. Goehring, P. J. Leibson, C. M. Weyand, and J. J. Goronzy. 2000. Killer cell activating receptors function as costimulatory molecules on CD4+CD28null T cells clonally expanded in rheumatoid arthritis. *J Immunol* 165:1138-1145.
210. Merck, E., R. B. Voyle, and H. R. MacDonald. 2009. Ly49D engagement on T lymphocytes induces TCR-independent activation and CD8 effector functions that control tumor growth. *J Immunol* 182:183-192.
211. Cerboni, C., M. Ardolino, A. Santoni, and A. Zingoni. 2009. Detuning CD8+ T lymphocytes by down-regulation of the activating receptor NKG2D: role of NKG2D ligands released by activated T cells. *Blood* 113:2955-2964.
212. Salih, H. R., H. G. Rammensee, and A. Steinle. 2002. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. *J Immunol* 169:4098-4102.
213. Salih, H. R., H. Antropius, F. Gieseke, S. Z. Lutz, L. Kanz, H. G. Rammensee, and A. Steinle. 2003. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. *Blood* 102:1389-1396.

214. Salih, H. R., D. Goehlsdorf, and A. Steinle. 2006. Release of MICB molecules by tumor cells: mechanism and soluble MICB in sera of cancer patients. *Human immunology* 67:188-195.
215. Holdenrieder, S., P. Stieber, A. Peterfi, D. Nagel, A. Steinle, and H. R. Salih. 2006. Soluble MICA in malignant diseases. *International journal of cancer* 118:684-687.
216. Holdenrieder, S., P. Stieber, A. Peterfi, D. Nagel, A. Steinle, and H. R. Salih. 2006. Soluble MICB in malignant diseases: analysis of diagnostic significance and correlation with soluble MICA. *Cancer Immunol Immunother* 55:1584-1589.
217. Osaki, T., H. Saito, T. Yoshikawa, S. Matsumoto, S. Tatebe, S. Tsujitani, and M. Ikeguchi. 2007. Decreased NKG2D expression on CD8+ T cell is involved in immune evasion in patients with gastric cancer. *Clin Cancer Res* 13:382-387.
218. Gendelman, M., N. Halligan, R. Komorowski, B. Logan, W. J. Murphy, B. R. Blazar, K. A. Pritchard, Jr., and W. R. Drobyski. 2005. Alpha phenyl-tert-butyl nitrone (PBN) protects syngeneic marrow transplant recipients from the lethal cytokine syndrome occurring after agonistic CD40 antibody administration. *Blood* 105:428-431.
219. Hixon, J. A., B. R. Blazar, M. R. Anver, R. H. Wiltrout, and W. J. Murphy. 2001. Antibodies to CD40 induce a lethal cytokine cascade after syngeneic bone marrow transplantation. *Biol Blood Marrow Transplant* 7:136-143.
220. Hixon, J. A., M. R. Anver, B. R. Blazar, A. Panoskaltsis-Mortari, R. H. Wiltrout, and W. J. Murphy. 2002. Administration of either anti-CD40 or interleukin-12 following lethal total body irradiation induces acute lethal toxicity affecting the gut. *Biol Blood Marrow Transplant* 8:316-325.
221. Uhlig, H. H., B. S. McKenzie, S. Hue, C. Thompson, B. Joyce-Shaikh, R. Stepankova, N. Robinson, S. Buonocore, H. Tlaskalova-Hogenova, D. J. Cua, and F. Powrie. 2006. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* 25:309-318.
222. Zhou, Q., R. A. Gault, T. R. Kozel, and W. J. Murphy. 2006. Immunomodulation with CD40 stimulation and interleukin-2 protects mice from disseminated cryptococcosis. *Infection and immunity* 74:2161-2168.
223. Kleinsmith, Lewis. 2006. *Principles of Cancer Biology*. Pearson. San Francisco, CA.
224. Weinberg, Robert. 2006. *The Biology of Cancer*. Garland Science. New York, NY.