THE UNIVERSITY OF CHICAGO

THE INNATE IMMUNE SYSTEM DRIVES T CELL TOLERANCE AGAINST ACUTE MYELOID LEUKEMIA

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES

AND THE PRITZKER SCHOOL OF MEDICINE

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

COMMITTEE ON IMMUNOLOGY

BY

DOUGLAS EVERETT KLINE

CHICAGO, ILLINOIS

AUGUST 2016

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to first thank Dr. Justin Kline for his tireless dedication to the advancement of this work and my progress as a young scientist. His incredible work ethic and scientific passion are inspirational. Even with his busy schedule, he would make time to help me harvest organs or talk about new experiments. He really took the time to invest in me as a scientist and as a person. Justin took a big risk on letting me join the lab after transferring from UNC and I did my best to repay his faith. Justin's mentorship is deeply appreciated and I am a much better scientist today because of him. Justin also created a lab environment where I could really thrive. Xiufen Chen is my lab mom. She is one of the kindest people I have ever known and was always willing to help, even at the sacrifice of her own time. The work presented here would not have been possible without her. Dom Fosco was instrumental in helping my progress when I joined the lab. He was not only willing to help, but also helped create an environment where coming to lab was fun. Annie Albright is an undergraduate in the lab who I have worked closely with over the last two years. Annie was always willing to assist in experiments and talk about projects and ideas. She has incredible scientific potential and will without doubt be successful in whatever path she chooses; I look forward to watching her progress as a scientist. Finally, I would to thank the rest of the Kline lab members, especially Emily Curran, who have made significant intellectual and experimental contributions to this work.

I would also like to thank Dr. Peter Savage for the guidance he gave me during my time at the University of Chicago. Pete is like a second mentor to me and was always available to discuss science or life in general. Members of the Savage lab have

been absolutely instrumental in the design and execution of this body of work. Dan Leventhal and Sven Malchow were previous lab members of the Savage lab who were vital in my success as a graduate student. Dana Gilmore, Victoria Lee and Jamie Chao were also very helpful with experiments and insightful comments in lab meeting and journal club. These individuals are all very talented and the future of the Savage lab is in good hands.

I would like to thank my thesis committee members, Dr. Thomas Gajewski, Dr. Seungmin (Sam) Hwang, Dr. Bana Jabri and Dr. Yang-Xin Fu. They always provided excellent scientific advice and I truly felt that they wanted me to succeed. The project reached an elevated intellectual level with their input.

We are grateful to Ryan Duggan, David Leclerc, and Mike Olson from the Flow Cytometry Core at the University of Chicago for help with cell sorting and analysis of Image Stream X data. Some of the analysis performed in this body of work would not have been possible without them.

I have been doing lab research for many years now and the training I had in the past has allowed me to be in this position today. First, Jeffery Shorer, my high school AP biology teacher, was instrumental in getting me excited about science. He is the one that really shaped by future towards science and is by far the best teacher I have ever had. He has had an amazing impact of the kids in New Berlin and I would not be where we are without his tireless work. Over a decade later, we still talk about that AP biology class and how it shaped our lives. I hope to make an impact like he has in guiding youth into science. I would like to thank Dr. Michael Carvan and Jesse Grzybowski. During my

final summer of high school I joined Mike's lab at the University of Wisconsin-Milwaukee where I learned what it was like to work in a lab and learned many basic techniques which helped me in the future. Jesse's kindness, patience, and willingness to take on a high school student was amazing and got me started in laboratory research. As an undergraduate at Michigan State University, I was truly fortunate to join the laboratory of Dr. Norbert Kaminski. Norb really gave me the opportunity to succeed and set me up with an incredible instructor, Robert Crawford. Bob trained me in many methods I still use weekly in the lab. His patience with me in the lab was instrumental and both he and Norb let me "take the reins" on an independent project that really pushed my scientific development. While working in Norb's lab, I have met several people I still keep in contact with today. Dr. Barbara Kaplan, Dr. Peer Karmaus, and Dr. Colin North were instrumental in my progress. I also would like to thank Dr. Roland Tisch, whose laboratory I joined after finishing my undergraduate degree. Dr. Aaron Martin provided me excellent training in cellular immunology in Roland's lab and is someone I consider a mentor and friend. Roland and Aaron were both very supportive, and my time in Roland's lab really molded my scientific creativity and progress.

Finally, I would like to thank my family and friends. My parents are amazing people and have facilitated my progress as a scientist and a person. My dad has always encouraged me to explore the unknowns of the biological world and his interest in academic science, even without formal training, is inspirational. My mom has been an enormous source of comfort in my life. When the PhD process seemed too daunting I always had someone I could call and I am truly grateful to both of them. My brother (Kevin) and sister (Katie), as well as their significant others, have always supported me

and I am glad I was able to come back to the Midwest and spend more time with them. I would also like to thank my grandparents. Eleanor and Jim both had positive impacts on my life and shaped me as a person; I am glad I got to spend precious time with them. I truly feel I get my scientific appetite from Bernie and Virginia and, although I did not get to spend much time with them, their passion for science lives through me. Additionally, my uncles, aunts, and cousins have provided me with times of great enjoyment during my PhD journey. Finally, I would like to thank my wonderful girlfriend Stephanie. Your unwavering belief and confidence in me has really allowed me to flourish and grow as a scientist and a person. None of this would have been possible without you.

For two years, I was supported by the Immunology training grant at the University of Chicago (T32 AI007090). This work was funded by R01 CA16670 to Justin Kline. Emily Curran was funded by was funded by T32 CA009566-27 and NIH/NIGMS Clinical Therapeutics Training Grant T32 GM007019.

ABSTRACT

While the host immune system can recognize and respond to antigens-derived from solid cancers, tumors are rarely spontaneously rejected. The discovery of immune evasion mechanisms activated by solid cancers has led to the development of immunotherapies aimed at unleashing anti-tumor immunity. These immunotherapies are now first line treatment in many solid cancers and capable of inducing potent and durable responses in human patients. In contrast to solid tumors, much less is known about immune escape mechanisms utilized by hematopoietic malignancies, such as acute myeloid leukemia (AML). Here we discovered a unique immune evasion mechanism in AML bearing mice where leukemia-specific $CD8^+$ T cells underwent abortive proliferation and were ultimately deleted from the host. T cell tolerance in mice with systemic leukemia was driven by immature splenic $CD8\alpha^+$ dendritic cells which acquire and cross-present leukemia-derived antigens to $CD8^+$ T cells in a tolerogenic context. The activation of the innate immune system with a stimulator of interferon genes (STING) agonist was able to prevent the T cell tolerant state and cure mice with established AML. Thus our work provides the framework to clinically investigate therapeutic agents which activate the innate immune system in patients with AML.

BACKGROUND AND SIGNIFICANCE

The innate immune system is the first line of defense against foreign invaders

The immune system is an ancient defense mechanism which functions to defend the host from invading pathogens. However, inappropriate immune activation can lead to tissue destruction and immunopathology. Therefore, the immune system must carefully balance pro- and anti-inflammatory signals to eradicate pathogens while maintaining tissue homeostasis. The immune system is generally divided into two distinct branches: the innate and adaptive immunes systems. The innate immune system serves as the first line of defense against invading pathogens and includes numerous cell types such as macrophages, dendritic cells (DCs), eosinophils, and others. To detect foreign invaders, innate immune cells express a series of germline-encoded pattern recognition receptors (PRRs) which sense the presence of pathogens and facilitate the generation of an appropriate immune response. After pathogen sensing, innate immune cells limit the spread of infection through several mechanisms including the phagocytosis of microorganisms and the production pro-inflammatory cytokines and anti-microbial effectors. Overall, the innate immune system serves to detect foreign pathogens and initiate an appropriate protective response in the host.

The adaptive immune system mediates pathogen control

While the innate immune system prevents the rapid spread microbes, it is often insufficient to completely eliminate an invading pathogen. Therefore, a critical role for

the innate immune system is also to facilitate the activation of the adaptive immune system, which is specifically tuned to eliminate pathogens and provide immunological memory. The adaptive immune system consists of two classes of lymphocytes known as T cells and B cells. In contrast to innate immune cells, which utilize germline encoded receptors to detect the presence of pathogens, each T cell is equipped with a unique T cell receptor (TCR) that imprints a single antigen specificity. In the case of classic αβ T cells, the TCR is a heterodimer consisting of an α-chain and a β-chain, which together recognize a short peptide bound to a major histocompatibility complex (MHC) molecule. Because each TCR recognizes a single peptide antigen, the total T cell pool must have a diverse TCR repertoire to enable the recognition of antigens derived from vastly divergent pathogens. The TCR β-chain consists of a variable (V), diversity, (D), and joining (J) gene segment, with each segment being selected from several potential genomic variants. In a quasi-random process, recombination-activating genes (RAGs) splice a single V, D, and J segment together to generate a functional TCR β-chain (known as combinatorial diversity). At the junction of each spliced gene segment, the enzyme terminal deoxynucleotidyl transferase (TdT) facilitates the random addition of a small number of nucleotides, further increasing the potential diversity of the β-chain (junctional diversity)[1]. A similar process occurs during rearrangement of the TCR α-chain, although it contains only a V and J segment. Overall, through combinatorial and junctional diversity, there are ~10^{15} possible unique T cell receptors allowing the total T cell pool to recognize nearly any antigen[2].

T cells develop in the thymus, and two main classes can be delineated based on the expression of CD4 and CD8 co-receptors. $CD4^+$ T cells are classically considered

helper T cells, and upon recognition of peptide in the context of an MHC class II molecule differentiate and produce cytokines which influence the nature of a developing immune response. In contrast, $CD8^+$ T cells recognize peptides on MHC class I and have the characteristic ability to kill target cells, including pathogen-infected and transformed cells. Overall, both $CD4^+$ and $CD8^+$ T cells function in concert to protect the host from pathogenic invaders.

The innate immune system orchestrates adaptive immune responses

The random nature in which TCRs are generated ensures that the T repertoire can recognize peptide antigens derived from nearly any potential pathogen. However, this diversity also results in generation of T cells capable of recognizing self-antigens. Therefore, tolerance mechanisms are required to prevent the development of autoimmunity. In the thymus, T cells with high affinity for self-antigens undergo clonal deletion, purging the repertoire of autoreactive specificities[3]. While thymic tolerance is efficient, it is often incomplete and autoreactive T cell clones can escape thymic deletion. Consequently, additional mechanisms of peripheral tolerance are required to avoid autoimmune pathology. For example, regulatory T cells (Tregs), a subset of naturally suppressive $CD4^+$ T cells, are critical in preventing the activation of autoreactive T cells[4, 5]. Another tolerance mechanism occurs at the level of antigen-presenting cells (APCs), particularly DCs. In the steady-state, DCs constantly acquire exogenous proteins from their local environment, and present self-antigens to T cells in secondary lymphoid organs. Autoreactive T cells encountering self-antigen presented

by “immature” DCs are ultimately rendered tolerant through a variety of mechanisms including T cell deletion or cell intrinsic anergy[6]. The presentation of autoantigens by immature DCs ensures that autoreactive T cells that encounter antigen in the absence of infection are rendered tolerant to “self”[7, 8, 9]. On the other hand, during infection, APCs acquire antigens derived from pathogens and, in this case, need to be able to mediate the activation of antigen-specific T cells. Therefore, DCs need to be able to sense local environmental cues in order to either induce tolerance to self-antigens, or to prime adaptive immunity in response to microbial or viral pathogenesis[10].

Two non-mutually exclusive theories have been proposed to explain how the innate immune system becomes activated in response to pathogen encounter. One theory postulates that innate immune cells directly detect bacteria or viruses via a series of germline-encoded PRRs, which bind conserved pathogen associated molecular patterns (PAMPs)[11]. While, it has been convincingly shown that some pathogenic moieties directly bind host PRRs, there are several documented forms of sterile inflammation, without an obvious pathogenic trigger. Therefore, it has also been proposed that the innate immune system can also be activated by danger associated molecular patterns (DAMPs) released upon cellular stress or aberrant cell death, as would be the case following pathogen encounter or in the tumor environment[12]. In either case, PAMPs and DAMPS bind PRRs and induce the activation of APCs, known as APC maturation, which is critical for the subsequent priming of adaptive immunity.

APC maturation is a complex biological process characterized by increased cell surface expression of MHC molecules as well as expression of T cell costimulatory molecules B7.1, B7.2, and others[13]. It is now well accepted that in addition to the TCR

binding peptide MHC complexes, APC-expressed costimulatory ligands and cytokines are required for full activation of T lymphocytes. Thus, for naïve T cells to be properly primed and acquire effector function, they need to encounter antigen on a mature APC expressing both cognate antigen and costimulatory molecules. Ultimately, this imprints innate immune cells with the ability to orchestrate adaptive immunity; under homeostatic conditions, immature DCs drive T cell tolerance whereas after pathogen sensing, mature DCs are able to stimulate productive T cell responses.

APCs present intra- and extra-cellular antigens

Pathogens can have intracellular and/or extracellular lifecycles and in order for T cells to be capable of detecting all potential threats, APCs display both endogenous and exogenous peptide antigens on MHC I and II molecules. The expression of MHC II is, for the most part, limited to “professional” APCs (DCs, macrophages, and B cells), and peptides presented on MHC II molecules are primarily derived from endocytosed extracellular material, although an endogenous MHC II loading pathway has also been described[14].

In contrast, MHC I is expressed by all nucleated cells and is loaded with peptides derived from endogenous proteins degraded by the proteasome. MHC I antigen presentation reveals the self-proteome of an cell, but in the case of an intracellular infection, also displays foreign peptides to $CD8^+$ T cells, flagging them for destruction. However, as discussed above, to acquire effector function, naïve $CD8^+$ T cells require encounter with a mature APC presenting cognate antigen along with costimulatory

molecules. How then are $CD8^+$ T cells activated when APCs are not directly infected by a virus or bacteria? Over 40 years ago in two seminal papers, Mike Bevan demonstrated the existence of a pathway which permitted the presentation of exogenous antigens on MHC I[15, 16]. This pathway was termed cross-presentation, and is essential for the activation of $CD8^+$ T cells against exogenous antigens, including those derived from malignant cells and extracellular micro-organisms[17, 18].

Dendritic cells are a heterogeneous population of innate immune cells

DCs are a heterogeneous population of highly phagocytic APCs with the characteristic ability to stimulate naïve T cells. DCs resident to secondary lymphoid tissues can be categorized as either plasmacytoid (pDCs) or conventional DCs (cDCs), based on unique ontogeny, transcriptional profiles, and functions[19]. pDCs are specialized producers of type I interferons to initiate anti-viral immune responses, but are less well adept at phagocytosis and antigen presentation in the steady state[20].

Conventional DCs are highly proficient at antigen uptake and presentation to naive T cells. In lymphoid tissues, the cDC pool can further be divided based on differential expression of CD8α[21, 22]. $CD8\alpha^+$ DCs (and the developmentally-related $CD103^+$ DCs)[23], are exceptionally efficient at the cross-presentation of exogenous antigens on MHC I[17, 24, 25, 26]. This ability is at least partially due to the expression of high levels of MHC class I processing machinery, along with specialized vesicular systems which delay antigen degradation to promote cross-presentation[26, 27, 28]. For example, the small GTPase Rac2 facilitates the recruitment of the NAPDH oxidase to early

endosomes in CD8α$^+$, but not CD8α$^-$ DCs, which actively promotes the alkalization of endocytic vesicles[29, 30]. Conversely, CD11b$^+$ DCs are proficient at the presentation of antigen to CD4$^+$ T cells through enhanced expression of proteins involved in MHC II processing[26, 31].

Evaluating the necessity of the CD8α$^+$ DC subset for the cross-presentation of cellular antigens has been difficult to study in vivo, as many transcription factors which control CD8α$^+$ DC development are also required by other DCs subsets[32]. However, in a landmark study, Murphy and colleges found that mice deficient in the basic leucine zipper transcription factor ATF-like3 (Batf3) specifically lack CD8α$^+$ and CD103$^+$ DCs, while containing normal numbers of other APC populations[33]. *Batf3*$^{-/-}$ mice exhibit markedly diminished anti-viral and anti-tumor T cell responses, demonstrating the requirement of CD8α$^+$ DCs in cross-presenting antigens to induce CD8$^+$ T cell responses in vivo. In the steady state, CD8α$^+$ DCs have been clearly shown to be highly proficient at cross-presenting cellular antigens; however, in an inflammatory environment, CD11b$^+$ DCs and pDCs have been shown to cross-present intestinal and viral antigens, respectively, demonstrating the plasticity of dendritic cell function[17, 34].

<u>The immune system can recognize antigens derived from malignant cells</u>

Because tumors develop from self-tissues, how antigens derived from transformed cells might be recognized by the host immune system has been the subject of intensive debate[35]. Some of the first evidence that the immune system was able to respond to tumor-derived antigens came from experiments in which carcinogen-induced

tumors were surgically resected and subsequently re-transplanted into the autologous host, or genetically identical secondary hosts. In many cases, mice spontaneously rejected these transplanted tumors[36, 37]. In a similar series of experiments, it was demonstrated that lethally irradiated tumor cells could induce protection from a secondary live tumor challenge, indicating that the immune system is capable of recognizing and responding to antigens derived from malignant cells[38]. The understanding that the immune system can detect a growing cancer was further supported by seminal work performed by Thierry Boon[39]. By treating a carcinoma cell line with a strong carcinogen, mutated tumor cell clones were generated which were spontaneously rejected upon implantation into immunocompetent mice, while the parental tumor cell line grew progressively. Moreover, a primary challenge with these "tum$^-$" variants resulted in protection from a secondary challenge with parental carcinoma cells[40, 41]. Thus, tumor cells contain "natural" antigens which can be recognized by host, although these antigens were poorly immunogenic in isolation. These revolutionary experiments established that antigens derived from malignant cells can be identified by the host immune system, and these observations stimulated further investigation into the type of tumor antigens which are recognizable to host T cells.

The nature of tumor antigens

In general, two classes of cancer antigens have been described. Tumor associated antigens (TAA) are generated from the over-expression or aberrant expression of self-proteins in cancer cells. A major class of TAA are cancer-testis

antigens (CTA), which are derived from proteins whose expression is normally restricted to germ cells, but are not expressed elsewhere in the body[42]. However, CTA are re-expressed in some cancers due to disrupted regulation of gene expression. Using a genetic approach where melanoma cDNA libraries were screened for their ability to stimulate a melanoma-specific $CD8^+$ T cell clone, Boon and colleges successfully cloned the first human CTA, melanoma antigen-1 (MAGE-A1), and $CD8^+$ T cell responses against MAGE-A1 were reported in a melanoma patient[43]. To date, more than 25 antigenic MAGE proteins have been characterized, many of which have served as targets for therapeutic vaccination[44]. In addition to the MAGE proteins, several other proteins of the CTA family have been discovered including NY-ESO1, synovial sarcoma X breakpoint 1 (SSX1), and SSX2[45, 46]. While both T cell and humoral responses are often mounted against CTA following vaccination, this approach has had limited clinical efficacy, although several studies are still ongoing[47, 48]. Because TAA are, by definition, derived from normal self-proteins, T cells capable of recognizing TAA with high affinity are likely subjected to tolerance mechanisms, both in the thymus and periphery. Additionally, while TAA-specific T cells may be expanded following antigen-vaccination, immune evasion mechanisms present in the tumor microenvironment may ultimately perturb their effector function.

The genomic instability of many types of cancer has the potential to generate nonsynonymous mutations that can be recognized by the adaptive immune system. These tumor neo-antigens, more broadly known as tumor-specific antigens (TSA), hold enormous potential for inducing tumor-specific immune responses, and because of this, there has been a concerted effort to identify and induce immunity against TSA[49].

Through exome sequencing approaches, it has become feasible to identify tumor-specific mutations from individual patients, and to screen mutant peptide libraries for their ability to stimulate autologous peripheral blood T cells or tumor infiltrating lymphocytes (TILs) in vitro[50]. Remarkably, in at least some patients, TILs were found reactive against neo-epitopes, and there is direct evidence that TSA-specific T cells can mediate disease remission when expanded in vitro and reinfused into patients[51, 52]. Importantly, the rate of nonsynonymous mutations varies greatly among human cancer subtypes. Carcinogen induced malignancies, such as smoking-related lung cancer and UV light-induced melanoma, typically contain thousands of potential neo-antigens, whereas other cancer types, including blood cancers, typically harbor very few nonsynonymous mutations[53]. However, even cancers with relatively low mutation rates can harbor tumor neo-antigens capable of eliciting T cell responses[54]. In summary, there is now indisputable evidence that the immune system can recognize and respond to a wide variety of tumor antigens derived from normal or mutated proteins.

Spontaneous immune responses can be generated against cancer

With the knowledge that the immune system can detect cancer antigens, the mechanisms through which spontaneous cancer immunity occurs have been intensely investigated. In order for the immune system to generate an effective anti-tumor immune response, a number of events must occur in succession, a process Chen and Melman have termed the cancer-immunity cycle[55]. First, dead or dying cancer cells must be sensed by the innate immune system in a context which promotes adaptive

immune activation, as opposed to tolerance. Activation of innate immunity in this setting is suggested to be promoted by "immunogenic cell death"[56]. In a hypoxic, nutrient-starved environment, tumor cells are thought to undergo aberrant cell death and release DAMPs which can mature host APCs. Next, cancer cell-derived antigens need to reach the tumor draining lymph node (tdLN), where they can be cross-presented by mature APCs to prime tumor antigen-specific $CD8^+$ T cells. Tumor-derived antigens can reach the LN either directly through the lymphatics or they can be carried by populations of migratory DCs. Regardless, following the antigen encounter on a mature APC, activated T cells must then proliferate, enter the bloodstream, and traffic back to the tumor to ultimately mediate tumor lysis.

Interestingly, a fraction of patients with solid tumors show spontaneous infiltration of $CD8^+$ T cells into the tumor microenvironment, indicating that the cancer-immunity cycle is initiated in some patients. These tumors are now described as having a "T cell inflamed" phenotype, and the intratumoral presence of $CD8^+$ T cells has been correlated with improved disease prognosis[57, 58]. That spontaneous priming against solid cancers can occur in a sterile environment has prompted investigation as to the tumor-derived signals which trigger the activation of innate immunity and, subsequently, tumor-specific $CD8^+$ T cells. In mice, localized tumor induction stimulates the production of type I interferon (IFN) by the host, and the sensing of type I IFN by DCs is required for the spontaneous priming of anti-tumor $CD8^+$ T cells[59, 60]. Importantly, several human tumor subtypes display a type I IFN gene signature, which has been correlated with the presence of tumor-infiltrating T cells[61, 62]. These findings support the data from animal models and demonstrate a critical role for type I IFN in bridging innate and adaptive

anti-cancer immune responses in mice and man. Recently, the mechanism by which type I IFN is induced in response to cancer was uncovered. Here, stimulator of interferon genes (STING) – mediated sensing of tumor-derived DNA by host DCs was required for the induction of type I IFN and the subsequent activation of adaptive immunity[63]. Collectively, these observations suggest that tumor-derived DNA activates the STING pathway, cumulating in the production of type I IFN and the cross-priming of tumor specific $CD8^+$ T cells[64].

Batf3-lineage DCs cross-prime $CD8^+$ T cells against solid tumors

The APCs that acquire and present tumor-derived antigens to support anti-tumor T cell responses in the tdLN and within the tumor itself have recently been characterized. In mouse models, $CD169^+$ macrophages lining the sub-capsular sinus of LNs have been shown to both phagocytose and cross-present antigens derived from apoptotic tumor cells[65]. Deletion of CD169-expressing cells resulted in ablation of adaptive immune responses following vaccination with dead tumor cells, and also was associated with more rapid growth following challenge with live tumor cells. However, in this experimental system, the role of other APC populations in generating anti-tumor immunity was not evaluated, and the role of $CD169^+$ macrophages in priming $CD8^+$ T cells against live tumor cells has not been explored. Interestingly, there is evidence that $CD169^+$ macrophages may shuttle antigens to $CD8\alpha^+$ DCs for cross-presentation, suggesting that this macrophage population may be involved in the initiation of T cell priming by increasing antigen availability to $CD8\alpha^+$ DCs[66].

Batf3-dependent DCs, which include CD8α$^+$ and CD103$^+$ DCs, have also been implicated in the activation of CD8$^+$ T cells against solid tumor antigens. For example, type I IFN signaling in CD8α$^+$ DCs was shown to be required for spontaneous priming of tumor antigen-specific CD8$^+$ T cells in an orthotopic melanoma model[60]. Additionally, Batf3-dependent CD103$^+$ DCs, although present in low frequencies when compared to other APCs present in autochthonous mammary tumors, were superior in antigen cross-presentation to tumor-specific CD8$^+$ T cells[67]. More recently, migratory CD103$^+$ DCs were found to cross-present tumor derived antigens in the tdLN and the administration of FMS-like tyrosine kinase 3 ligand (FLT3L) led to the expansion of CD103$^+$ DCs and stunted tumor growth[68]. Similarly, intra-tumoral injection of FLT3L-induced bone marrow dendritic cells (BMDCs) was able to facilitate T cell priming and limit tumor growth in a genetically driven melanoma model[69]. Moreover, several groups have demonstrated severely defective T cell priming and more rapid outgrowth of immunogenic solid tumors in the absence of Batf3-dependent DCs[33, 59, 60]. Together, these data suggest that Batf3-dependent DCs play a dominant role in generating and sustaining adaptive anti-tumor immune responses against solid cancers.

Tumors evade immune-mediated destruction

While a fraction of human cancers are infiltrated by T cells, this is rarely associated with immune-mediated control of disease progression. This observation suggests that while CD8$^+$ T cells may be primed against a developing neoplasm, immune evasion mechanisms prevent its elimination.

Over the last 20 years, the discovery of numerous immune co-inhibitory receptors, including the CD28 family members programmed death 1 (PD-1) and cytotoxic lymphocyte antigen-4 (CTLA-4), has enhanced our understanding of the mechanisms which modulate T cell function[70]. Both PD-1 and CTLA-4 are expressed on activated T cells, including TILs, and serve to dampen TCR signaling, which ultimately inhibits effector function. While, these receptors likely evolved to protect the host from overt immunopathology following the clearance of infection, their expression in the tumor microenvironment may limit the destruction of cancer. PD-1 has two well defined ligands, programmed death ligands 1 and 2 (PD-L1/PDL-2), whose expression pattern is distinct[71]. PD-L1 is broadly expressed on immune and non-immune cells, whereas PD-L2 expression is limited to immune cells, including DCs and macrophages. Fascinatingly, PD-L1 is often upregulated on malignant cells following their exposure to IFN-γ, suggesting that tumors can co-opt immune inhibitory mechanisms to evade destruction[72, 73].

CTLA-4 is also highly expressed on activated T cells in the tumor microenvironment and binds the B7 costimulatory molecules expressed on mature APCs[74]. CTLA-4 has been shown to limit T cell activation via both T cell intrinsic and extrinsic mechanisms. For example, CTLA-4 has a high affinity for B7 and can directly out-compete CD28 for binding, thereby limiting T cell costimulation and subsequent survival signals[75]. Interestingly, because of the high affinity of CTLA-4 for B7 molecules, activated T cells can also “strip” costimulatory molecules off of APC membranes, rendering them less well-equipped for subsequent T cell activation[76]. While PD-1 and CTLA-4 have somewhat analogous functions, they may serve as immune inhibitory

checkpoints at different steps of the cancer-immunity lifecycle. Because expression of B7 molecules is restricted to professional APCs in the tdLN, CTLA-4 likely inhibits the priming of tumor specific T cells. However, PD-L1 is expressed not only in the tdLN, but also in the tumor itself, as tumor cells can express PD-L1. Therefore, PD-1/PD-L1 interactions may actively limit T cell function at the effector phase of the immune response in the tumor microenvironment[77].

Solid tumors also recruit populations of suppressor cells, including Tregs and myeloid derived suppressor cells (MDSC) which restrict anti-tumor immunity and, in at least some cancer types, the frequency of intratumoral Tregs negatively correlates with disease prognosis[78]. The developmental origin and antigen-specificity of tumor-associated Tregs has been of intense debate. Tregs can develop in the thymus (tTregs) in response to elevated TCR and interleukin 2 (IL-2) signaling or in the periphery (pTreg) following exposure to transforming growth factor-β (TGF-β)[79, 80]. Because the tumor microenvironment often contains high amounts of TGF-β, it has been suggested that many intra-tumoral Tregs may be extra-thymically derived[81]. Recently, mouse prostate tumors were shown to recruit thymically-derived Tregs specific for a prostate-associated self-antigen[82]. Regardless of their developmental origins, tumor-associated Tregs may promote tumorigenesis via multiple distinct mechanisms, most importantly being the direct suppression of an ongoing anti-tumor immune response[83]. Moreover, evidence indicates that Tregs can promote tumor neo-angiogenesis, providing enhanced delivery of oxygen and nutrients to a normally hypoxic tumor environment[84].

Another potential immune evasion strategy activated in solid tumors is the loss of immunogenic antigens or the downregulation of MHC I molecules, which can prevent

the killing of malignant cells. In a carcinogen-induced sarcoma model, Robert Schreiber and colleagues conclusively demonstrated that adaptive immune pressure on a developing neoplasm promotes the emergence of “antigen loss variants” , tumor cells that have lost the expression of immunogenic antigens, in a process termed immunoediting[85, 86]. The concept of immunoediting is based on the hypothesis that tumor cells which express immunogenic antigens are recognized by $CD8^+$ T cells and are eliminated. However, tumor cells which have down-regulated the expression of immunogenic antigens, are able to avoid immune-mediated destruction and progressively grow. The immunoediting hypothesis is further supported by the fact that many human cancers contain mutations in MHC I or beta 2 microglobulin, a protein required for the cell surface expression of MHC I[87, 88]. Therefore, tumors can avoid immune destruction through in a process of microevolution, where malignant cells lose immunogenic antigens or MHC I expression to avoid T cell mediated destruction.

As highlighted here, a developing malignancy can avoid immune detection and destruction through several distinct mechanisms which likely function in concert to promote tumor growth. The discovery of these immune evasion mechanisms has led to the development of immunotherapies aimed at unleashing the immune system against cancer.

Igniting a smoldering anti-tumor immune response

The crowning achievement in cancer immunotherapy thus far has been the development of monoclonal antibodies which block the coinhibitory receptors PD-1 and

CTLA-4. In studies of mice with solid tumors, blockade of PD-1 or CTLA-4 with monoclonal antibodies, termed checkpoint blockade, leads to drastically enhanced $CD8^+$ T cell responses and, in some cases, elimination of established tumors[89]. With these exciting results in hand, checkpoint blockade was advanced into the clinic and has demonstrated remarkable efficacy. In melanoma patients, CTLA-4 blockade leads to long term remission in a small fraction of subjects, although some patients develop severe immune-related side-effects[90]. PD-1 blockade also has therapeutic efficacy and is now FDA approved for the treatment of melanoma, lung cancer, bladder cancer and Hodgkin lymphoma[91, 92]. Interestingly, the efficacy of checkpoint blockade often correlates with the presence of intratumoral T cells, indicating that a spontaneous immune response needs to be generated prior to the blockade of PD-1, and that PD-1/PD-L1 interactions limit T cell function at the effector phase[93, 94]. The fact that "releasing the breaks" on the immune system is capable of inducing tumor remission is definitive evidence that the immune system recognizes tumor antigens, and can be manipulated in a clinically meaningful way.

Adoptive T cell therapy (ACT) is another therapeutic approach to enhance anti-tumor immunity. Classically, this approach has been utilized in melanoma patients, but has shown efficacy in other cancers as well[95]. Here, TILs are isolated from tumor biopsy specimens, and expanded in-vitro with tumor lysates or anti-CD3 antibodies and IL-2. Typically, patients are treated with lymphodepleting chemotherapy or radiation prior to ACT in order to enhance homeostatic proliferation of adoptively-transferred T cells and to deplete regulatory cell populations[96]. As with checkpoint blockade, this approach requires that a spontaneous immune response is generated against a

developing cancer, and only a fraction of patients display a T cell inflamed tumor subtype, which limits the use of this therapy. Additionally, this process is expensive and time consuming.

Because Tregs are enriched in a wide-variety of solid cancers, and the number of intra-tumoral Tregs has been inversely correlated with prognosis in some cancers, a number of strategies to deplete Tregs in animal models have been successful in restoring effective anti-tumor immunity[83]. Tregs constitutively express high levels of IL-2Rα (CD25), and CD25 depleting antibodies administered to tumor-bearing mice, alone or in combination with other immunotherapies, have shown promise in preclinical cancer models[97, 98]. However, Treg depletion with anti-CD25 antibodies, or an IL-2Rα immunotoxin (denileukin diflitox) has been relatively ineffective in humans[99]. Currently, approaches aimed at modulating the suppressive function of tumor-associated Tregs, rather than their depletion, are being evaluated.

Inducing endogenous immunity against solid cancers

Although some tumors display an inflamed phenotype, and are ripe with T cells and immune-related gene transcripts, a large fraction of human cancers are largely devoid of immune cells (non-inflamed). Inflamed tumors, in which a spontaneous immune response has been generated, appear to be more likely to respond to checkpoint blockade therapy and cancer vaccines, whereas non-inflamed cancers, which lack evidence of spontaneous immunity, often fail to respond to such treatments. Recently, there has been interest in identifying the tumor intrinsic factors that mediate T

cell exclusion from a growing malignancy. It was discovered that the WNT/β-catenin signaling pathway is activated in a subset of human cancers, which strongly correlates with a non-T cell inflamed phenotype[69]. Genetically driven murine tumors engineered to express stabilized β-catenin show decreased levels of CCL3 and CCL4, which prevented the accumulation of intratumoral, Batf3-dependent $CD103^+$ DCs. In these mice, intratumoral administration of FLT3L-induced BMDCs was sufficient to support the accrual of TILs and prevented tumor outgrowth. This result was confirmed in a transplantable melanoma model where systemic administration of FLT3L expanded intratumoral $CD103^+$ DCs and was associated with tumor rejection[68]. Overall, these results demonstrate that Batf3-dependent DCs are critical regulators of the anti-tumor T cell response and suggest that the activation or adoptive transfer of mature DCs may be able to induce anti-tumor immunity, even in previously “non-T cell-inflamed” tumors.

ACT was originally performed using the transfusion of autologous TILs following in vitro expansion, as discussed above. However, this approach requires the patients to harbor a pool of activated, tumor-specific TILs which can be expanded ex vivo. More recently, an elegant protocol has been established to “reprogram” peripheral blood T cells with tumor specificity. Chimeric antigen receptors (CARs) consist of an extracellular, antibody-based, recognition domain linked to the intracellular TCR signaling machinery[100]. The specificity of the CAR comes from the antibody domain and thus CARs can be designed with nearly any antigen specificity. Autologous T cells are harvested from a patient’s blood, retrovirally transduced with the CAR of interest, and then transfused back into the patient. The most successful use of CARs comes from patients with B cell lymphoma, in which CARs target the B cell markers CD19 or CD20.

In many patients, CAR treatment led to durable responses and was capable of inducing remission[101]. However, these patients are severely immunocompromised, as B cells are completely eliminated, and need to be given passive antibody infusion. Because of the efficacy of CAR T cells, care needs to be used when choosing potential antigen targets. Practically, the use of CARs is restricted to tumor-specific proteins or TAA antigens expressed by cells whose presence is not required to the health of the patient. Another drawback of CAR therapy is that the targeted antigens need to be extracellular, as CARs use antibody domains to trigger activation. Overall CARs hold tremendous potential to eradicate certain tumor subtypes and recently, more sophisticated CARs are being developed to increase both tumor specificity and efficacy.

The pathogenesis of and immunogenicity of acute myeloid leukemia

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and is characterized by the infiltration of poorly differentiated myeloid cells into the bone marrow, blood, and peripheral tissues[102]. Aggressive chemotherapy regimens can often induce complete remission in patients with AML and is curative in ~35% of patients under the age of 60. However, following chemotherapy, most patients will relapse and AML eventually overwhelms the host; this is especially true for patients over the age of 60. For patients who relapse following chemotherapy or for those with high-risk AML in first complete remission, allogenic stem cell transplantation (SCT) can be curative. The efficacy of allogeneic SCT is thought to rely on the induction of a graft-versus leukemia effect, where donor cells recognize minor histocompatibility antigens expressed on host

leukemia cells. However, due to the serious risks involved with SCT, only a fraction of patients are candidates for this procedure. Therefore, there has been considerable interest in the development of immunotherapies aimed at stimulating immune-mediated eradication of AML[103].

Potentially immunogenic antigens have been identified in AML, and include peptides derived from Proteinase 3 (PR3), Wilm's tumor protein 1 (WT1), and others[104, 105]. WT1 is highly expressed in AML cells and its expression is restricted to select healthy tissues, including gonads, kidneys, and progenitor cells in various tissues. Thus, WT1 was a promising leukemia-associated antigen for vaccine-based immunotherapy approaches in AML. WT-1 peptide vaccination increased the frequency of WT1-specific T cells in peripheral blood and, in some cases, was correlated with disease free progression, however these results need further validation[106]. There has yet to be the characterization of a true AML-specific antigen, and these neo-antigens may be limited due to a relatively low number of nonsynonymous mutations in AML[53]. However, it has been suggested that even cancers with low mutation burdens can harbor immunogenic neo-antigens[54]. Together, it has become clear that AML contains antigens which can be recognized by the host immune system. However, like solid tumors, AML is rarely rejected by the host and it is likely that immune evasion mechanisms exist to promote disease progression.

Immune evasion by hematopoietic malignancies

While the immune evasion mechanisms which operate in solid cancers have been well-characterized, much less is known about how the immune system interacts with hematological cancers, such as AML. As these malignancies tend to grow in a disseminated pattern, lack the complex stromal network induced by solid tumors, and do not drain into a classical tumor-draining lymph node, it is likely that the manner in which AML cells interact with host immune system differs greatly from their solid tumor counterparts. Therefore, mechanisms of immune evasion utilized by solid tumors may not completely overlap with tolerance mechanisms operating in hematopoietic malignancies.

Tregs are known to be expanded in AML patients and may play a role in suppressing anti-leukemia immunity[107, 108]. In these studies, the frequency of Tregs was negatively correlated with response to chemotherapy and overall survival, as has been seen in many solid cancers. Tregs also accumulate in the spleen and livers of mice harboring advanced stage AML, and Treg depletion in combination with ACT or PD-1 blockade led to a significant reduction in leukemia burden in AML bearing mice[108, 109]. However, Treg depletion alone had no effect on leukemia progression. These results suggest that AML-specific Tregs may play a role in limiting anti-leukemia immunity.

There is also evidence that the expression of coinhibitory ligands, either by AML cells or by host immune cells, may prevent anti-leukemia immunity. For example, wildtype mice succumb to disease more rapidly than *PD-1*$^{-/-}$ mice harboring systemic AML[109, 110]. Additionally, blockade of PD-L1 improved survival in AML bearing mice,

although this effect was relatively minor and all mice ultimately succumb to disease[110]. Overall, a better understanding of the interactions between the immune system and AML will aid in the development of effective immunotherapies to treat this difficult disease.

Summary

Over the past two decades, the general understanding of immunity and cancer has advanced significantly, to the point where immune-based therapies are regularly and effectively used to treat a growing number of malignancies. It is now universally accepted that the immune system can recognize tumor antigens, which in some cases, results in the generation of endogenous immune responses. However, it is also known that solid tumors activate a variety of immune evasion pathways which effectively inhibit anti-cancer immunity. Although less well-studied, emerging evidence indicates that hematological cancers, including AML, also activate immune escape mechanisms. Because the pathogenesis and behavior of leukemia is fundamentally different than that of solid tumors, our laboratory has focused its efforts on the characterization of immune evasion mechanisms operational in leukemia-bearing mice.

To study immune responses generated against leukemia antigens, we utilized the C1498 cell line which was generated from an AML that spontaneously arose in a C57BL/6 mouse. Here, we discovered a novel mechanism of T cell tolerance in AML-bearing animals in which leukemia-specific $CD8^+$ T cells encountered antigen, underwent abortive proliferation, and were ultimately deleted from the host[111]. The few

leukemia-specific $CD8^+$ T cells that escaped deletion were dysfunctional, consistent with an anergic phenotype. Interestingly, T cell tolerance could be prevented upon the maturation of host APCs, implicating APCs as the drivers of the tolerant state in leukemia-bearing animals.

The nature of the APCs which might have been regulating the T cell tolerant state in AML-bearing mice was investigated. Using fluorescently labeled leukemia cells, we demonstrated that leukemia-derived proteins were exclusively acquired and cross-presented by splenic $CD8\alpha^+$ DCs. Moreover, in the steady state, $CD8\alpha^+$ DCs were found to actively drive AML-specific T cell tolerance. Interestingly, the same subset of Batf3-dependent DCs has been shown to be required to productively prime $CD8^+$ T cells against solid tumors. Thus, we postulated that the maturation status of $CD8\alpha^+$ DCs ultimately determines if tolerance or immunity ensues in mice with solid or blood cancers.

With the knowledge that type I IFN is a critical regulator of APC activation in the setting of solid cancer, whether the lack of a type I IFN response was associated with T cell tolerance in mice with disseminated leukemia was investigated. Interestingly, we found that AML failed to induce production of IFN-β from host DCs, in contrast to what has been described in solid tumor models[112]. Additionally, mice unable to sense type I IFNs showed no defect in AML-specific immune responses or survival following intravenous tumor challenge, suggesting that the type I IFN pathway is not activated in AML-bearing mice. Keeping in mind the requirement for type I IFN in DC-mediated priming of anti-tumor immunity, we utilized pharmacological approaches aimed at activating type I IFN signaling in DCs to induce productive T cell immunity. Treatment of

AML-bearing mice with the STING agonist DMXAA lead to the robust induction of type I IFN, and an impressive increase in mouse survival. Overall, this work provides insight as to how the immune system interacts with a circulating leukemia, and specifically, how the innate immune system drives T cell tolerance against a disseminated hematopoietic malignancy.

MATERIALS AND METHODS[1]

Mice

C57BL/6 (H-2^b) mice, aged 6–12 weeks, were purchased from Jackson Laboratories or Taconic Laboratories. *Batf3*$^{-/-}$, *Rag2*$^{-/-}$, *Tap1*$^{-/-}$, *H-2K*$^{b-/-}$, and IL-12 YFP reporter mice, all on the C57BL/6 background, were purchased from Jackson Laboratories and bred in our animal facility[33, 113, 114]. 2C TCR transgenic mice (2C T cells recognize the SIY (SIYRYYGL) peptide antigen in the context of H-$2K^b$) have been described previously, and were bred in our animal facility[115]. Bcl-X_L transgenic mice, in which BCL-X_L expression is controlled by the LCK promoter, have been reported previously[116], and were a gift from A. Sperling (University of Chicago). $2C^{BCL\text{-}XL}$ double-transgenic mice were generated through cross-breeding. $Nur77^{GFP}$ transgenic mice, where GFP expression directly correlates with TCR signal strength, were purchased from Jackson Laboratories, and were crossed with 2C mice to generate 2C $Nur77^{GFP}$ animals[117]. FoxP3-DTR animals were obtained from A. Chervonsky (University of Chicago), with permission from A. Rudensky[5]. *Tmem173*$^{-/-}$ and *Ifnar*$^{-/-}$ mice have been reported previously[118, 119], and were provided by Y.X. Fu (University of Chicago). *Clec9a*$^{-/-}$ mice were provided by Caetano Reis e Sousa (The Francis Crick Institute)[120]. Animals were maintained in a specific pathogen-free environment and used according

[1] Parts of this section are reproduced here, with minimal modification, from Zhang L et al. CD40 ligation reverses T cell tolerance in acute myeloid leukemia. The Journal of clinical investigation 2013, 123(5): 1999-2010 and Curran E et al. STING Pathway Activation Stimulates Potent Immunity against Acute Myeloid Leukemia. Cell reports 2016, 15(11): 2357-2366.

to protocols approved by the Institutional Animal Care and Use Committee at the University of Chicago, according to NIH guidelines for animal use.

Tumor cell lines and inoculation

The C1498 murine AML cell line was purchased from ATCC[110]. C1498 cells were cultured in complete DMEM supplemented with 10% fetal calf serum. C1498.GFP cells were engineered by retroviral transduction using the pLEGFP plasmid; C1498.SIY cells were engineered by retroviral transduction using the pLEGFP plasmid expressing cDNA for the SIY model peptide antigen in frame with eGFP. Cell surface expression of the SIY peptide is K^b restricted, and thus can be recognized by a small fraction of endogenous C57BL/6 $CD8^+$ T cells, and is also specifically recognized by 2C TCR Tg $CD8^+$ T cells. B16.OVA cells, expressing the full-length chicken ovalbumin (OVA) protein, were a gift from Y.X. Fu (University of Chicago). The FBL cell line is an MHC class I^+, MHC class II^- AML cell line expressing the FMuLV gag peptide (CCLCLTVFL), presented in the context of K^b, which was donated by Dr. Ryan Teague (St. Louis University). To generate the *Cbfb-MYH11/Mpl*-induced mouse leukemia model (CMM^+), polyinosinic-polycytidylic acid (poly(I:C)) was administered to *Cbfb*$^{+/56M}$*/Mx1-Cre*$^+$ mice to induce expression of core-binding factor β-smooth muscle myosin heavy chain[121]. Two weeks later, bone marrow cells were harvested and transduced with the retroviral *MIG-Mpl* vector and GFP genes to generate a transplantable *Cbfb-MYH11/Mpl*$^+$ mouse AML, as previously described[122].

C1498, C1498.GFP, C1498.SIY, and B16.OVA cells were washed 3 times with PBS to remove FCS and resuspended in PBS at a concentration of 10^6–10^7 cells/ml. For intravenous (IV) challenge, a volume of 0.1 ml (10^5–10^6 tumor cells) was injected into the lateral tail vein of each mouse. For SC challenge, a volume of 0.1 ml (10^6 tumor cells) was injected under the skin of the right lower lateral abdominal wall. For experiments with FBL, 10^5 cells were inoculated IV or SC.

ELISPOT and ELISA

ELISPOT was conducted with the BD Bioscience mouse IFN-γ ELISPOT kit according to the provided protocol. Briefly, ELISPOT plates were coated with anti-mouse IFN-γ Ab and stored overnight at 4°C. Plates were then washed and blocked with DMEM supplemented with 10% FCS for 2 hours at room temperature. Splenocytes or LN cells (tdLN for SC inoculation; pooled inguinal and axillary LNs for IV inoculation) from individual tumor-challenged mice were harvested at various time points and plated in triplicate at between 5×10^5 and 1×10^6 cells/well. Unless otherwise indicated, stimulation was performed with irradiated (150 Gy) C1498 cells (5×10^4 cells/well) or SIY peptide (100 nM). Stimulation with media alone, or with PMA (50 ng/ml) and ionomycin (500 nM) served as negative and positive controls, respectively. Plates were stored at 37°C in an 8% CO_2 incubator overnight, washed, and coated with detection Ab for 2 hours at room temperature. Plates were again washed and coated with avidin peroxidase for 1 hour at room temperature, then washed and developed by addition of AEC substrate. Developed plates were dried overnight, read using an ImmunoSpot Series 3 Analyzer, and analyzed with ImmunoSpot software.

For cytokine ELISAs, C57BL/6 mice were treated with DMXAA or vehicle control, and serum was collected 6 hours later. ELISA was performed for IFN-β, TNF-α and IL-6 using pre-coated plates (Biolegend) according to the manufacturer's protocol.

Flow Cytometry and pentamer analysis

Organs were harvested and passed through a 70μm filter. Red blood cells were lysed prior to flow cytometric analysis. Fc receptors were blocked with anti-CD16/32 antibodies to eliminate non-specific staining. Samples were then stained with the following directly conjugated antibodies (BD Bioscience, eBioscience, or Biolegend): CD11c (clone:HL3), Thy1.2 (53-2.1), CD205 (205yelka), DNGR-1 (10B4), CD11b (M1/70), Siglec H (551), TCRβ (H57-597), CD4 (GK1.5), CD8α (53-6.7), CD69 (H1-2F3), I-A/I-E (M5/114.15.2), H-2K^{b} (AF6-88.5), IFN-γ (XMG1.2), TNF-α (MP6-XT22), CD80 (16-10A1), CD86 (IT2.2) and B220 (RA3-6B2). TLR-3 (11F8) expression was analyzed via intracellular staining after fixation and permeabilization (eBioscience). The SIY and negative control OVA peptide pentamers were purchased from Proimmune. After cell surface staining with anti-CD4 and anti-B220 (for exclusion of CD4^{+} T cells and B cells, respectively), as well as an anti-CD8 antibody, pentamer staining was performed on spleen or LN cells from individual mice according to the manufacturer's protocol. Dead cells were excluded using fixable viability dyes (Invitrogen). Samples were run on a LSRII or LSRFortessa (BD Bioscience) and analysis was performed using FlowJo (treestar). Image Stream samples were run on an ImageStreamX (Amnis) and analyzed via IDEAS software (Amnis).

Adoptive T cell transfers

Splenic $CD8^+$ T cells were isolated from 2C TCR transgenic mice via magnetic separation (Miltenyi). Purified 2C T cells were labeled with 5 μM CFSE or CellTrace Violet (CTV) (as per manufactures instructions - Invitrogen) and 1 x 10^6 were inoculated IV into recipient mice. The next day, mice received 1 x 10^6 C1498.SIY cells IV or SC, in the flank. Six days later, spleens were harvested and stained with antibodies against Thy1.2, CD8α, and an antibody that specifically recognizes the 2C TCR (1B2). In some experiments, 2C T cells expressing the congenic marker CD45.1 were used and transferred 2C T cells were identified as $1B2^+CD45.1^+$ cells by flow cytometry. Numbers of 2C cells were calculated by multiplying the total number of live spleen or LN cells by percent $CD8^+$ T cells present and, finally, by percent $CD45.1^+$ or $1B2^+CD8^+$ cells present per sample.

For 2C and OT-I cotransfers, T cells were isolated from OT-I ($Thy1.1^+$) and 2C ($Thy1.2^+$) mice using a CD8 microbead kit (Miltenyi). Purified OT-I and 2C T cells were mixed at a 1:1 ratio, and 2 x 10^6 T cells (10^6 2C and 10^6 OT-I T cells) were co-transferred into groups of C57BL/6 mice. Twenty-four hours later, half of the mice received C1498.SIY cells IV, while the other half remained leukemia-free. DMXAA or vehicle was administered on day five, and seven days later, spleen cells from each group of mice were analyzed by flow cytometry after cell surface staining with anti-CD8, anti-Thy1.2, anti-Thy1.1, and anti-1B2 antibodies in order to identify the frequencies of OT-I and 2C T cells present.

Intracellular cytokine staining

Six days following C1498.SIY cell challenge, ~5 x 10^6 spleen cells isolated from leukemia-bearing animals were cultured in the presence or absence of 500 nM SIY peptide, or with phorbol 12-myristate 13-acetate (PMA) and ionomycin for five hours at 37°C. GolgiPlug (BD bioscience) was added to the cultures for the final four hours at a final concentration of 1 μg/ml. Cells were then harvested and stained with antibodies against Thy1.2 or TCRβ and CD8α before fixation and permeabilization (eBioscience) and subsequent staining with an anti-IFN-γ antibody.

In vivo administration of agonistic anti-CD40 and Poly(I:C)

Groups of C57BL/6 mice were challenged with 10^6 C1498.SIY cells IV or SC on day 0, or remained tumor free. On days 0, 2, and 4, mice received intraperitoneal (i.p.) injection of agonistic anti-CD40 Ab (FGK45; 100 μg- Bio X cell) or isotype control antibody. On day 6, spleen and LN cells from tumor-challenged and naive mice treated with anti-CD40 or isotype control antibody were isolated, analyzed by flow cytometry after SIY or OVA pentamer staining (as above), and also restimulated using IFN-γ ELISPOT assay (as above). For poly(I:C) survival experiments, 10^6 C1498 or C1498.SIY cells were inoculated IV into C57BL/6 or *Batf3*$^{-/-}$ mice. On days 0, 3, 6, 9, and 12, mice received 100 μg of Poly(I:C) (Sigma) or PBS IP.

Isolation of dendritic cells

Dendritic cells were isolated from lymphoid organs by digestion followed by mechanical disruption. Briefly, organs were harvested, injected with ~5 ml of 1mg/ml Collaganase IV (Sigma) and 20 µg/ml DNAse I (Roche), and incubated at 37°C for 30 minutes. Organs were then crushed through a 70 µm filter and red blood cells were lysed before DC enrichment or flow cytometric analysis. CD3ε (145-2C11) and CD19 (eBio1D3) biotinylated antibodies were used followed by secondary streptavidin staining to eliminate T and B cells from cytometric analysis of DC populations.

In vivo phagocytosis and cross-presentation assays

4 x 10^6 C1498 cells were labeled with CellTrace Violet according to the manufacturer's protocol, washed three times with PBS, and injected IV through the lateral tail vein. 1-24 hours later, organs were harvested, collagenase digested, and stained with the indicated antibodies in preparation for flow cytometry to identify APC populations that had acquired C1498 cells or cell fragments. In some uptake experiments, C1498 cells were labeled with 10 µM EdU overnight. The next day AML cells were labeled with CTV and three hours post IV injection tumor derived DNA was detected in DCs after permeabilization as per the manufactures instructions (Click-iT EdU kit - Thermo Fischer Scientific).

For cross-presentation assays, splenic DC populations were purified by FACS three hours after IV injection of 4 x 10^6 C1498 or C1498.SIY cells. Before FACS separation of splenic DCs, T and B cells were depleted by incubating spleen cells with

anti-CD3ε (145-2C11) and anti-CD19 (eBio1D3) biotinylated antibodies followed by anti-biotin microbeads (Miltenyi). Sorted DC populations were cultured (1:1 or 1:2) with purified $CD8^+$ CTV-labeled 2C T cells for 65-72 hours in RPMI 1640 (Invitrogen) supplemented with 10% FBS, 2-mercaptoethanol, essential amino acids and antibiotics (complete RPMI). Subsequently the CTV dilution of cultured 2C T cells was analyzed by flow cytometry.

IFA Vaccination

5 x 10^6 C1498.SIY cells were inoculated IV into groups of C57BL/6 or *Batf3*$^{-/-}$ mice. Six days later, leukemia-bearing or naïve mice received a SC vaccination with IFA (Sigma) or with SIY peptide (25 µg) emulsified in IFA as previously described[123]. Five days later, vaccine-draining lymph node cells were isolated, stained with SIY/K^b pentamer, along with anti-Thy1.2 and anti-CD8α antibodies, and the frequencies of SIY-reactive $CD8^+$ T cells in each group were assessed by flow cytometry.

Quantitative real-time PCR analysis

C57BL/6, *Tmem173*$^{-/-}$ or *Ifnar*$^{-/-}$ mice were treated with DMXAA or vehicle control, and spleen cells harvested 6 hours later. For measurement of cytokine expression in leukemia-bearing mice, 5 x10^6 C1498 cells were injected IV or SC. Spleen or lymph node cells, respectively, were harvested 72 hours later. All samples were re-suspended in Trizol (Life Technologies) and total RNA isolated via chloroform extraction. cDNA

was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qPCR) reactions were performed using TaqMan Gene Expression Master Mix (A&B) and a 7300 Real Time PCR system (A&B) was then performed for *Ifnb*, *Tnfa* and *Il6*. The primers used for PCR of *Ifnb*, *Tnfa* and *Il6* have been described previously[124].

In vivo administration of STING agonists

DMXAA (5,6-dimethylxanthenone-4-acetic acid) (Sigma-Aldrich) was dissolved in sterile 7.5% $NaHCO_3$. 450 μg was injected IV on the day(s) indicated in each experiment. dithio-(R_P, R_P)-[cyclic[A(2',5')pA(3',5')p]] (ML-RR-CDA) was provided by Aduro Biotech and diluted in sterile PBS and 100 μg was injected IV.

Statistical analysis

Multivariable data were analyzed via 2-way ANOVA with Bonferroni post-tests, and Student's t tests were used to compare two groups. Survival differences were compared using a log-rank test. Data are presented as mean ± SEM. A p-value of <0.05 was considered to be statistically significant.

RESULTS I: CD40 LIGATION REVERSES T CELL TOLERANCE IN ACUTE MYELOID LEUKEMIA[1]

Summary

Spontaneous antigen-specific T cell responses can be generated in hosts harboring a variety of solid cancers, but are subverted by immune evasion mechanisms active within the tumor microenvironment. In contrast to solid tumors, the mechanisms that regulate T cell activation versus tolerance to malignant cells of hematopoietic origin have been under-explored. A murine AML model was utilized to investigate antigen-specific T cell responses against AML cells inoculated intravenously (IV) versus subcutaneously (SC). Robust antigen-specific T cell responses were generated against AML cells following SC but not IV inoculation. In fact, IV AML cell inoculation prevented functional T cell activation in response to a subsequent SC AML cell challenge. This T cell dysfunction was antigen-specific, and was independent of regulatory T cells or myeloid-derived suppressor cells. Antigen-specific T cell receptor transgenic $CD8^+$ T cells proliferated but failed to accumulate, and expressed low levels of effector cytokines in hosts following IV AML induction, consistent with abortive T cell activation and peripheral tolerance. Administration of agonistic anti-CD40 antibody enhanced accumulation of functional T cells and prolonged mouse survival. Our results suggest that antigen-specific T cell tolerance is a potent immune evasion mechanism in hosts with AML which can be prevented upon activation of host APCs via CD40 engagement.

[1] Parts of this section are reproduced here, with minimal modification, from Zhang L et al. CD40 ligation reverses T cell tolerance in acute myeloid leukemia. The Journal of clinical investigation 2013, 123(5): 1999-2010

Introduction

In contrast to the translational research progress being made uncoupling immune inhibitory mechanisms in the setting of solid tumors, the negative regulatory mechanisms orchestrated by hematologic malignancies, such as acute myeloid leukemia (AML), are much less understood. Because hematological malignancies differ greatly in their growth rate and pattern and stromal milieu when compared to tumors which progress locally as a solid mass, it seemed likely that the interaction with the host immune response might be distinct. Recent observations from solid tumor models have suggested that local inflammation generated by tumor cell death can result in the elaboration of "danger signals" which activate host innate immune cells[125, 126], including $CD8\alpha^+$ dendritic cells (DCs)[60]. Activated DCs can consequently cross-present tumor-derived antigens and initiate $CD8^+$ T cell activation, resulting in a spontaneous anti-tumor immune response. However, in the case of a systemic leukemia, it is conceivable that this immunogenic cell death might not occur, for example, because the hypoxia and nutrient deprivation that contribute to death of a subset of cancer cells in a growing solid tumor mass might not be a major factor in a setting in which cancer cells are disseminated widely throughout the host. Therefore, the nature of the major immune evasion mechanisms active in hosts with leukemia also might be distinct. Understanding these mechanisms should point towards the most logical strategies for immunotherapy for patients with hematologic malignancies.

With these notions in mind, we utilized a transplantable model of AML in which leukemia cells were introduced intravenously (IV) versus subcutaneously (SC) into mice to analyze both spontaneous immune responses and mechanisms of immune escape.

In fact, IV inoculation of AML cells prevented the generation of an antigen-specific T cell response induced by SC inoculation in the same mouse, indicating a rapid induction of peripheral tolerance. This tolerance appeared to be due to the intrinsic dysfunction and deletion of anti-tumor T cells, and was reversed by administration of an agonistic anti-CD40 antibody. Our findings suggest that dominant peripheral tolerance is a major mechanism of immune escape with hematogenous dissemination of leukemia, and that anti-CD40 may have a therapeutic benefit that could be translated clinically.

Results

Diminished survival in C57BL/6 mice after IV versus SC challenge with C1498 AML

To investigate the role of adaptive immunity in the control of AML progression, we challenged cohorts of C57BL/6 and T cell/B cell–deficient *Rag2*$^{-/-}$ hosts IV or SC with 10^6 C1498.SIY cells (C1498 cells engineered by retroviral transduction to express the SIYRYYGL model peptide antigen), and survival was assessed. Whereas no difference in survival was seen after inoculation of C1498.SIY cells IV versus SC in *Rag2*$^{-/-}$ hosts, C57BL/6 mice challenged SC with C1498.SIY cells survived significantly longer compared to those challenged with the same number of C1498.SIY cells IV, and approximately 20% of mice survived long-term **(Figure 1A)**. These results suggested that a partial adaptive immune response was generated when C1498 cells were implanted SC, but not IV. Furthermore, the similar survival observed in C1498.SIY challenged *Rag2*$^{-/-}$ mice, regardless of inoculation route, argued that the "antigen" load to which mice were exposed was similar when comparing SC and IV routes of inoculation.

Minimal functional antigen-specific T cell responses are generated in mice harboring C1498.SIY cells IV

To test directly whether antigen-specific T cell responses were occurring in C57BL/6 mice after IV versus SC C1498 cell inoculation, spleens and LNs were harvested from groups of C57BL/6 mice at various time points after either IV or SC C1498.SIY cell inoculation, and the number and function of SIY-reactive $CD8^+$ T cells

was analyzed using SIY/K^b pentamers and IFN-γ ELISPOT. SIY pentamer–reactive $CD8^+$ T cells were more numerous in the spleens of C57BL/6 mice challenged with C1498.SIY cells SC versus IV on day 10 after C1498.SIY cell challenge **(Figure 1B and C)**. Furthermore, when the function of SIY-specific T cells was analyzed with IFN-γ ELISPOT, significantly higher numbers of IFN-γ spot-forming cells were observed in mice 5 and 10 days after SC C1498.SIY cell challenge **(Figure 1D)**. In contrast, in C57BL/6 mice challenged with C1498.SIY cells IV, only minimal functional responses were detected at all time points analyzed. A similar, although slightly delayed, kinetic pattern of functional activation of endogenous C1498–specific T cells was seen in mice challenged with control C1498.GFP cells **(Figure 1E)**, which indicates that the impaired priming or activation of tumor antigen-specific T cells in hosts harboring leukemia cells systemically was not limited to T cells specific for the model SIY antigen.

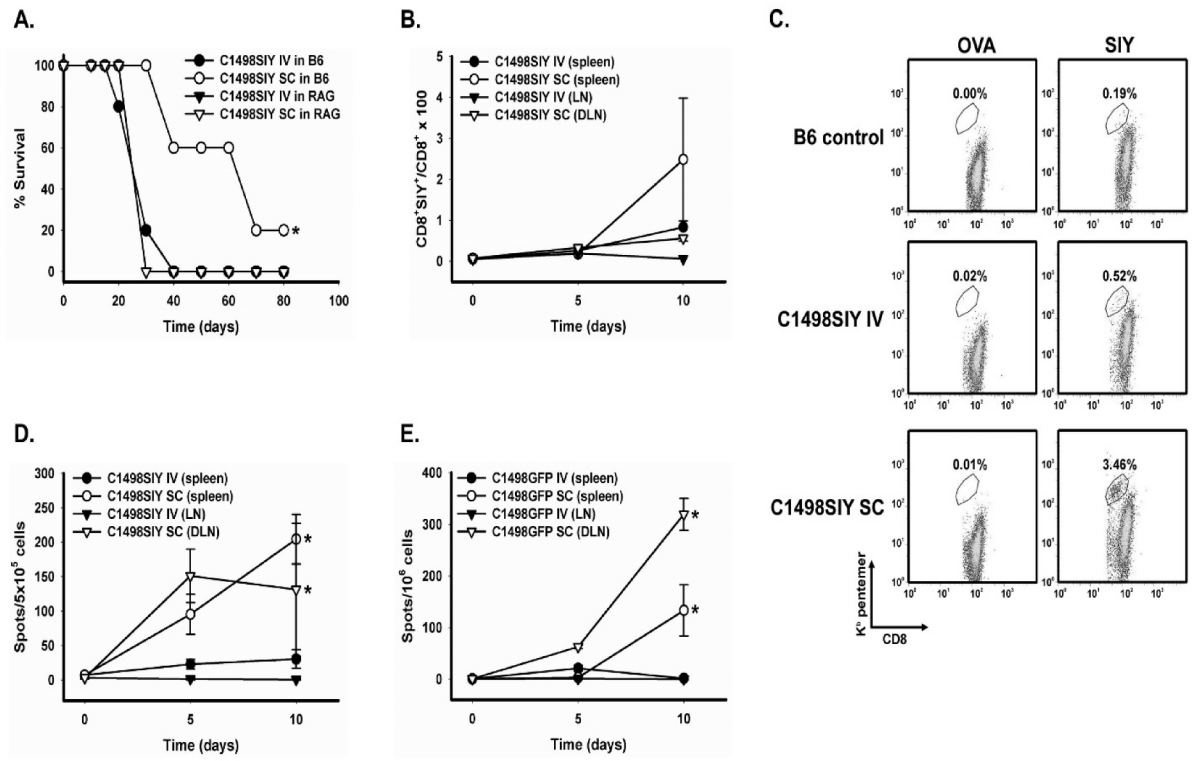

Figure 1. Decreased survival and tumor antigen-specific T cell responses following intravenous versus subcutaneous C1498.SIY cell challenge. A) Groups of 5 RAG2$^{-/-}$ or C57BL/6 mice received 10^6 C1498.SIY cells IV or SC and survival was assessed (*p = 0.009 for survival of C57BL/6 mice with C1498.SIY SC versus IV). **B)** C57BL/6 mice (10/group) received 10^6 C1498.SIY cells IV or SC. On days 5 and 10, spleen and lymph node cells from 5 mice (tdLN from SC-challenged mice and pooled axillary and inguinal LN from IV-challenged mice) were analyzed for SIY-reactive CD8$^+$ T cells following SIY/K^b pentamer staining and flow cytometry (p = 0.09 for comparison of percentages of SIY-reactive CD8$^+$ T cells in spleens of mice harboring C1498.SIY cells SC versus IV on day 10). **C)** Representative FACS plots of SIY and OVA pentamer staining from mice in B. **D)** IFN-γ ELISPOT analysis of spleen and lymph node cells from mice in B. following in vitro restimulation with media or SIY peptide (*p < 0.001 for comparison of number of IFN-γ spot-forming cells from both spleens and lymph nodes of mice challenged with C1498.SIY SC versus IV on days 5 and 10). **E)** Mice received 10^6 C1498.GFP cells IV or SC as in B. On days 5 and 10, an IFN-γ ELISPOT assay was performed as in B. following in vitro restimulation with media or irradiated C1498.GFP cells (*p < 0.05 for comparison of numbers of IFN-γ spot-forming cells from spleens and lymph nodes of mice challenged with C1498.GFP SC versus IV on day 10). Data are representative of 2 experiments.

Generation of antigen-specific T cell dysfunction after IV C1498.SIY cell inoculation.

Given the equivalent antigen load after IV versus SC inoculation of an identical number of C1498.SIY cells, it was conceivable that the IV-disseminated leukemia cells not only failed to prime a specific T cell response, but might have actively induced peripheral tolerance. To determine whether this was the case, mice received IV C1498.SIY cell inoculation on day –6, followed by SC C1498.SIY cell challenge on day 0 (a dual-challenge approach referred to herein as IV/SC). In fact, strikingly diminished functional SIY-specific T cell responses were observed in the spleens and tumor-draining LNs of mice subjected to IV/SC administration **(Figure 2A and B)**. Similar findings were observed in parallel experiments in which control C1498 cells were used **(Figure 2C)**, which suggests that T cell dysfunction induced by IV C1498 cells was not dependent upon their expression of the SIY antigen. Thus, hematogenous dissemination of AML cells actively promoted the induction of T cell dysfunction in C57BL/6 mice.

To ensure that the T cell tolerance to IV-disseminated leukemia was not an artifact of an individual cell line, parallel experiments were performed using murine FBL cells that naturally express the retroviral Gag protein. C57BL/6 mice received IV, SC, or IV/SC inoculation of FBL cells as above, and Gag-specific $CD8^+$ T cell responses were analyzed by IFN-γ ELISPOT after ex vivo restimulation with Gag peptide. Strikingly diminished functional Gag-specific endogenous $CD8^+$ T cell responses were again observed in mice that received IV/SC inoculation **(data not shown)**. These results argue that induction of peripheral T cell tolerance is a common mechanism of immune evasion in hosts with disseminated AML.

To determine whether the ability of IV C1498 cell inoculation to induce peripheral tolerance was dose dependent, a range of cell numbers was introduced IV. Indeed, increasing numbers of IV C1498.SIY cells led to progressively diminished functional SIY-specific T cell responses after subsequent SC inoculation with 10^6 C1498.SIY cells 6 days later **(Figure 2D)**. To determine whether the induction of peripheral tolerance was unique to the IV setting, groups of C57BL/6 mice were inoculated with C1498.SIY cells IV, or SC on the left flank on day –6; on day 0, both groups received a second inoculation of SC C1498.SIY cells on the right flank. As expected, the IV/SC C1498.SIY challenged recipients failed to generate a functional SIY-specific T cell response. In sharp contrast, enhanced SIY-specific T cell responses were seen in spleens of SC/SC C1498.SIY challenged recipients **(Figure 2E)**. This suggests that the initial SC C1498.SIY cell inoculation on day –6 actually promoted antigen-specific T cell priming, similar to what might be expected with a tumor cell based vaccine. To determine whether a functional antigen-specific T cell response after SC C1498.SIY cell inoculation could be inhibited by subsequent IV C1498.SIY cell inoculation, groups of C57BL/6 mice were challenged with SC C1498.SIY cells on day –6, and some received subsequent IV C1498.SIY cell inoculation on day 0. Functional SIY-specific T cell responses were analyzed in the spleens of these mice 6 days later, which demonstrated that antigen-specific T cell responses in mice receiving SC/IV C1498.SIY cell inoculation were similar to those in mice receiving SC C1498.SIY cell inoculation alone **(data not shown)**. This result suggested that once antigen-specific $CD8^+$ T cells were functionally primed after SC C1498.SIY cell challenge, they were no longer sensitive to tolerization with a subsequent IV C1498.SIY cell challenge.

It was important to exclude the possibility that global immune suppression as a result of advanced tumor burden was responsible for the defective antigen-specific T cell responses seen in mice after IV C1498.SIY cell inoculation. To address this possibility, C57BL/6 mice were challenged with live or irradiated (150 Gy) C1498.SIY cells IV on day –6, followed by SC C1498.SIY cell challenge on day 0. This dose of radiation was found to be nearly 100% lethal to C1498.SIY cells, as assessed by trypan blue exclusion **(data not shown)**. Diminished SIY-specific T cell responses against SC C1498.SIY tumors were observed whether live or irradiated C1498.SIY cells were previously introduced IV **(Figure 2F)**, which argues that systemic immune suppression from a rapidly growing tumor was not the cause of peripheral tolerance induced after IV C1498.SIY cell inoculation.

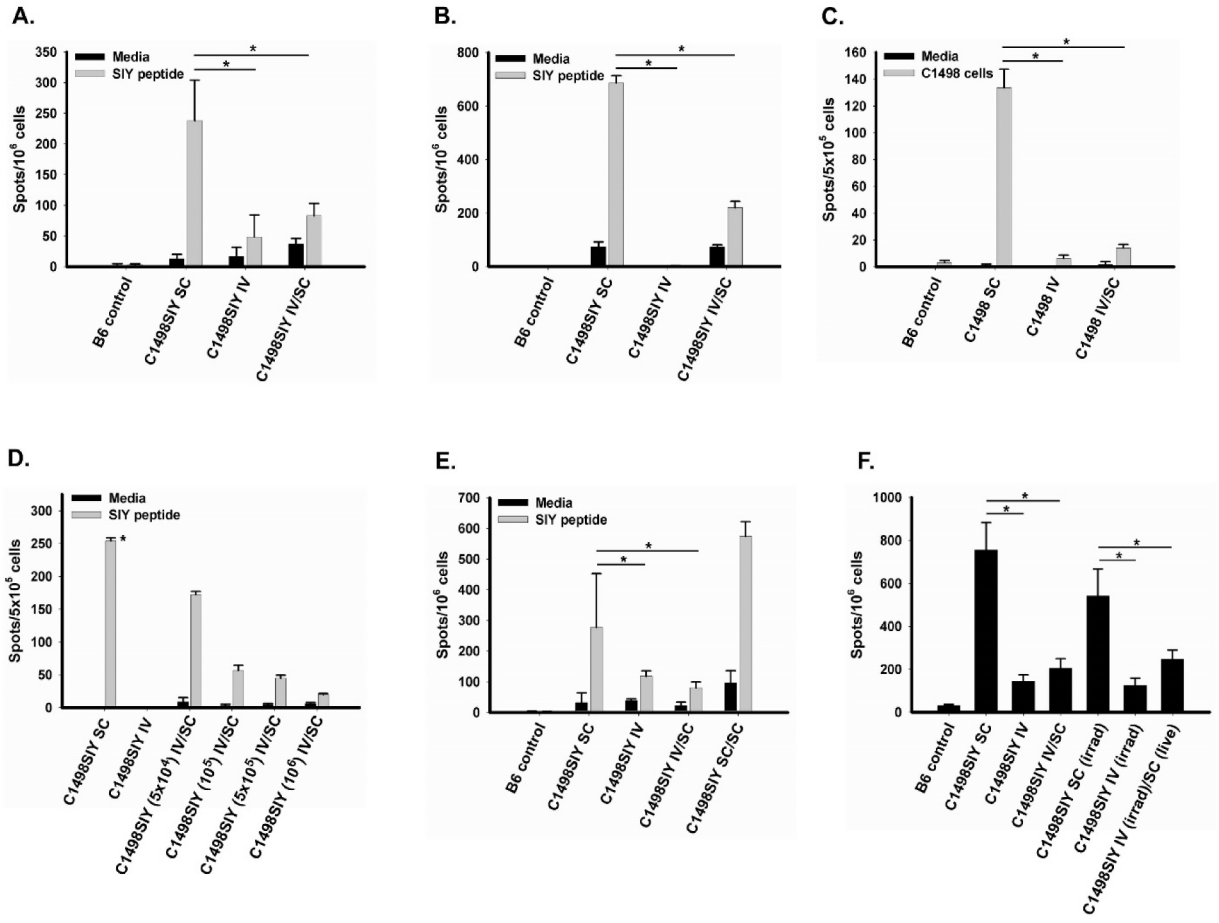

Figure 2. Intravenous inoculation of C1498.SIY cells rapidly generates a T cell dysfunctional state. A and B) Mice (3/group) received C1498.SIY cells IV or SC on day 0. A third group received C1498.SIY cells IV on day -6, followed by a SC challenge with C1498.SIY cells on day 0 (IV/SC). On day 6, spleen (A.) and lymph node (B.) cells from individual mice were restimulated with media or SIY peptide in an IFN-γ ELISPOT assay (*p=<0.05 for comparison of C1498.SIY SC versus IV and IV/SC). **C)** Control C1498 cells were inoculated into mice (3/group) as in A. On day 6, lymph node cells were restimulated with media or irradiated C1498 cells in an IFN-γ ELISPOT assay (*p < 0.05 for comparison of C1498 SC versus IV and IV/SC). **D)** Indicated numbers of C1498.SIY cells were introduced IV into mice (2-3/group) on day -6, and 10^6 C1498.SIY cells were inoculated SC on day 0. Controls received C1498.SIY cells (10^6) IV or SC on day 0. On day 6, spleen cells were restimulated as in A. in an IFN-γ ELISPOT assay (*p < 0.05 for comparison between mice challenged with C1498.SIY cells SC and all other groups). **E)** Mice (3/group) received C1498.SIY cells as in A. A fourth group received C1498.SIY cells SC in one flank on day -6, followed by C1498.SIY cells SC in the opposite flank on day 0. (SC/SC). An IFN-γ ELISPOT analysis was performed on day 6 (*p < 0.05 for comparison of C1498.SIY SC versus IV and IV/SC).

Figure 2 Continued: F) Live or irradiated C1498.SIY cells were inoculated IV, SC or IV/SC into mice (3/group) as in A., and an IFN-γ ELISPOT assay was performed on day 6 (*$p < 0.05$ for comparison of C1498.SIY SC (live) versus IV and IV/SC, and also for C1498.SIY SC (irrad) versus IV (irrad) and IV (irrad)/SC (live)). Data are representative of 2-3 experiments.

T cell dysfunction in mice bearing IV C1498.SIY cells occurs in an antigen-specific manner.

To determine whether the T cell dysfunction induced by IV C1498.SIY cells was specific to the antigens expressed on the tumor cells, 2 experiments were performed. First, groups of C57BL/6 mice were challenged IV with either C1498.GFP or C1498.SIY cells on day –6. On day 0, these mice received SC C1498.SIY cells, and 6 days later, spleen cells from these mice were restimulated ex vivo with the SIY peptide in an IFN-γ ELISPOT assay. Surprisingly, IV inoculation of either C1498.GFP or C1498.SIY cells led to a severely blunted SIY-specific T cell response against a subsequent SC C1498.SIY cell inoculation **(data not shown)**. We speculated that a state of "shared tolerance" to unknown antigens on C1498 cells might explain this result. Thus, we next used a different cancer cell line expressing a different model antigen to determine whether T cell tolerance in IV-challenged mice was antigen specific. Groups of C57BL/6 mice were challenged with IV C1498.SIY cells on day –6 and received a subsequent SC challenge on day 0 with C1498.SIY cells or B16.OVA cells (B16 melanoma cells engineered to express the full-length chicken OVA protein). On day 6, spleen cells were restimulated ex vivo with either the SIY peptide or a K^b-restricted OVA-derived peptide (SIINFEKL) in an IFN-γ ELISPOT assay. SIY-specific $CD8^+$ T cell responses were reduced as before, whereas OVA-specific T cell responses remained intact **(Figure 3A)**. This result suggests that T cell dysfunction in mice inoculated with IV C1498.SIY cells occurred in an antigen-specific manner.

T cell dysfunction in mice harboring IV C1498.SIY cells is not reversed after depletion of Tregs or MDSCs.

As Tregs and MDSCs have been shown to suppress antitumor T cell responses in murine cancer models[127, 128], we sought to clarify whether they were regulating T cell dysfunction in mice harboring IV C1498.SIY cells. To address this possibility, Tregs and MDSCs were depleted from FoxP3-DTR mice, or via administration of an anti–Ly-6G Ab to C57BL/6 mice, respectively, which had received dual IV/SC C1498.SIY cell inoculation. Depletion of FoxP3$^+$ Tregs upon administration of diphtheria toxin to FoxP3-DTR mice did not restore functional SIY-specific T cell responses in C1498.SIY IV/SC dual-challenged mice **(Figure 3B)**, which suggests that Tregs were dispensable for the induction of T cell tolerance in this setting. Furthermore, although the anti–Ly-6G antibody effectively depleted splenic and LN CD11b$^+$Gr-1$^+$ cells **(data not shown)**, its administration did not reverse the T cell dysfunction induced in IV/SC C1498.SIY cell dual-challenged mice **(Figure 3C)**. Identical results were obtained when an anti–Gr-1 Ab was administered in vivo to deplete MDSCs **(data not shown)**. Thus, despite meaningful depletion of these potentially suppressive cell populations, functional SIY-specific T cell responses were not restored, which argues that neither Tregs nor MDSCs were required for the early induction of tolerance in mice with IV C1498.SIY cells.

Antigen-specific T cells proliferate, but fail to accumulate in hosts bearing IV C1498.SIY cells.

To further clarify the mechanism underlying the induction of T cell dysfunction in mice inoculated with IV C1498.SIY cells, we employed an adoptive transfer model using SIY antigen–specific TCR transgenic $CD8^+$ T cells (referred to herein as 2C T cells). Purified 2C T cells (4×10^6) were CFSE-labeled and adoptively transferred into C57BL/6 mice; 1 day later, mice received 10^6 C1498.SIY cells IV or SC, or no tumor as a control. At 6 days after C1498.SIY cell inoculation, the absolute numbers, percentages, and CFSE dilution profiles of 2C T cells present in spleens and LNs were analyzed **(Figure 4A–D)**. 2C T cells both proliferated and accumulated in spleens and LNs of mice receiving SC C1498.SIY cell challenge. In contrast, whereas 2C T cells from mice harboring IV C1498.SIY cells were induced to proliferate, they did not accumulate, and were recovered in significantly lower numbers than those in mice after SC C1498.SIY cell challenge **(Figure 4A)**. Functional analysis of 2C T cells from tumor-bearing mice revealed decreased production of IFN-γ by 2C T cells from mice with IV C1498.SIY **(Figure 4E)**. This difference was further accentuated by comparison of absolute numbers of IFN-γ–producing 2C T cells from mice challenged with IV versus SC C1498.SIY cells **(Figure 4F)**. Collectively, these data confirmed the results of experiments examining the endogenous response to an IV C1498.SIY challenge, and suggest that deletion and/or anergy of antigen-specific T cells may occur in hosts inoculated with IV C1498.SIY.

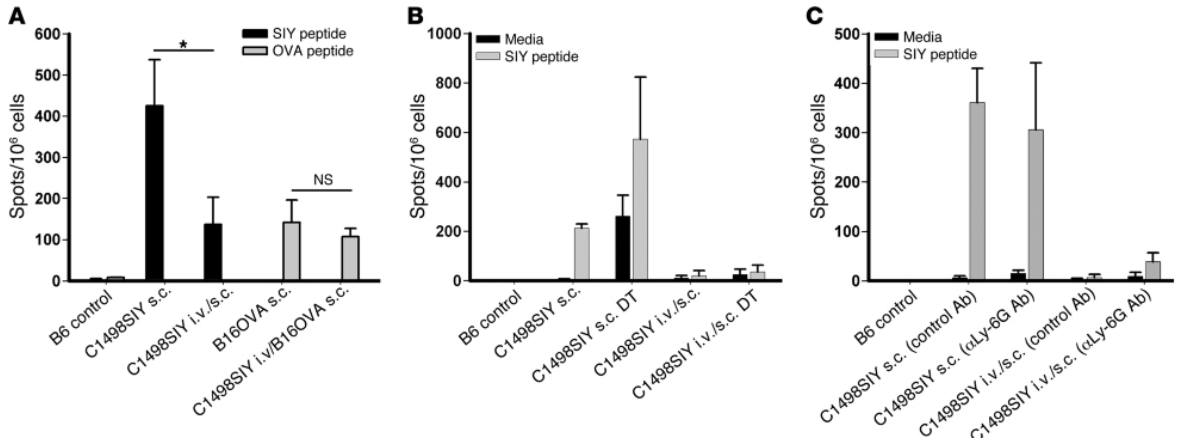

Figure 3. T cell dysfunction in mice after IV C1498.SIY cell inoculation is antigen specific, and is not regulated by Tregs or MDSCs. A) C57BL/6 mice received 10^6 C1498.SIY or B16.OVA cells SC only. Additional cohorts of mice received 10^6 C1498.SIY cells IV on day –6, followed by either C1498.SIY or B16.OVA cells SC on day 0. On day 6, spleen cells were restimulated with SIY or OVA peptide in an IFN-γ ELISPOT assay. *P < 0.05. **B)** FoxP3-DTR mice received C1498.SIY cells SC or IV/SC and were treated with diphtheria toxin (DT; 1 μg in 0.1 ml per mouse) or PBS as follows: SC C1498.SIY cell–challenged mice, days -2, -1, 0, 2, and 5; IV/SC C1498.SIY cell–challenged mice, days -8, -7, –4, -1, 2, and 5. On day 6, an IFN-γ ELISPOT assay was performed. **C)** C57BL/6 mice received SC or IV/SC C1498.SIY cells and received either the anti–Ly-6G Ab 1A8 or isotype control Ab (300 μg i.p. on days 0 and 3 for SC challenge and on days -6, -3, 0, and 3 for IV/SC challenge). On day 6, spleen cells were restimulated with media or SIY peptide in an IFN-γ ELISPOT assay. **(A–C)** Data are representative of 2 experiments with 3 mice/group.

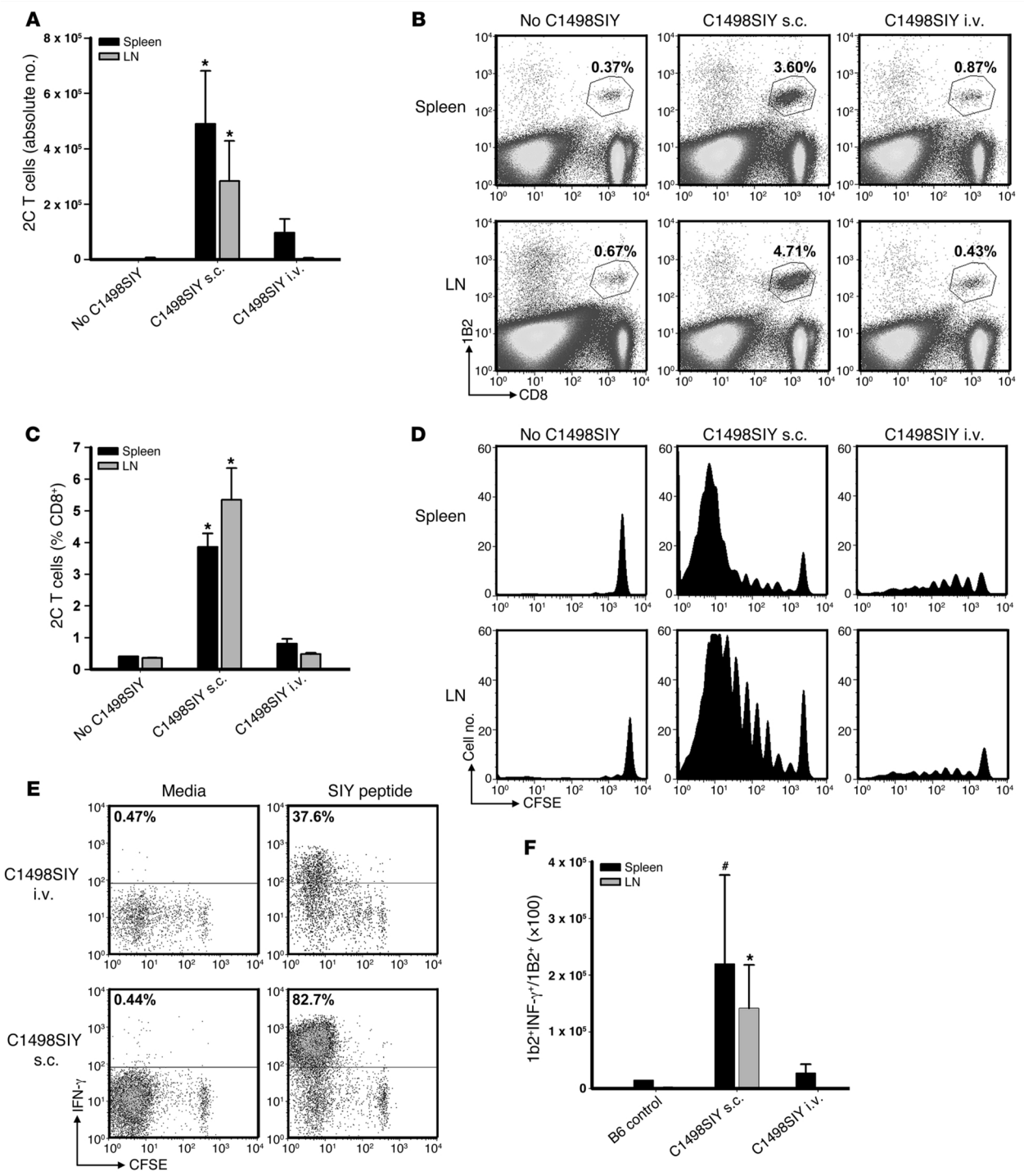

Figure 4. **SIY-specific 2C T cells undergo abortive peripheral tolerance in mice with IV C1498.SIY.** CFSE-labeled 2C T cells (4×10^6) were adoptively transferred into C57BL/6 mice, followed 1 day later by inoculation with IV or SC C1498.SIY.

Figure 4 Continued: A) On day 7, 2C T cells were enumerated. *p < 0.05, IV versus SC Data are representative of 4 experiments with 3–5 mice/group. **B)** Representative FACS plots from mice in A. Gated areas represent percent 2C T cells among the entire CD8 T cell population. **C)** Mean percent 2C T cells from mice in A. *p < 0.05, IV versus SC **D)** CFSE dilution of 2C T cells from mice in A. **E.** Mice received 2C T cells and C1498.SIY challenge as in A. On day 7, spleen and LN cells were restimulated with media or SIY peptide. Production of IFN-γ by 2C T cells was analyzed. Numbers represent percent IFN-γ 2C T cells. **F)** Numbers of IFN-γ–producing 2C T cells after IV or SC C1498.SIY cell challenge. # p = 0.10, * p < 0.05, IV versus SC **(E and F)** Data are representative of 3 experiments with 3 mice/group.

Transgenic expression of the anti-apoptotic BCL-X_L protein in 2C T cells restores their ability to accumulate in hosts harboring IV C1498.SIY cells.

The above data demonstrating the failure of 2C T cells to accumulate in hosts harboring disseminated C1498.SIY cells raised the possibility that they were being specifically targeted for deletion. To test this possibility, we interbred 2C mice with *Bcl-X_L* transgenic mice, in which Bcl-X_L expression is directed within the T cell compartment (referred to herein as $2C^{BCL\text{-}XL}$ mice). CFSE-labeled 2C or $2C^{BCL\text{-}XL}$ T cells were adoptively transferred into groups of C57BL/6 mice, which were inoculated the following day IV or SC with C1498.SIY cells, or remained tumor free. On day 6, the absolute numbers and extent of CFSE dilution of 2C versus $2C^{BCL\text{-}XL}$ T cells were analyzed. As shown in **Figure 5A**, the ability of 2C T cells to both proliferate and accumulate in mice inoculated with C1498.SIY cells IV was restored upon transgenic expression of Bcl-X_L. Both 2C and $2C^{BCL\text{-}XL}$ T cells failed to proliferate in leukemia-free hosts and were recovered in similar numbers **(Figure 5A and B)**, arguing against an intrinsic advantage of $2C^{BCL\text{-}XL}$ T cells to survive and proliferate after adoptive transfer into hosts in which their cognate antigen was not present. Furthermore, $2C^{BCL\text{-}XL}$ T cells were recovered in significantly higher numbers from mice with IV C1498.SIY cells compared with control 2C T cells **(Figure 5B)**, and $2C^{BCL\text{-}XL}$ T cells produced higher levels of IFN-γ and TNF-α than did control 2C T cells upon ex vivo restimulation with SIY peptide, although not to the level of control 2C T cells isolated from hosts with SC C1498.SIY cell challenge **(Figure 5C)**. When spleens and livers (a primary location of C1498 cell progression) of mice were analyzed 3–4 weeks after 2C versus $2C^{BCL\text{-}XL}$ adoptive transfer and IV C1498.SIY challenge, 6- and 20-fold increases in the percentage of $2C^{BCL\text{-}XL}$ versus 2C

T cells were observed in the livers and spleens, respectively **(Figure 5D)**. In fact, almost no 2C T cells could be identified in the spleens and livers of mice after IV C1498.SIY cell inoculation at this later time point. Together, these data argue that T cell deletion represents a potent mechanism of tolerance induced in hosts with AML. Despite restored accumulation and enhanced early effector function of $2C^{BCL\text{-}XL}$ T cells in mice harboring IV C1498.SIY cells, their adoptive transfer did not lead to improved control of leukemia cell progression or significantly enhanced survival compared with adoptive transfer of control 2C T cells **(data not shown)**. In fact, when analyzed at this later time point after IV C1498.SIY cell inoculation, $2C^{BCL\text{-}XL}$ T cells produced low levels of IFN-γ **(data not shown)**, which suggests that additional negative regulatory mechanisms are involved in leukemia-specific T cell tolerance during the course of disease progression.

Endogenous antigen-specific T cell responses are restored, and mouse survival is prolonged after administration of agonistic anti-CD40 Ab.

In other peripheral tolerance models, for example through the use of costimulatory ligand blockade[129], T cell deletion and anergy appear to operate in concert to induce and maintain the tolerant state. It seemed plausible that a similar process might be occurring with IV dissemination of leukemia, where antigen cross-presentation by immature host DCs might be occurring. Although $CD11c^{+}$ cells from spleens and LNs of mice after IV versus SC C1498.SIY cell inoculation did not differ significantly in their expression of MHC class I or classical costimulatory molecules **(data not shown)**, we nevertheless hypothesized that there might be a qualitative

defect in the ability of DCs from IV-challenged mice to functionally prime leukemia antigen-specific T cells. As CD40 ligation has previously been shown to activate DCs in vivo[130, 131, 132], we investigated whether administration of an agonistic anti-CD40 Ab in mice inoculated with IV C1498.SIY cells would restore T cell activation and persistence, leading to improving leukemia control and mouse survival. In the 2C T cell adoptive transfer system, anti-CD40 treatment led to a markedly enhanced ability of 2C T cells to proliferate and accumulate in hosts harboring IV C1498.SIY cells **(Figure 6A)**, which suggests that deletion of antigen-specific T cells was prevented. CD40 ligation also led to markedly enhanced production of IFN-γ and TNF-α by antigen-specific 2C T cells **(Figure 6B)**.

We then examined the effect of anti-CD40 mAb on the endogenous T cell response to IV C1498.SIY cells. Anti-CD40 mAb induced markedly higher frequencies and absolute numbers of endogenous SIY-specific $CD8^+$ T cells in C57BL/6 mice with IV C1498.SIY cell challenge compared with those seen in isotype control Ab–treated mice **(Figure 6C and data not shown)**. In contrast, anti-CD40 treatment had no significant effect on the frequency of SIY-reactive $CD8^+$ T cells in mice after SC C1498.SIY cell challenge **(Figure 6C)**. Similarly, functional SIY-specific T cell responses were strikingly enhanced in mice after IV C1498.SIY cell inoculation and anti-CD40 treatment. Again, anti-CD40 treatment did not significantly augment the already robust functional SIY-specific T cell responses that occurred naturally after SC C1498.SIY cell challenge **(Figure 6D)**. Furthermore, anti-CD40 treatment prevented the T cell tolerance induced by IV C1498.SIY cell inoculation in IV/SC C1498.SIY cell dual-challenged mice, as measured by functional SIY-specific T cell responses **(Figure 6E)**.

In keeping with augmented SIY-specific T cell responses, significantly prolonged survival, and in some cases, disease cure, was observed in mice after IV C1498.SIY cell inoculation and treatment with anti-CD40 mAb **(Figure 6F)**, even when IV C1498.SIY cells were established 8 days prior to initiation of anti-CD40 treatment **(Figure 6G)**. To determine whether anti-CD40 treatment could prolong survival in a second transplantable AML model, groups of C57BL/6 mice received IV challenge with FBL cells. Because of the aggressive nature of FBL (death within 2.5 weeks of IV challenge with 10^5 FBL cells), C57BL/6 mice were inoculated with IV FBL cells and treated with anti-CD40 or isotype control Ab 5 days later. Similar to what was observed in the C1498 model, anti-CD40 treatment of C57BL/6 mice harboring IV FBL cells led to an impressive prolongation of survival **(Figure 6H)**. Collectively, these results argue that the T cell tolerant state generated in mice with IV C1498.SIY cells is likely regulated by tolerogenic host APCs, in a way that can be prevented and, more importantly, reversed in vivo after treatment with an agonistic anti-CD40 mAb.

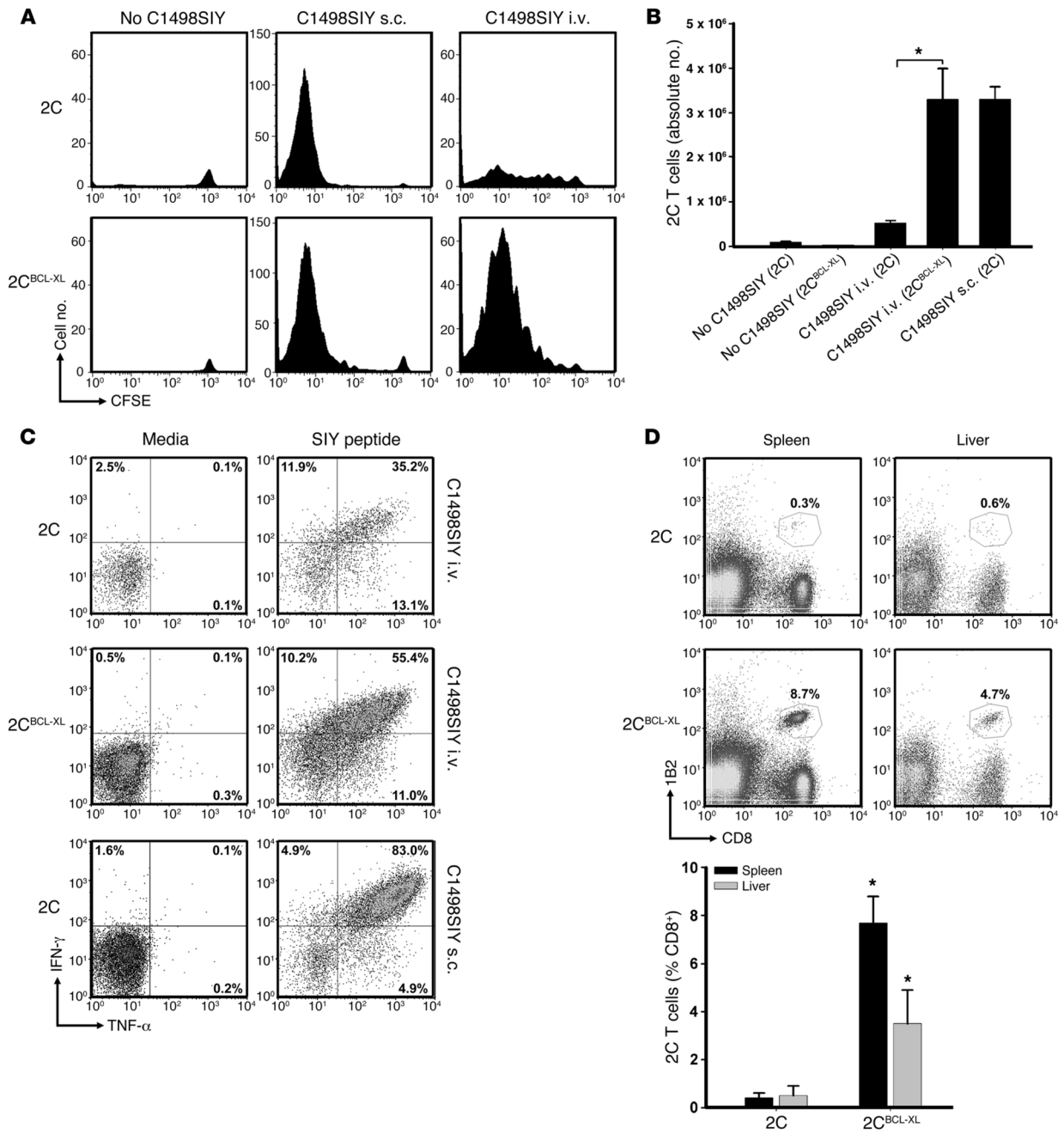

Figure 5. Transgenic expression of Bcl-X_L in 2C T cells rescues them from deletion in hosts with IV C1498.SIY cells. A) CFSE-labeled 2C or 2C $^{BCL\text{-}XL}$ T cells were transferred into C57BL/6 mice. On day 1, mice received IV or SC C1498.SIY cells. On day 7, CFSE dilution of splenic 2C and 2C $^{BCL\text{-}XL}$ cells was analyzed. Representative CFSE dilution profiles are shown. **B)** Absolute numbers of 2C T cells in spleens of mice in A. *$p < 0.05$.

Figure 5 continued: C) 2C or $2C^{BCL\text{-}XL}$ T cells were transferred into mice and subsequently challenged with IV or SC C1498.SIY cells as in A. On day 7, spleen cells were restimulated with media or SIY peptide, and production of IFN-γ and TNF-α was analyzed. Numbers represent percent 2C T cells producing the indicated cytokines. **B and C)** are representative of 2 experiments with 3 mice/group. **D.** Percent 2C and $2C^{BCL\text{-}XL}$ T cells in spleens and livers of mice 24 days after IV C1498.SIY cell challenge. Representative plots are shown. Gated areas represent percent 2C or $2C^{BCL\text{-}XL}$ T cells among the entire $CD8^+$ T cell population. Mean percent 2C and $2C^{BCL\text{-}XL}$ T cells in groups of 3 mice are also shown. *$p < 0.05$, 2C versus $2C^{BCL\text{-}XL}$. Data are representative of 2 experiments.

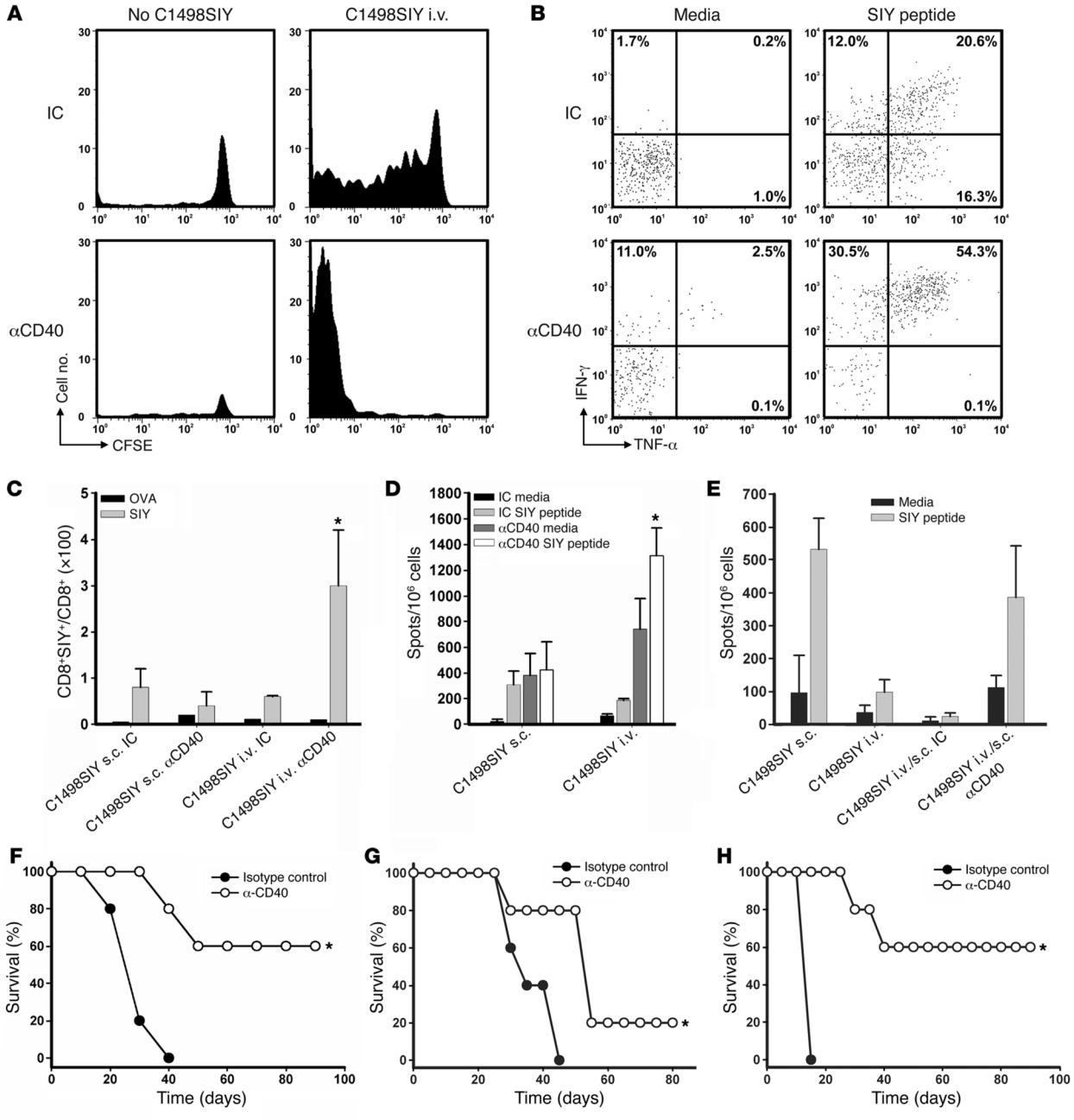

Figure 6. **Agonistic CD40 ligation prevents T cell deletion, priming large numbers of activated T cells, in mice harboring C1498.SIY cells IV A)** CFSE dilution of 2C T cells 7 days after transfer into C57BL/6 mice challenged with IV C1498.SIYcells and treated with anti-CD40 or isotype control Ab(IC). **B)** Splenocytes from mice in A) were restimulated with media or SIY peptide, and IFN-γ and TNF-α production by 2C T cells was assessed. Numbers represent percent cytokine-producing 2C T cells.

Figure 6 Continued: C) C57BL/6 mice received IV or SC C1498.SIYcells and were treated with anti-CD40 and the number of $SIY^{+}CD8^{+}$T cells was analyzed. A negative control OVA tetramer was also used. *p < 0.05 versus all other groups. **(D)** IFN-γ ELISPOT analysis of splenocytes from mice in C. *p < 0.05 versus control Ab. **E)** C57BL/6 mice received C1498.SIY cells IV on day –6 and were treated with anti-CD40 or isotype control Ab on days –6 and –3. On day 0, these mice were challenged with C1498.SIY cells SC. Control mice received C1498.SIY cells IV or SC on day 0 only. IFN-γ ELISPOT analysis was performed on day 6. **F)** C57BL/6 mice received C1498.SIY cells IV On days 0, 2, and 4, anti-CD40 or isotype control Ab was administered, and survival was assessed. *p = 0.002 versus control Ab. **G)** C57BL/6 mice received IV C1498.SIY cells on day 0. On days 8, 10, 12, 17, 22, and 27, anti-CD40 or CD40 or isotype control Ab. *p = 0.05 versus control Ab. **H)** C57BL/6 mice received FBL cells IV on day 0. On days 5, 7, 9, 13, and 18, anti-CD40 or isotype control Ab was administered, and survival was assessed. *p < 0.05 versus control Ab. Data are representative of 2 independent experiments with 3 (A–E) or 5 (F–H) mice/group.

Conclusions

Here, we have identified a unique immune evasion mechanism in which leukemia-specific $CD8^+$ T cells undergo abortive proliferation and are ultimately deleted from mice harboring systemic AML. The T cells which escape deletion appear dysfunctional and produce low levels of the effector cytokines IFN-γ and TNF-α. We postulated that leukemia-specific T cell tolerance could be mediated by direct interactions between AML cells and $CD8^+$ T cells, as C1498.SIY cells express SIY and $H\text{-}2K^b$. Alternatively, host APCs could be driving the tolerant phenotype by cross-presenting leukemia-derived antigens in a context not favorable for T cell activation. The fact that treatment of mice harboring systemic AML with an agonistic anti-CD40 mAb was able to prevent the T cell tolerant state suggested that host APCs may ultimately be regulating T cell tolerance. We therefore sought identify the APC(s) which promote T cell tolerance in leukemia-bearing mice, with the understanding that targeting the maturation of this APC subset may provide therapeutic benefit in mice with AML.

RESULTS II: CD8α$^+$ DCS INDUCE LEUKEMIA-SPECIFIC T CELL TOLERANCE

Summary

Antigen presenting cells (APCs) are critical for the acquisition of tumor-derived antigens and the orchestration of anti-tumor T cell responses. Batf3-dependent CD8α$^+$ and CD103$^+$ dendritic cells (DCs) have been implicated as the critical APCs that initiate and maintain spontaneous CD8$^+$ T cell priming against solid tumors. In contrast, little is known about the APCs that regulate immunity against malignancies of hematopoietic origin. Using a murine model of acute myeloid leukemia (AML) previously shown to induce a dense T cell tolerant state, we demonstrate that leukemia-derived antigens are exclusively acquired and cross-presented by CD8α$^+$ DCs. In the steady state, CD8α$^+$ DCs rapidly induce leukemia-specific T cell tolerance, which can be prevented upon their maturation by engagement of TLR3. Together, our data reveal that the same DC lineage can imprint disparate T cell fates in mice with solid verses hematopoietic malignancies. In the context of solid tumors, Batf3-dependent DCs stimulate productive effector responses; however, in mice harboring disseminated AML, CD8α$^+$ DCs actively drive T cell tolerance.

Introduction

Acute myeloid leukemia (AML) develops in the bone marrow but progresses to a systemic disease with a growth rate and pattern quite different than those of a typical solid malignancy. Because of its disseminated nature and lack of a classical tumor draining lymph node, the mechanisms that regulate immunity verses tolerance to AML may be distinct from those observed in solid cancers[103]. While much progress has been made in identifying immune evasion mechanisms activated in solid tumors[55], the mechanisms of immune escape employed by AML and other systemic hematological cancers have only begun to be characterized[110, 133, 134]. Recently, we identified a unique $CD8^+$ T cell tolerant state in mice with disseminated leukemia in which antigen-specific T cells underwent abortive proliferation and were rapidly deleted. Deletion of leukemia-specific $CD8^+$ T cells in this setting occurred independently of regulatory T cells and myeloid-derived suppressor cells, and could be prevented through conditional over-expression of the anti-apoptotic protein Bcl-X_L in T cells[111]. Moreover, the few leukemia-specific $CD8^+$ T cells that escaped deletion were functionally impaired, consistent with a hyporesponsive or anergic state. Interestingly, administration of an agonistic anti-CD40 antibody could reverse T cell tolerance and restore functional immunity against AML antigens. This result suggested that host antigen presenting cells (APCs) were driving the T cell tolerant phenotype; however, a role for direct antigen presentation by leukemia cells in mediating T cell tolerance was not excluded. Furthermore, the identity of the APC(s) that acquire and present leukemia-derived antigens to potentially promote T cell tolerance was unknown.

Dendritic cells (DCs) are a heterogeneous population of APCs with the characteristic ability to either coordinate adaptive immune responses or mediate the induction of T cell tolerance, depending on specific environmental cues. DCs resident to secondary lymphoid tissues are categorized as either plasmacytoid (pDCs) or conventional DCs (cDCs) based on unique developmental, functional, and transcriptional profiles[19]. The cDC population can be further divided based on differential expression of CD8α[21, 22]. $CD8\alpha^+$ DCs are highly efficient at the presentation of exogenous antigens on major histocompatibility complex (MHC) class I molecules - a phenomenon known as antigen cross-presentation[17, 24, 26]. In contrast, $CD8\alpha^-CD11b^+$ cDCs are proficient at presenting antigens to $CD4^+$ T cells in the context of MHC class II molecules[26, 31]. Until recently, the function of the $CD8\alpha^+$ DC subset has been difficult to study in isolation, as many transcription factors which control $CD8\alpha^+$ DC development are shared among DC subsets. However, the discovery that mice deficient in the basic leucine zipper transcription factor ATF-like3 (Batf3) have substantially reduced numbers of $CD8\alpha^+$ DCs, has facilitated investigation into the role of this DC subset in regulating immunity[33]. Anti-viral and anti-tumor $CD8^+$ T cell responses against immunogenic solid cancers are severely diminished in *Batf3*$^{-/-}$ mice, demonstrating the importance of $CD8\alpha^+$ DCs in cross-priming $CD8^+$ T cells in vivo.

Primary and metastatic human tumors are often spontaneously infiltrated with immune cells, and in some cancers, the density of intra-tumoral T cells has been correlated with improved prognosis[57]. Therefore, there has been considerable interest in identifying the APCs that acquire and present tumor-derived antigens to initiate and maintain anti-tumor T cell responses both in the tdLN and the tumor environment.

Recently, Batf3-dependent DCs have been implicated in $CD8^+$ T cell priming against solid tumor antigens. For example, type I IFN signaling in $CD8\alpha^+$ DCs was required for spontaneous priming of tumor antigen-specific $CD8^+$ T cells in an orthotopic melanoma model[60]. Furthermore, several groups have reveled defective cross-priming and rapid outgrowth of immunogenic solid tumors in *Batf3*$^{-/-}$ mice[33, 59, 60, 67]. Overall, these data suggest that Batf3-dependent DCs are critical for in generating and maintaining $CD8^+$ T cells responses against solid tumors.

In contrast to solid malignancies, the APCs which orchestrate adaptive immune responses against disseminated hematopoietic cancers have not been defined. In this study we sought to identify the APC(s) involved in the regulation of leukemia-specific $CD8^+$ T cell responses, particularly those which were presumably inducing leukemia-specific T cell tolerance.

Results

$CD11c^+$ cells acquire AML-derived proteins in vivo

We have previously described a unique $CD8^+$ T cell tolerant state in mice with systemic AML, in which leukemia-specific T cells encountered antigen, proliferated, but were ultimately deleted from the host. Interestingly, when the same AML cells were allowed to grow as a localized tumor, a robust leukemia antigen-specific $CD8^+$ T cell response ensued, demonstrating that host immune responses against localized versus disseminated tumor cells are markedly different[111]. It was initially presumed that disseminated leukemia cells might induce T cell tolerance through direct antigen presentation to peripheral $CD8^+$ T cells, which might be expected to result in deletion as has been seen in systems of TCR ligation without costimulation[8]. However, T cell tolerance in animals with systemic AML could be reversed upon administration of an agonistic anti-CD40 antibody, implicating host APCs as the cellular mediators of T cell tolerance[111]. To identify APC(s) capable of engulfing circulating AML cells or cellular material in vivo, and thus those which could be regulating the T cell tolerant state, C1498 AML cells were labeled with a fluorescent protein-binding dye, CellTrace Violet (CTV), and were inoculated IV into C57BL/6 mice. Three hours later, spleens were analyzed by flow cytometry for CTV fluorescence within known APC populations. As shown in **Figure 7**, CTV fluorescence was observed almost exclusively within the $CD11c^+$ cell compartment **(Figure 7A and 7B)**. When the total population of splenic CTV^+ cells was analyzed following IV C1498 cell inoculation, ~75% were $CD11c^+$, while only ~5% were $CD11b^+$ **(Figure 7C)**. As a non-tumor control, CTV-labeled, syngeneic $CD4^+$ T cells were inoculated similarly and no CTV signal was identified within $CD11c^+$

or CD11b$^+$ cell populations. **(Figure 7A, middle panels)**. Furthermore, no significant CTV signal was identified among MHC class II$^+$ cells in the lung or liver, indicating that acquisition of AML cellular material occurred largely within the spleen **(data not shown)**. It was important to evaluate whether the CTV signal derived from labeled AML cells was truly acquired by CD11c$^+$ cells as opposed to representing cellular conjugates or fragments of fused plasma membranes. To this end, single cell imaging was performed using the Amnis ImageStream instrument. Indeed, the CTV fluorescence signal visualized was intracellular, indicating that CD11c$^+$ cells actually took up AML-derived cellular material in vivo **(Figure 7D)**. Taken together, these results demonstrate that splenic CD11c$^+$ cells are those which predominantly acquire circulating AML cellular material in vivo.

Because we observed a high degree of uptake of leukemia-derived material by three hours post injection, we hypothesized that this uptake by CD11c$^+$ cells may be due to overt cell death by our AML cell line. However, either by trypan blue exclusion or propidium iodide and Annexin V staining, only ~5% of AML cells were undergoing apoptosis/necrosis in culture **(Figure 8A)**. If dead AML cell material was being selectively acquired by CD11c$^+$ cells, we hypothesized that chemotherapy treatment to induce death of cultured AML cells prior to their IV inoculation would result in enhanced uptake by splenic CD11c$^+$ cells. As expected, treatment of C1498 cells with gemcitabine led to a time-dependent increase in the frequency of cell death, as shown by PI and annexin V staining **(Figure 8A)**. Strikingly however, the IV inoculation of dead AML cells resulted in a dramatic increase in their uptake within the CD11b$^+$ compartment. This

result suggested that AML cell engulfment by $CD11c^+$ cells occurred independently of overt cell death **(Figure 8B and C)**.

$CD8\alpha^+$ DCs selectively acquire leukemia cell-derived material in vivo

Splenic DCs can be subdivided into distinct subsets based on defined cell surface markers. cDCs express high levels of CD11c, and can be further delineated based on presence or absence of CD8α expression, whereas pDCs are characterized by a $CD11c^{int}$Siglec-H^+ phenotype. To determine whether a specific $CD11c^+$ population was uniquely capable of leukemia cell phagocytosis, or whether this property was shared among different DC subsets, additional flow cytometric phenotyping was performed following IV inoculation of CTV-labeled C1498 cells. As shown in **Figure 9A**, CTV fluorescence was not associated with pDCs. Interestingly, among the cDC populations, CTV fluorescence was entirely restricted to the $CD8\alpha^+$ compartment **(Figure 9A and B)**. ImageStream analysis confirmed that leukemia cell-derived CTV fluorescence was found exclusively within $CD8\alpha^+$ DCs **(Figure 9C)**. A phenotypic analysis of the $CD8\alpha^+$ DCs that engulfed AML cells revealed co-expression of DEC-205 and DNGR-1, as well as high levels of MHC class I and II, but not CD4 or Sirpα **(Figure 9D and data not shown)**. This property of uptake of tumor material by $CD8\alpha^+$ DCs was not restricted to C1498 cells, as similar results were obtained with a second independent AML cell line, FBL **(Figure 10A)**, and also was observed with IV-inoculated B16 melanoma cells **(Figure 10B)**. These data indicate that uptake by $CD8\alpha^+$ DCs is a general occurrence with circulating tumor cells.

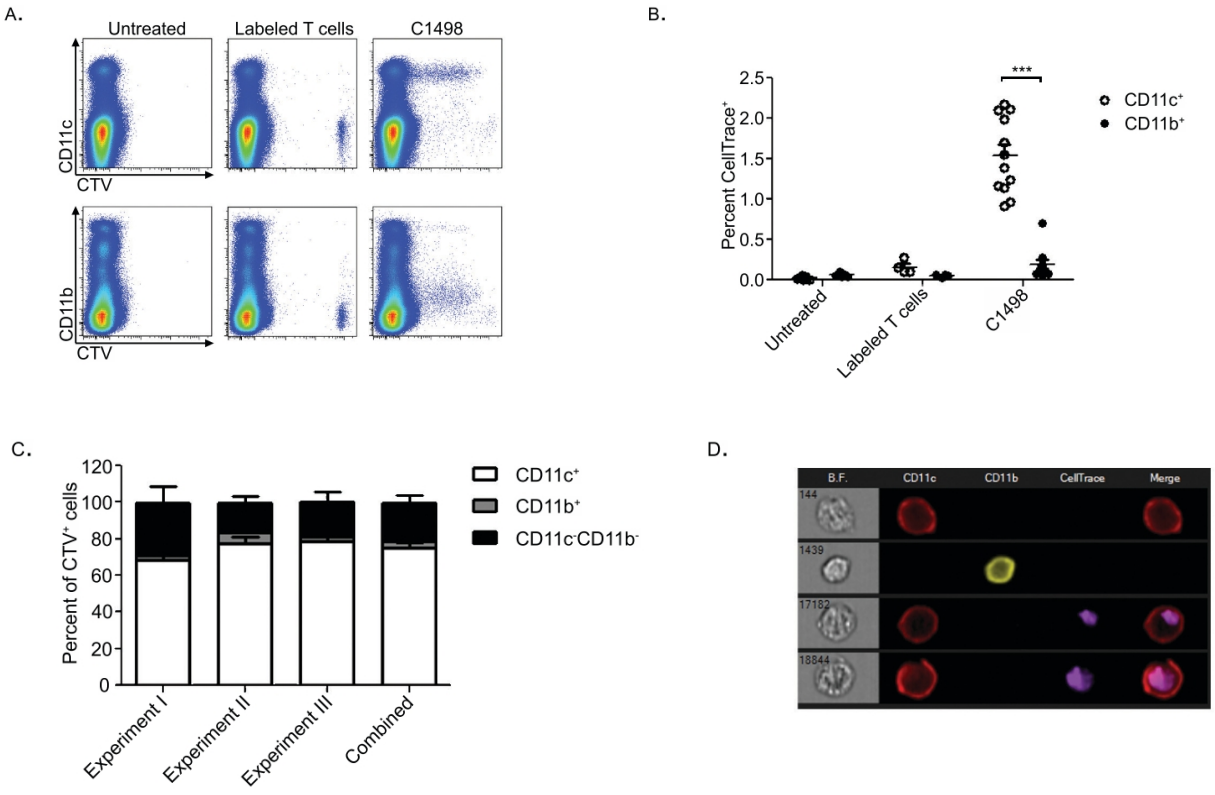

Figure 7. CD11c$^+$ cells acquire AML-derived proteins in vivo. (A-C) 1 x 10^6 syngeneic CD4$^+$ T cells or 4 x 10^6 C1498 AML cells were labeled with CTV and inoculated IV into C57BL/6 mice. Three hours later, splenic CD11b$^+$ or CD11c$^+$ cells were analyzed for CTV fluorescence. In A, representative plots are shown after gating on viable cells. In C, gating was performed on all CTV$^+$ events and shown is the expression of CD11c or CD11b on the population of CTV$^+$ cells. **D)** Three hours after IV inoculation of CTV-labeled C1498 cells, CTV fluorescence was analyzed via Image Stream cytometry. Data are pooled from (B and C) or representative of (A and D) at least 3 independent experiments, and are shown as mean ± SEM. *** $p<0.001$

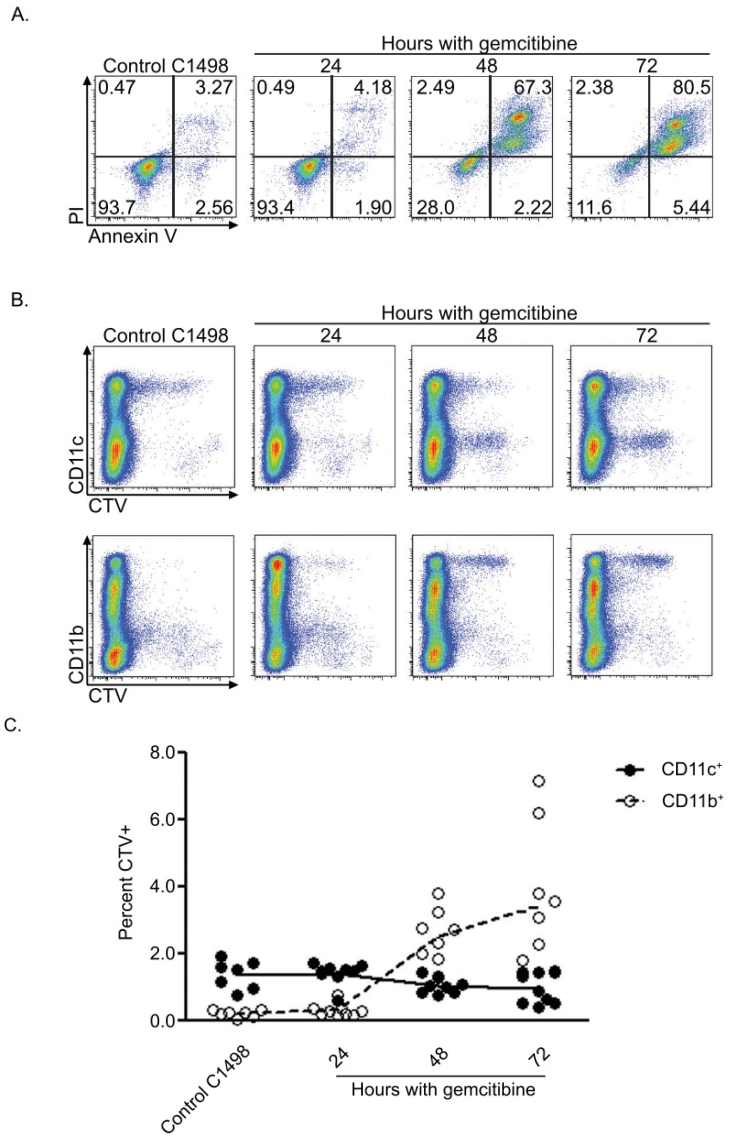

Figure 8. Gemcitabine-treated AML cells are engulfed by CD11b^{+} cells. C1498 cells were treated with 10μM gemcitabine for 24-72 hours prior to labeling with CTV and inoculation into C57BL/6 mice. **A)** C1498 cell death was confirmed via staining with propidium iodide (PI) and Annexin V. **B and C)** Three hours post injection, spleens were harvested and stained with antibodies against CD11c and CD11b. FACS plots shown are gated on CD3^{-}CD19^{-} cells. Data are representative of, or combined from 3 independent experiments.

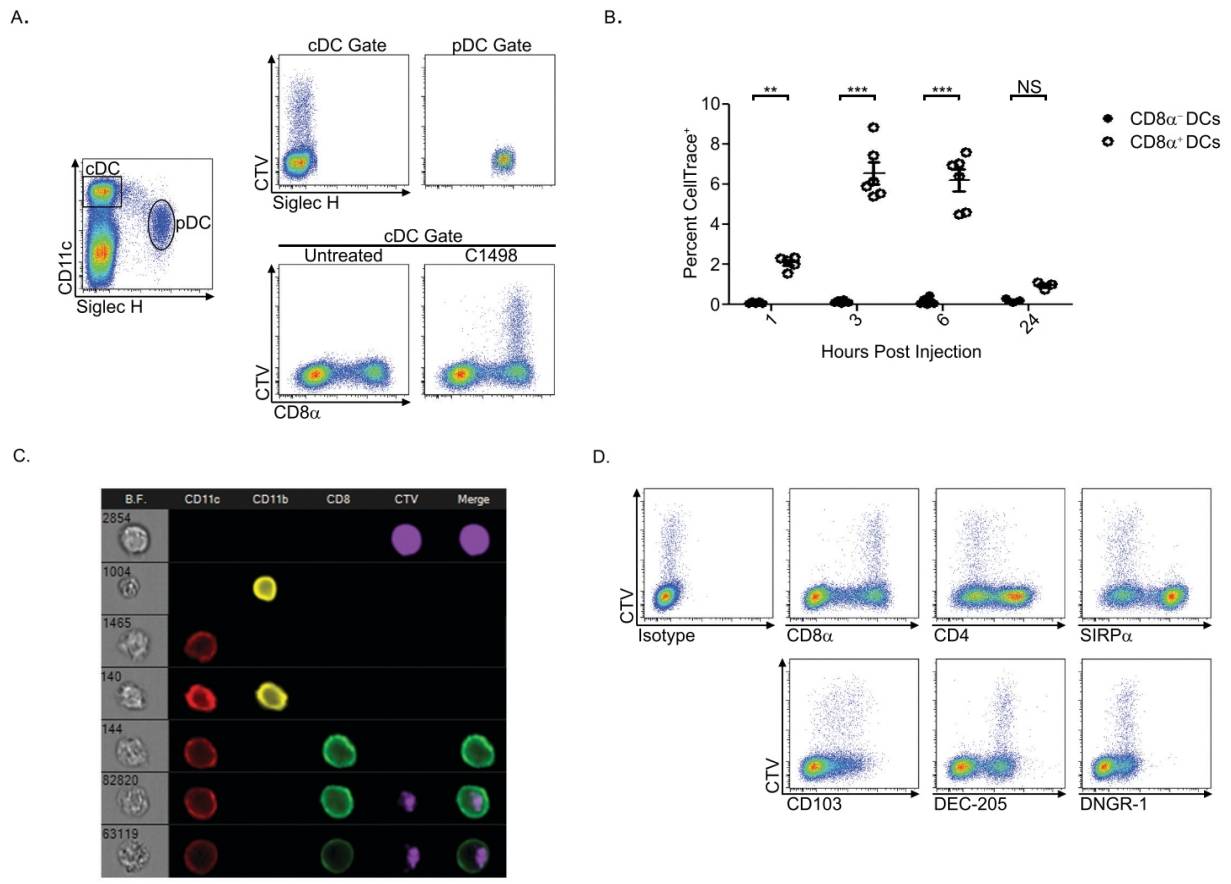

Figure 9. CD8α$^+$ DCs selectively acquire leukemia cell-derived material in vivo. (A and B) 4 x 10^6 CTV-labeled C1498 cells were inoculated IV into C57BL/6 mice. At the various time points indicated in (B), splenic DC subsets were analyzed for CTV fluorescence. Shown in A are plots representative of DCs analyzed three hours following leukemia cell inoculation. C) Image steam analysis of DC subsets three hours after CTV-labeled C1498 cell injection. The top row depicts a whole tumor cell in the spleen of an IV-challenged C57BL/6 mouse. Rows 2-5 depict a CD11c$^+$ cell, a CD11b$^+$ cell, a CD11c$^+$CD11b$^+$ cell and a CD8α$^+$ DC, all negative for CTV fluorescence. The last two rows are representative images of CD8α$^+$ DCs which contain a clear intracellular CTV fluorescent signal. D) The cell surface phenotype of CTV$^+$ splenic cDCs analyzed three hours after CTV-labeled C1498 inoculation is depicted in representative plots. Data are pooled (B) or representative (A, C and D) of at least 2 independent experiments, and are shown as mean ± SEM. ** p <0.01, *** p<0.001, NS - Not significant.

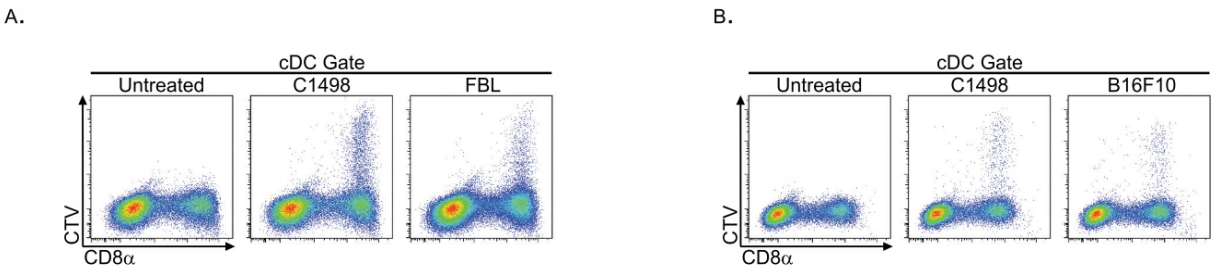

Figure 10. CD8α^{+} DCs phagocytose circulating tumor cells. A) 4 x 10^{6} CTV-labeled C1498 or friend virus-induced erythroleukemia (FBL) cells were inoculated IV and three hours later splenic DC populations were analyzed for CTV fluorescence. **B)** 1 x 10^{6} C1498 or B16F10 melanoma cells were CTV-labeled and injected IV. Splenic DC populations were analyzed for CTV fluorescence three hours after injection. All plots are gated on live conventional dendritic cells. Data are representative of 2 or 3 independent experiments.

CD8α$^+$ DCs cross-present antigens derived from circulating leukemia cells

Given that CD8α$^+$ DCs exclusively acquired leukemia-derived cellular material, their ability to cross-present leukemia antigens to CD8$^+$ T cells was next examined. To that end, C1498 cells expressing the model H-2K^b-restricted SIY peptide antigen(C1498.SIY) were inoculated IV into groups of C57BL/6 mice. Three hours later, CD8α$^-$ and CD8α$^+$ DCs were FACS-purified and cultured with CTV-labeled, CD8$^+$ SIY-specific T cells (2C T cells). 2C T cell proliferation, measured by CTV dilution, was used as an indicator of leukemia antigen cross-presentation. Strikingly, CD8α$^+$, but not CD8α$^-$ DCs isolated from mice challenged with C1498.SIY cells induced the proliferation of 2C T cells in the absence of exogenous SIY peptide **(Figure 11A and B)**. When pulsed with SIY peptide prior to culture, both CD8α$^+$ and CD8α$^-$ DCs were equally effective at inducing 2C T cell proliferation, indicating that both populations are capable of supporting T cell division ex vivo **(Figure 11A, bottom)**. As expected, 2C T cells did not divide when cultured with CD8α$^-$ or CD8α$^+$ DCs isolated from mice challenged with parental C1498 cells (SIY negative) **(Figure 11A, top left)**.

We next aimed to characterize the cellular requirements for CD8α$^+$ DCs to support ex vivo 2C proliferation following systemic AML challenge. After phagocytosis, protein antigens are shuttled to the cytoplasm where they undergo proteasome-mediated degradation. Subsequently, peptides traffic back to an MHC I-containing organelle, either endosomes or the ER, via the transporter associated with antigen processing 1 (TAP1)[18, 135]. As shown in **Figure 11C and D**, 2C proliferation was severely blunted upon co-culture with CD8α$^+$ DCs isolated from IV C1498.SIY challenged *TAP1*$^{-/-}$ mice, indicating that a classical antigen cross-presentation pathway

requiring TAP1 was utilized by host CD8α$^+$ DCs to cross-present leukemia antigens. Furthermore, 2C stimulation mediated by CD8α$^+$ DCs from leukemia-bearing mice required their expression of H-2K^b, suggesting that direct acquisition of antigen-loaded MHC I molecules from leukemia cells by DCs was not occurring to any meaningful degree **(Figure 11E and F)**. These results demonstrate that CD8α$^+$ DCs are exclusive in their ability to acquire and cross-present leukemia-derived antigens, and suggests that they may be the cellular mediators of AML-specific T cell tolerance.

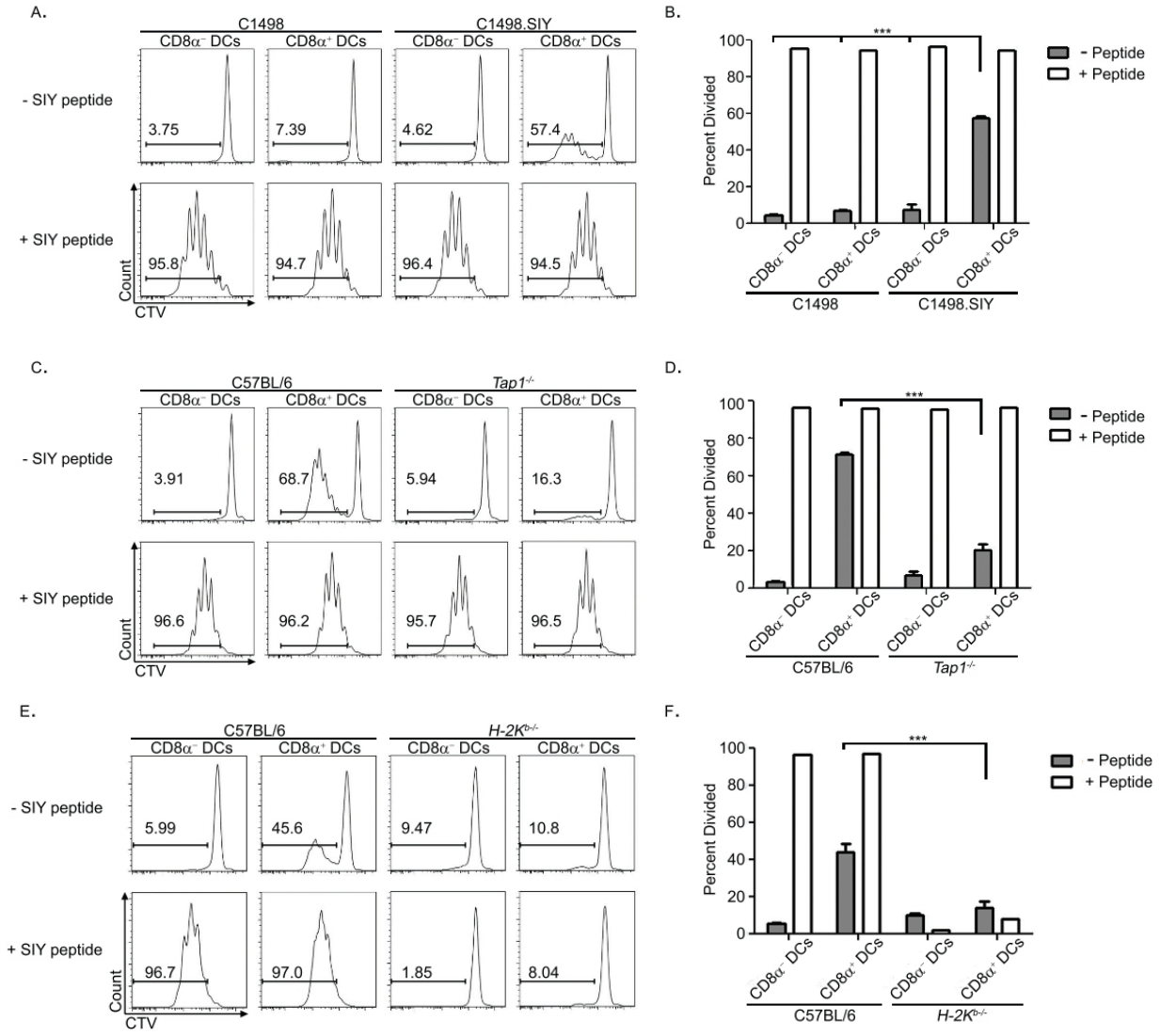

Figure 11. CD8α$^+$ DCs cross-present leukemia derived antigens. A and B) 4 x 10^6 C1498 or C1498.SIY cells were inoculated IV into C57BL/6 mice and three hours later, splenic CD8a$^-$ or CD8α$^+$ DC were FACS purified and cultured with CTV-labeled CD8$^+$ 2C T cells for 72 hours. Subsequently, 2C T cell division, assessed by CTV dilution, was monitored via flow cytometry. Where indicated, DCs were pulsed with 100nM SIY peptide prior to culture. Data are representative of more than 3 independent experiments and are shown as mean ± SEM. *** p<0.001. **C-F)** 4 x 10^6 C1498.SIY cells were inoculated into C57BL/6 and *Tap1*$^{-/-}$ **(C and D)** or *H-2K*$^{b-/-}$ **(E and F)** mice and cross-presentation assays were performed as in **A.** Data represent at least 2 independent experiments and are shown as mean ± SEM. *** p<0.001

CD8α$^+$ DCs mediate the recognition of leukemia antigens by CD8$^+$ T cells

It has recently been demonstrated that Batf3-dependent CD8α$^+$ and CD103$^+$ DCs are required for the spontaneous priming of functional CD8$^+$ T cell responses against solid tumors, which progress much more rapidly in Batf3-deficient hosts[33]. Our previous data has revealed that, similar to other solid tumor models, productive antigen-specific CD8$^+$ T cell responses are generated against SC C1498 cell tumors[111]. To confirm that Batf3-dependent DCs were also required for the priming of CD8$^+$ T cells against localized C1498 tumors, C57BL/6 or *Batf3*$^{-/-}$ mice were challenged with C1498.SIY cells SC. As expected, robust SIY-specific T cell responses were generated in C57BL/6 mice, as measured by IFN-γ production following SIY peptide re-stimulation **(Figure 12A and B)**. In contrast, numbers of functional SIY-specific CD8$^+$ T cells were significantly diminished in *Batf3*$^{-/-}$ mice harboring localized C1498.SIY tumors **(Figure 12A and B)**. In addition, adoptively-transferred leukemia-specific 2C T cells significantly expanded in C57BL/6 mice, but not in *Batf3*$^{-/-}$ mice, following SC C1498.SIY challenge **(Figure 12C and D)**. These data are consistent with previous reports, and demonstrate an essential role for Batf3-dependent DCs in priming CD8$^+$ T cell responses against localized tumors.

The observation that CD8α$^+$ DCs uniquely acquired and cross-presented leukemia antigens in animals with systemic AML suggested that in this setting, they may be inducing leukemia-specific T cell tolerance. CD8$^+$ T cell tolerance following IV leukemia challenge results from abortive T cell proliferation, where leukemia-specific T cells recognize their cognate antigen and divide, but are then rapidly deleted[111]. Thus, to monitor the fate of leukemia-specific CD8$^+$ T cells in the presence or absence of

CD8α$^+$ DCs, CTV-labeled CD8$^+$ 2C T cells were adoptively-transferred into C57BL/6 or *Batf3*$^{-/-}$ mice that were challenged with C1498.SIY cells IV one day later. As expected, 2C T cells proliferated in leukemia-bearing C57BL/6 mice **(Figure 12E and F)**, and many upregulated CD69, consistent with antigen encounter in vivo **(Figure 12G and H)**. Strikingly, following transfer into *Batf3*$^{-/-}$ hosts, most AML-specific T cells remained undivided **(Figure 12E and F)**, and far fewer expressed CD69 **(Figure 12G and H)**. To directly test if AML-specific CD8$^+$ T cells were encountering cognate antigen in *Batf3*$^{-/-}$ mice, we crossed 2C mice to Nur77^{GFP} reporter mice, where GFP expression is directly proportional to TCR signal strength[117]. Here again, GFP was upregulated upon IV tumor challenge in C57BL/6 mice, but not *Batf3*$^{-/-}$ mice **(Figure 12I)**. These data demonstrate that in the absence of host CD8α$^+$ DCs, the ability of leukemia-specific T cells to encounter cognate antigen in vivo was significantly impaired, consistent with a state of immunological ignorance. This result also revealed the relative inefficiency with which C1498.SIY cells and/or other APC populations presented the SIY antigen to 2C T cells in vivo. Furthermore, frequencies of endogenous **(Figure 12A and B)** and adoptively-transferred **(Figure 12C and D)** leukemia-specific CD8$^+$ T cells were similar in naïve and leukemia-bearing *Batf3*$^{-/-}$ mice, lending further support to the conclusion that CD8$^+$ T cells are largely ignorant of leukemia-derived antigens in the absence of CD8α$^+$ DCs. Lastly, in *Batf3*$^{-/-}$ mice, AML cell phagocytosis by another compensatory splenic DC or macrophage population was not observed **(Figure 13)**. Collectively, these data reveal that CD8α$^+$ DCs are required to mediate the recognition of leukemia antigens by CD8$^+$ T cells, and that in their absence, CD8$^+$ T cells remain effectively ignorant of a progressing leukemia.

CD8α$^+$ DCs tolerize leukemia-specific T cells in vivo

The results presented thus far strongly suggest that CD8α$^+$ DCs are the APC population which induces leukemia-specific T cell tolerance. If this is the case, then ignorant (naïve) leukemia-specific T cells in AML-bearing *Batf3*$^{-/-}$ mice would be expected to expand more vigorously than tolerized T cells in AML-bearing C57BL/6 mice following a secondary antigenic challenge. To test this hypothesis, we developed a vaccination strategy capable of stimulating a robust SIY-specific T cell response in leukemia-naïve mice that occurred independently of CD8α$^+$ DCs. As shown in **Figures 14A and B**, SIY peptide emulsified in incomplete Freund's adjuvant (IFA) induced an equivalent expansion of SIY-specific CD8$^+$ T cell in naïve C57BL/6 and *Batf3*$^{-/-}$ hosts. The frequency of SIY-reactive CD8$^+$ T cells was very minimally higher in leukemia-bearing C57BL/6 mice vaccinated with IFA plus SIY peptide compared to IFA alone, suggesting that an immunogenic vaccine was incapable of restoring antigen-specific CD8$^+$ T cell expansion in leukemia-bearing C57BL/6 mice **(Figure 14C and D)**. In stark contrast, SIY-specific CD8$^+$ T cells expanded significantly in leukemia-bearing *Batf3*$^{-/-}$ mice following SIY vaccination, similar to what was observed in naïve mice **(Figure 14B and D)**. Together, these data conclusively demonstrate that CD8α$^+$ DCs induce leukemia-specific T cell tolerance in vivo.

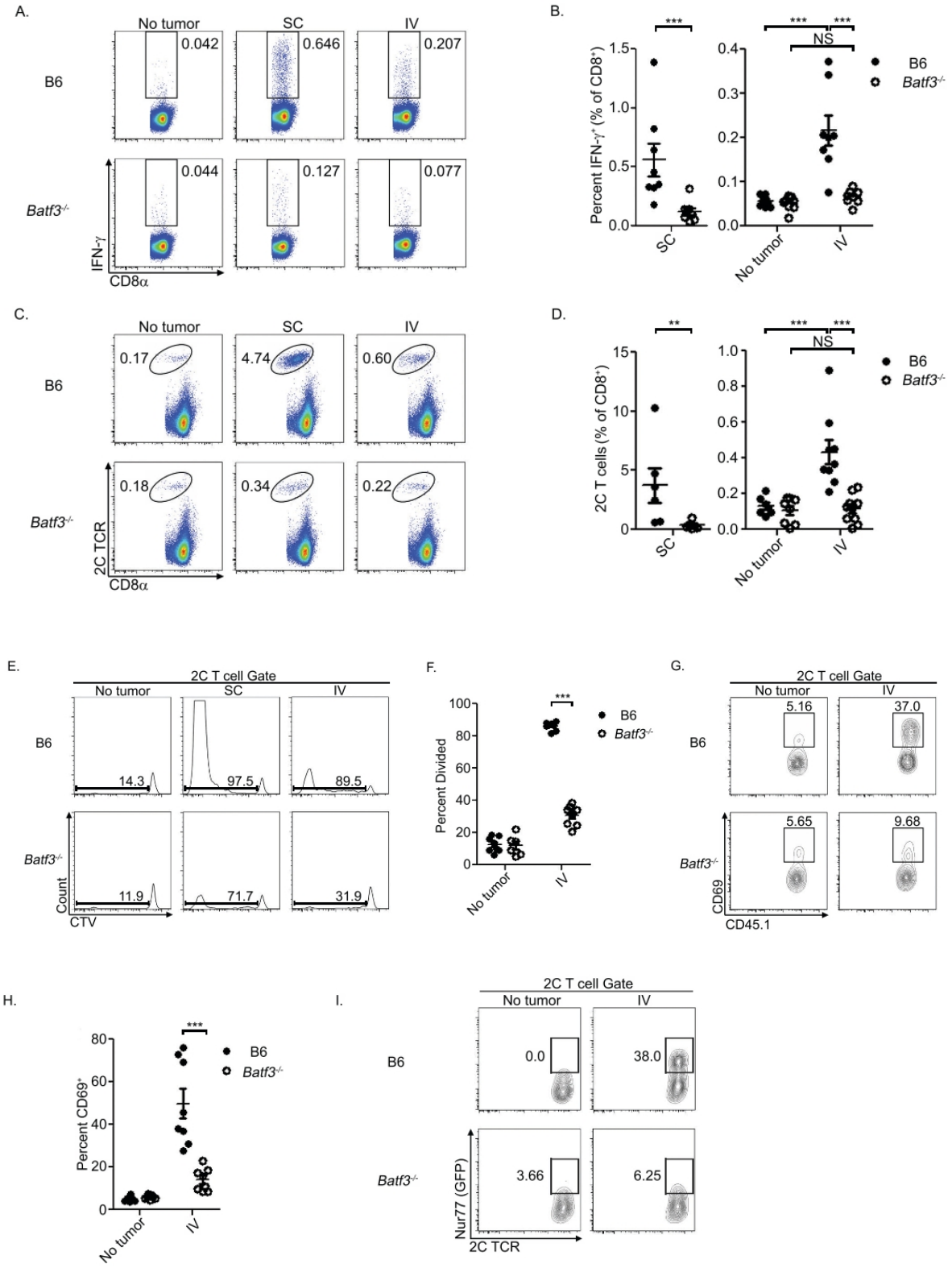

Figure 12. CD8α$^+$ DCs mediate the recognition of AML- derived antigens by CD8$^+$ T cells.

Figure 12 Continued: (A and B) 1 x 10^6 C1498.SIY cells were inoculated SC or IV into C57BL/6 and *Batf3*$^{-/-}$ mice. Six days later, IFN-γ production by endogenous SIY-specific T cells was analyzed after in vitro re-stimulation of spleen cells with SIY peptide. **(C-F)** 1 x 10^6 CTV-labeled CD8$^+$ 2C T cells were transferred into C57BL/6 or *Batf3*$^{-/-}$ mice which received 1 x 10^6 C1498.SIY cells one day later. Six days following tumor challenge, the frequency and division of the transferred 2C cells was monitored. The representative plots shown have been gated on CD8$^+$ T cells (C) or transferred CD8$^+$ 2C T cells (E) as described in Materials and Methods. **(G and H)** CD45.1$^+$ 2C T cells were transferred into B6 or *Batf3*$^{-/-}$ mice (CD45.2$^+$) followed by IV inoculation of C1498.SIY cells one day later. 36 hours after leukemia cell inoculation, CD69 expression on transferred 2C T cells was analyzed. **I)** 2C mice were crossed to Nur77^{GFP} reporter mice and 2C Nur77^{GFP} CD8$^+$ T cells were inoculated into C57BL/6 mice which received 10^6 C1498.SIY cells 1 day later. 24 hours post tumor inoculation, GFP expression was monitored via flow cytometry. **A-H) Data** are pooled from at least 3 independent experiments, and are shown as mean ± SEM. **I)** Data represent 2 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS - Not significant.

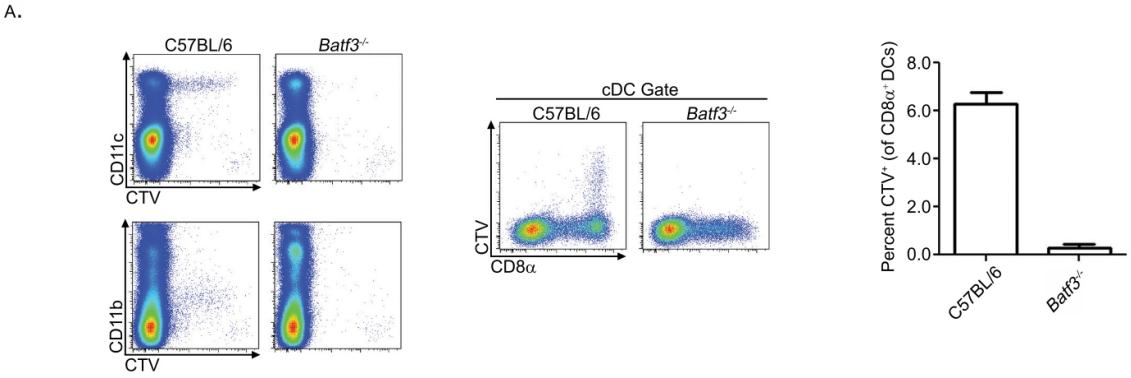

Figure 13. Lack of compensatory AML cell uptake by other APC populations in *Batf3*$^{-/-}$ mice. 4 x 10^6 C1498 cells were labeled with CTV and injected into groups of C57BL/6 or *Batf3*$^{-/-}$ mice. Three hours later, spleens were harvested and stained with CD11c, CD11b and CD8α. Plots in the left panel are gated on live cells and the middle panels is pre-gated on conventional DCs. Data represent or are combined from 2 independent experiments.

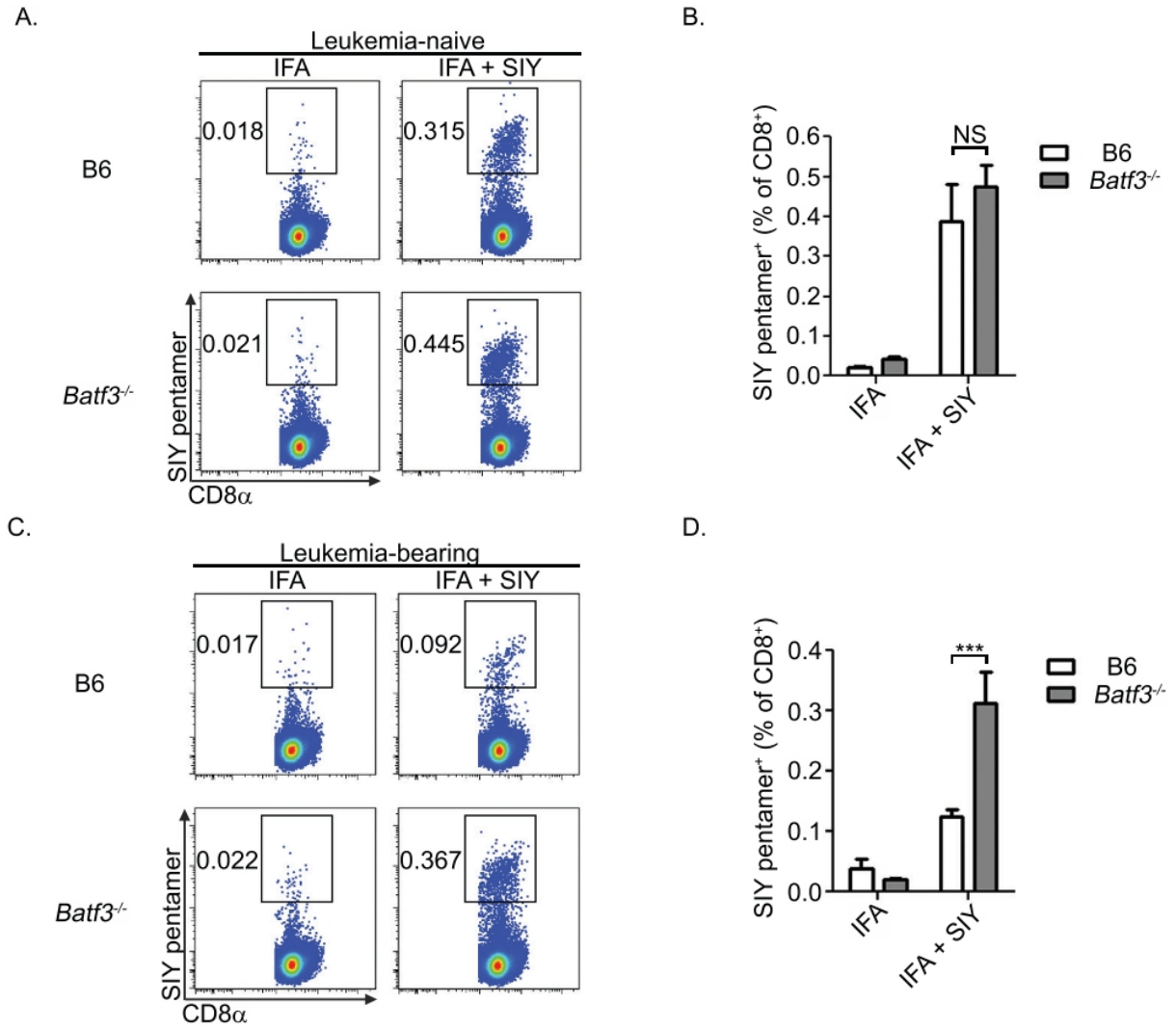

Figure 14. CD8α$^+$ DCs tolerize leukemia-specific T cells in vivo. A and B) Naïve C57BL/6 or *Batf3*$^{-/-}$ mice were vaccinated SC with IFA or IFA + SIY peptide on day 0. On day 5, the frequency of SIY pentamer-reactive CD8$^+$ T cells from the vaccine-draining lymph node was analyzed. Representative plots are shown in (A) and have been gated on CD8$^+$ T cells. **C and D)** C57BL/6 or *Batf3*$^{-/-}$ mice received 5 x 10^6 C1498.SIY cells IV on day 0. Six days later, mice were vaccinated with IFA or IFA + SIY peptide as above. On day 5 following vaccination, the frequency of SIY pentamer-reactive CD8$^+$ T cells from the vaccine-draining lymph node was analyzed. Representative plots are shown in **(C)** and have been gated on CD8$^+$ T cells. Data from B and D are pooled from two independent experiments, and are shown as mean ± SEM. *** p<0.001, NS - Not significant.

Activation of CD8α$^+$ DCs prevents AML-induced T cell tolerance

The fact that CD8α$^+$ DCs actively promoted T cell tolerance in mice with disseminated AML suggests that the maturation state of these DCs might regulate their ability to either activate or tolerize leukemia-specific T cells in vivo. To test the hypothesis that activation of CD8α$^+$ DCs in AML-bearing mice would prevent the tolerant T cell phenotype, we took advantage of their unique expression of pattern recognition receptors[19]. CD8α$^+$ DCs have been shown to express high levels of Toll-like receptor 3 (TLR3), which naturally recognizes viral double-stranded RNA[136]. In agreement with published results, we observed very high TLR-3 expression in CD8α$^+$ DCs, but not on other DC subsets **(Figure 15A)**. To stimulate TLR-3-expressing CD8α$^+$ DCs in vivo, the synthetic TLR-3 agonist, poly(I:C), was utilized. Administration of poly(I:C) promoted the generation of robust leukemia antigen-specific T cell responses, and prolonged survival in leukemia-bearing C57BL/6 mice, but not in *Batf3*$^{-/-}$ mice **(Figure 15B and C)**. Importantly, administration of poly(I:C) was also sufficient to prolong the survival of mice harboring parental C1498 AML, suggesting that TLR-3-induced activation of CD8α$^+$ DCs led to enhanced immunity to naturally-expressed AML antigens **(Figure 15D)**.

We have previously demonstrated that maturation of host APCs via CD40 ligation was sufficient to prevent T cell tolerance in AML-bearing mice[111]. However, whether maturation of a specific APC subset was required for the efficacy of anti-CD40 therapy was not examined. Therefore, we sought to determine if the ability of anti-CD40 treatment to prevent T cell tolerance required CD8α$^+$ DCs. While anti-CD40 treatment resulted in a significant survival advantage in AML-bearing C57BL/6 mice, there was no

therapeutic effect in *Batf3*$^{-/-}$ mice **(Figure 15E)**. These data indicate that the activation of the CD8α^{+} DC subset is required for the effectiveness of anti-CD40 immunotherapy, and further suggests that the activation state of CD8α^{+} DCs ultimately dictates whether functional immunity or immune tolerance develops in leukemia-bearing hosts.

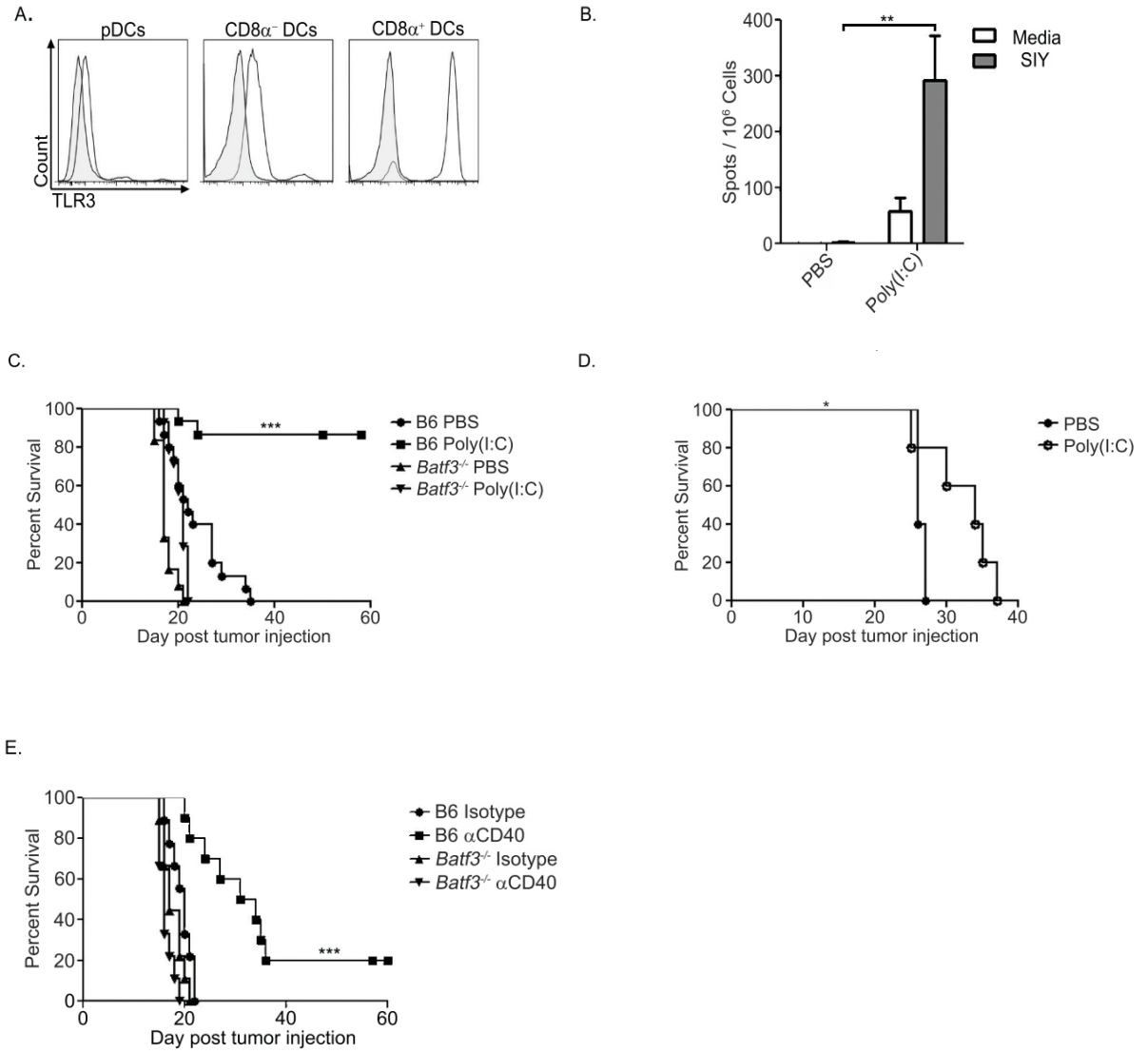

Figure 15. Activation of CD8α$^+$ DCs prevents AML-induced T cell tolerance. A) Representative plots depict expression of TLR-3 on different spleen cell populations. Shaded histograms represent isotype control staining. **B)** C57BL/6 mice received 10^6 C1498.SIY cells IV. On days 0 and 3, poly(I:C) (100 µg) or PBS were administered (IP). On day six, 10^6 splenocytes were stimulated with media or 100 nM SIY peptide in an IFN-γ ELISPOT assay. **C)** C57BL/6 or *Batf3*$^{-/-}$ mice were given 10^6 C1498.SIY cells IV on day 0, and were administered poly(I:C) or PBS every 3 days for 15 days, starting on day 0. **D)** C57BL/6 mice were given 10^6 C1498 cells IV and either 100 µg poly(I:C) or PBS I.P. every 3 days for 15 days, starting on day 0. **E)** C57BL/6 or *Batf3*$^{-/-}$ mice were given 10^6 C1498.SIY cells IV and on days 0, 2 and 4, received anti-CD40 or isotype control antibody IP. Results represent at least 2 independent experiments (A, B, and D), or were pooled from at least 2 experiments (C and E). * $p<0.05$ ** $p<0.01$ *** $p<0.001$

Conclusions

The observation that agonistic anti-CD40 antibody was able to prevent the T cell tolerant state in AML bearing mice prompted us to investigate the host APCs which were inducing leukemia-specific T cell tolerance. We found that splenic $CD8\alpha^+$ DCs were exclusive in their ability to cross-present antigens derived from circulating leukemia cells to $CD8^+$ T cells. When AML cells were growing as a solid tumor, Batf3-dependent DCs were required for the priming of adaptive immunity, as previously described. However, in mice with systemic AML, the same subset of $CD8\alpha^+$ DCs was required to drive T cell tolerance. Thus the same subset of DCs can imprint disparate T cells fates in mice with solid tumors verses mice with disseminated hematopoietic malignancies.

RESULTS III: STING PATHWAY ACTIVATION STIMULATES POTENT IMMUNITY AGAINST ACUTE MYELOID LEUKEMIA[1]

Summary

Type I IFN, essential for spontaneous T cell priming against solid tumors, is generated through recognition of tumor DNA by STING. Interestingly, we observe that type I IFN is not elicited in animals with disseminated AML. Further, survival of leukemia-bearing animals is not diminished in the absence of type I IFN signaling, suggesting that STING may not be triggered by AML-derived DNA. However, the STING agonist, DMXAA, induces expression of IFN-β and other inflammatory cytokines, promotes dendritic cell (DC) maturation, and results in the striking expansion of leukemia-specific T cells. Systemic DMXAA administration significantly extends survival in two AML models. The therapeutic effect of DMXAA is only partially dependent on host type I IFN signaling, suggesting that other cytokines are important. A synthetic cyclic dinucleotide that also activates human STING provided a similar anti-leukemic effect. These data demonstrate that STING is a promising immunotherapeutic target in AML.

[1] Parts of this section are reproduced here, with minimal modification, from Curran E et al. STING Pathway Activation Stimulates Potent Immunity against Acute Myeloid Leukemia. Cell reports 2016, 15(11): 2357-2366.

Introduction

Anti-tumor T cell responses develop spontaneously in a fraction of cancer patients, and the presence of tumor-infiltrating T cells has prognostic implications[58, 137, 138, 139]. How the sterile tumor environment supports tumor-specific T cell priming has been of significant interest in recent years. Gene expression profiling of human melanomas has revealed a type I IFN signature in tumors heavily infiltrated by $CD8^+$ T cells[140], suggesting that type I IFN might be important for bridging innate and adaptive anti-tumor immune responses. Direct evidence in support of this hypothesis has come from animal models in which type I IFN signaling in host cells was necessary to promote spontaneous anti-tumor $CD8^+$ T cell priming[59, 60]. Furthermore, several groups demonstrated that transplanted and carcinogen-induced solid tumors grow more rapidly in type I IFN receptor-deficient (*Ifnar*$^{-/-}$) mice[60, 141]. Collectively, these observations have established an essential role for type I IFN in generating adaptive immune responses against solid cancers.

The cancer cell-derived signals that induce host type I IFN production remained elusive until it was shown that tumor DNA triggered IFN-β production by dendritic cells (DCs) in vivo through activating the cytosolic DNA-sensing STING (Stimulator of Interferon Genes) pathway[63]. In the native STING pathway, cyclic dinucleotides known as cyclic GMP-AMP (cGAMP) are generated from cytosolic DNA by the enzyme cGAMP synthase (cGAS)[142, 143]. Upon cGAMP binding, the STING homodimer undergoes a significant conformational change and traffics from the endoplasmic reticulum to the Golgi, where it recruits TANK-binding kinase 1 (TBK1), resulting in its phosphorylation, activation of interferon regulatory factor 3 (IRF3) and transcription of

type I IFN[119, 144]. STING also activates the STAT6 and nuclear factor kappa B (NF-κB) pathways, inducing the expression of a number of inflammatory cytokines and chemokines, including CCL20, TNF-α and IL-6[142, 143, 145, 146].

Recent work indicates that, in the setting of a localized tumor, spontaneous anti-tumor $CD8^+$ T cell responses are abrogated in STING-deficient hosts, but occur normally in mice deficient in other nucleic acid-sensing receptors[63], suggesting that tumor-derived DNA and STING are critical for generating adaptive anti-tumor immunity. However, in contrast to solid malignancies, hematological cancers, such as AML, are typically disseminated at inception and lack classical draining lymph nodes. While it has been demonstrated that AML cells can be recognized by the host immune system[105], the mechanisms that regulate immunity and immune tolerance against this disease and, specifically, the role of STING and type I IFN, are relatively unknown. Interestingly, our recent work has implicated antigen-presenting cells (APCs), producers of type I IFN, in generating a unique T cell tolerant state in AML-bearing animals[111], suggesting that the host type I IFN response may not be activated in this disease. If this is the case, strategies aimed at stimulating type I IFN production in AML-bearing hosts, such as through STING activation, might lead to effective adaptive immunity against leukemia-derived antigens.

Here, we demonstrate that a host type I IFN response is not generated in mice with systemic AML. Further, the survival of leukemia-bearing mice is similar in the presence or absence of host type I IFN signaling and STING, in sharp contrast to what has been observed in solid tumor models[59, 60, 63]. However, administration of the STING agonist, 5,6-demethylxanthenone-4-acetic acid (DMXAA), to animals with established

AML, induces type I IFN and TNF-α production, leads to APC maturation, and culminates in extremely potent activation of leukemia antigen-specific $CD8^+$ T cells. DMXAA treatment significantly prolongs the survival of, and in some cases, cures mice with AML. Collectively, these results provide strong rationale for the therapeutic development of STING agonists as immunotherapy for AML.

Results

Disseminated AML fails to induce a host type I IFN response

Host type I IFN signaling is necessary for the generation of spontaneous $CD8^+$ T cell responses against solid tumors[59, 60]. To determine whether type I IFN was induced in mice with systemic leukemia, C1498 AML cells were inoculated IV into C57BL/6 mice and *Ifnb* expression was measured in bulk spleen cells. *Ifnb* expression was also analyzed in the tdLN of mice given a localized (SC) C1498 challenge as a positive control[60]. *Ifnb* mRNA levels were similarly low in spleen cells from leukemia-free and IV C1498 cell-challenged animals. In contrast, *Ifnb* expression could be readily detected in tdLN cells from SC C1498 cell-challenged mice, as expected **(Figure 16A)**. To determine whether type I IFN signaling was important for the generation of functional immune responses to systemic AML, survival of wild-type and *Ifnar*$^{-/-}$ mice given an IV challenge with C1498 cells was compared, and found to be quite similar **(Figure 16B)**. Conversely, and in agreement with published data, tumors derived from SC-implanted C1498 cells progressed more rapidly in *Ifnar*$^{-/-}$ compared to wild-type mice **(Figure 16C)**. Moreover, whereas *Ifnar*$^{-/-}$ mice mount severely diminished immune responses against solid tumors, leukemia-specific 2C T cells proliferated similarly and were recovered at equal numbers in IV challenged C57BL/6 and *Ifnar*$^{-/-}$ mice **(Figures 16D-F)**. Collectively, these results indicate that a disseminated leukemia fails to stimulate a type I IFN response in the host.

The STING agonist, DMXAA, induces IFN-β, TNF-α and IL-6 expression in vivo

Various STING agonists, including synthetic cyclic dinucleotides (CDNs) and DMXAA, have been used therapeutically with success in mouse models, either when injected intra-tumorally, or when administered as part of a localized cancer vaccine[124, 146]. Because IFN-β was not induced in animals with systemic AML, it was of interest to determine if a systemically-delivered STING agonist could generate a host type I IFN response sufficient to mediate control or rejection of AML. Following IV administration, DMXAA induced *Ifnb* expression in spleen cells in a STING-dependent manner, as demonstrated by lack of *Ifnb* expression in DMXAA-treated *Tmem173*$^{-/-}$ (STING deficient) mice **(Figure 17A)**. STING also activates NF-κB through a poorly understood mechanism. Consistent with this, enhanced *Tnfα* and *Il6* expression levels were observed in the spleens of DMXAA-treated mice, also in a STING-dependent manner **(Figure 17B and C)**. Serum levels of IFN-β, TNF-α and IL-6 were also elevated following DMXAA treatment **(Figure 17D-F)**.

DMXAA treatment activates host antigen-presenting cells

Type I IFN has been shown to directly activate APCs[147]. Because DMXAA administration induced type I IFN, an in vivo effect on DCs and macrophages was next investigated through an analysis of their cell surface expression of co-stimulatory and MHC class II molecules, as well as IL-12 production. Increased expression of CD80, CD86 and MHC class II was observed on DCs and, to a lesser extent, on macrophages from DMXAA-treated animals, again in a STING-dependent manner **(Figure 18A and**

B). Furthermore, IL-12 production was 2-3 fold higher in DCs following DMXAA treatment. Macrophages did not produce any detectable IL-12 at baseline or following STING activation **(Figure 18C and D)**. Collectively, these data demonstrate that DMXAA treatment activates APCs, and DCs in particular, which may enhance their capability to stimulate adaptive anti-leukemia immune responses.

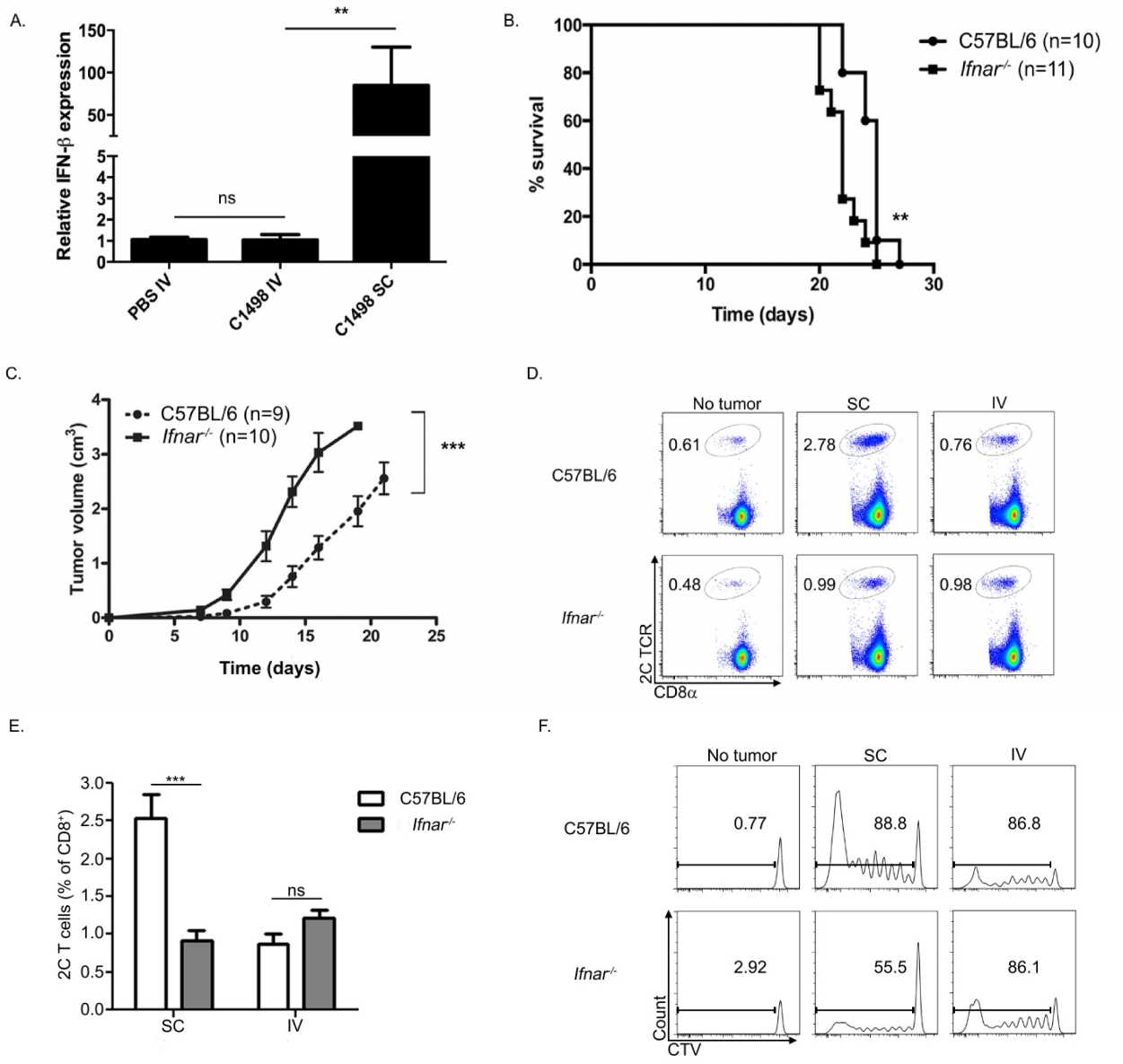

Figure 16. Systemic AML fails to induce a host type I IFN response. (A) 5 x 10^6 C1498 cells were inoculated IV or SC into C57BL/6 mice. Control mice received PBS IV. Three days later, *ifnb* expression in bulk spleen cells (IV C1498 or PBS mice) or in draining lymph node cells (SC C1498 mice) was analyzed by qRT-PCR and normalized to controls that received PBS IV. Data are pooled from 3 independent experiments each with 3 mice per group and represented as mean ± SEM. **(B)** C57BL/6 and *Ifnar*$^{-/-}$ mice were challenged with 10^6 C1498 cells IV and survival assessed. **(C)** C57BL/6 and *Ifnar*$^{-/-}$ mice received 10^6 C1498 cells SC and tumor volume was assessed over time. Data are pooled from 2 independent experiments each with 4-5 mice per group and represented as mean ± SEM. n.s. = not significant; **p < 0.01; ***p < 0.001.

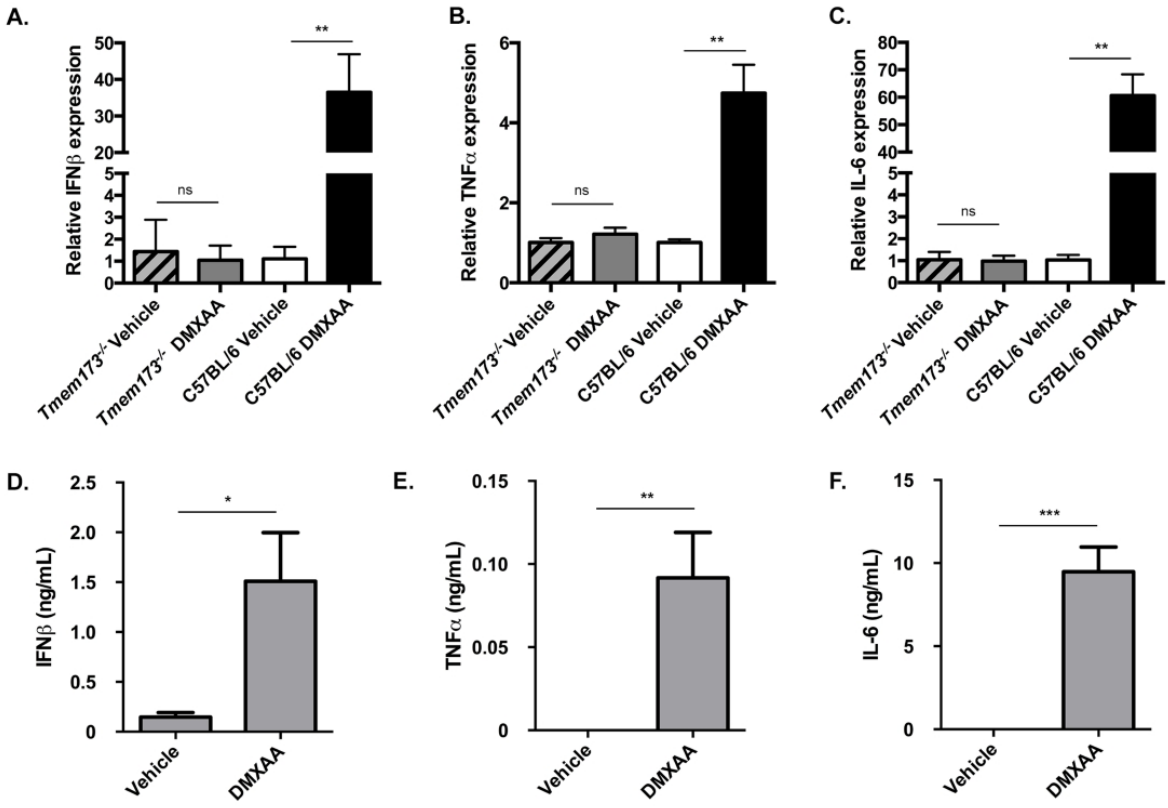

Figure 17. DMXAA induces *ifnb*, *tnfa* and *il6* expression in a STING-dependent manner. C57BL/6 or *Tmem173*$^{-/-}$ mice received DMXAA (450µg) or vehicle (NaHCO) IV. **(A-C)** Spleen cells were harvested 6 hours later, and expression of *infb* **(A)**, *tnfa* **(B)** or *il6* **(C)** were measured by qRT-PCR and relative normalized expression was determined by comparison to vehicle control-treated mice. **p < 0.01 for *ifnb*, *tnfa* and *il6* expression in DMXAA versus vehicle control-treated mice. p = n.s. for differences in *ifnb*, *tnfa* and *il6* expression in DMXAA versus vehicle control-treated *Tmem173*$^{-/-}$. Data are pooled from 2 independent experiments each with 3 mice per group and represented as mean ± SEM. **(D-F)** Serum obtained 6 hours later. IFN-β **(D)**,TNF-a **(E)**, and IL-6 **(F)** were measured by ELISA. *p<0.05, **p<0.01, or ***p<0.001 for IFN-β, TNF-α, and IL-6 levels in DMXAA versus vehicle-treated mice. Data are pooled from 2 independent experiments each with 3 mice per group and represented as mean ± SEM.

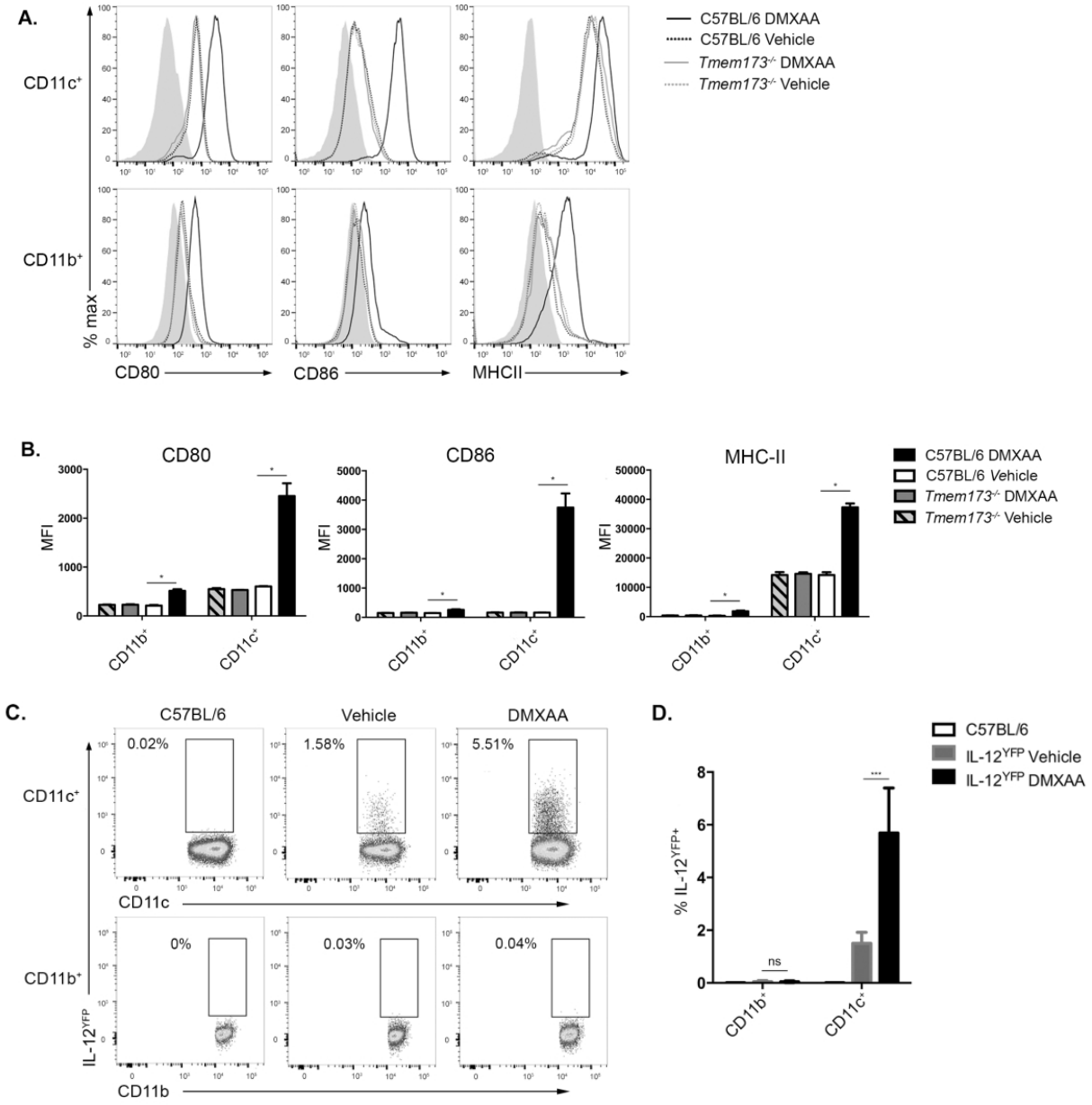

Figure 18. DMXAA activates APCs. C57BL/6 and *Tmem173*$^{-/-}$ **(A and B)** or IL-12^{YFP} reporter mice **(C and D)** received a single IV injection of DMXAA or vehicle control. 12 hours later, cell surface CD80, CD86 and MHC-II levels were analyzed on live splenic CD11c^{+}or CD11b^{+}CD11c^{-}cells. Representative histograms are shown in **(A)**. Median fluorescence intensity (MFI) for CD80, CD86 and MHC-II are quantified in **(B)**. *$p < 0.05$ for comparison between C57BL/6 DMXAA and vehicle treated. Data presented in **(B)** are representative of 2 independent experiments each with 3 mice per group and represented as mean ± SEM.

Figure 18 Continued: (C) Representative plots of$IL\text{-}12^{YFP}$ expression in splenic $CD11c^{+}$or $CD11b^{+}CD11c^{-}$ cells 12 hours after treatment with DMXAA or vehicle control. Numbers in each plot indicate the frequency of $IL\text{-}12^{YFP+}$ cells among total $CD11c^{+}$ (top row) or $CD11b^{+}CD11c^{-}$(bottom row) cell populations. Data from **(C)** are quantitated in **(D)**. ***$p < 0.001$ for comparison between DMXAA and vehicle-treated $IL\text{-}12^{YFP}$ mice. Data in **(D)** are pooled from 2 independent experiments each with 2-4 mice per group and represented as mean ± SEM.

STING activation stimulates potent leukemia-specific $CD8^+$ T cell responses

Having shown that DMXAA treatment induces type I IFN expression and APC activation, its effect on leukemia-specific $CD8^+$ T cell responses was next investigated. C57BL/6 mice were challenged IV with C1498.SIY cells, and were subsequently treated with DMXAA or vehicle control. One week later, endogenous SIY-specific $CD8^+$ T cell responses were analyzed in the spleen and bone marrow of leukemia-bearing animals. As shown in **Figure 19A-C**, a striking expansion of SIY-specific $CD8^+$ T cells occurred in DMXAA- versus vehicle control-treated mice. Importantly, the effect of DMXAA on the expansion of SIY-specific T cells required their exposure to cognate antigen, as no increase in the frequency or number of antigen-specific $CD8^+$ T cells occurred in DMXAA-treated, leukemia-free animals **(Figure 19A-C)**. Antigen-specific $CD8^+$ T cells in DMXAA-treated, leukemia-bearing animals were functional, and produced IFN-γ following ex vivo re-stimulation **(Figure 19D and E)**. In contrast, very few IFN-γ^+ $CD8^+$ T cells were generated in vehicle control-treated mice with AML **(Figure 19D and E)**. Also important was the observation that DMXAA did not induce IFN-γ production by polyclonal $CD8^+$ T cells in naïve mice **(Figure 19D and E)**. The effect of DMXAA on enhanced leukemia-specific $CD8^+$ T cell priming was completely STING-dependent and did not occur in leukemia-bearing *Tmem173*$^{-/-}$ hosts treated with DMXAA **(data not shown)**. These data demonstrate that STING activation promotes the robust expansion of endogenous leukemia antigen-specific T cells.

In order to directly track the proliferation and expansion of AML-specific $CD8^+$ T cells following STING activation, TCR transgenic $CD8^+$ 2C T cells were CTV-labeled and adoptively-transferred into C57BL/6 mice. C1498.SIY AML cells were then

inoculated IV, and DMXAA or vehicle control was administered. In leukemia-free mice, 2C T cells remained largely undivided, whether or not DMXAA was administered, indicating that STING activation did not stimulate leukemia-specific T cells in the absence of antigen **(Figure 20A and B)**. 2C T cells proliferated but failed to expand significantly in leukemia-bearing mice treated with vehicle control, as we have reported previously **(Figure 20A and B)**[111]. Interestingly, DMXAA treatment led to the accumulation of large numbers of fully-divided 2C T cells in leukemia-bearing animals **(Figure 20A and B)**. The frequency of 2C T cells isolated from AML-bearing mice that produced IFN-γ was also enhanced following DMXAA treatment **(Figure 20C and D)**. As shown in **Figure 20E**, the amount of IFN-γ produced on a per-cell basis was also higher in 2C T cells from DMXAA versus vehicle control-treated mice with AML. Together, these data demonstrate that activation of the STING pathway leads to an impressive expansion of functional leukemia-specific $CD8^+$ T cells following adoptive transfer into mice with established AML.

To confirm that DMXAA-induced STING activation did not cause antigen-independent $CD8^+$ T cell expansion in vivo, 2C and OT-I T cells (the later which recognize an irrelevant antigen derived from chicken ovalbumin) were co-transferred into mice. The following day, mice received C1498.SIY cells IV or remained leukemia-free. DMXAA or vehicle control was administered, and the frequencies of 2C and OT-1 T cells were subsequently analyzed. OT-I T cells failed to expand in any group, regardless of AML cell inoculation or DMXAA administration **(Figure 20F and G)**. This result conclusively demonstrates that STING activation results in activation of $CD8^+$ T cells in an antigen-specific manner.

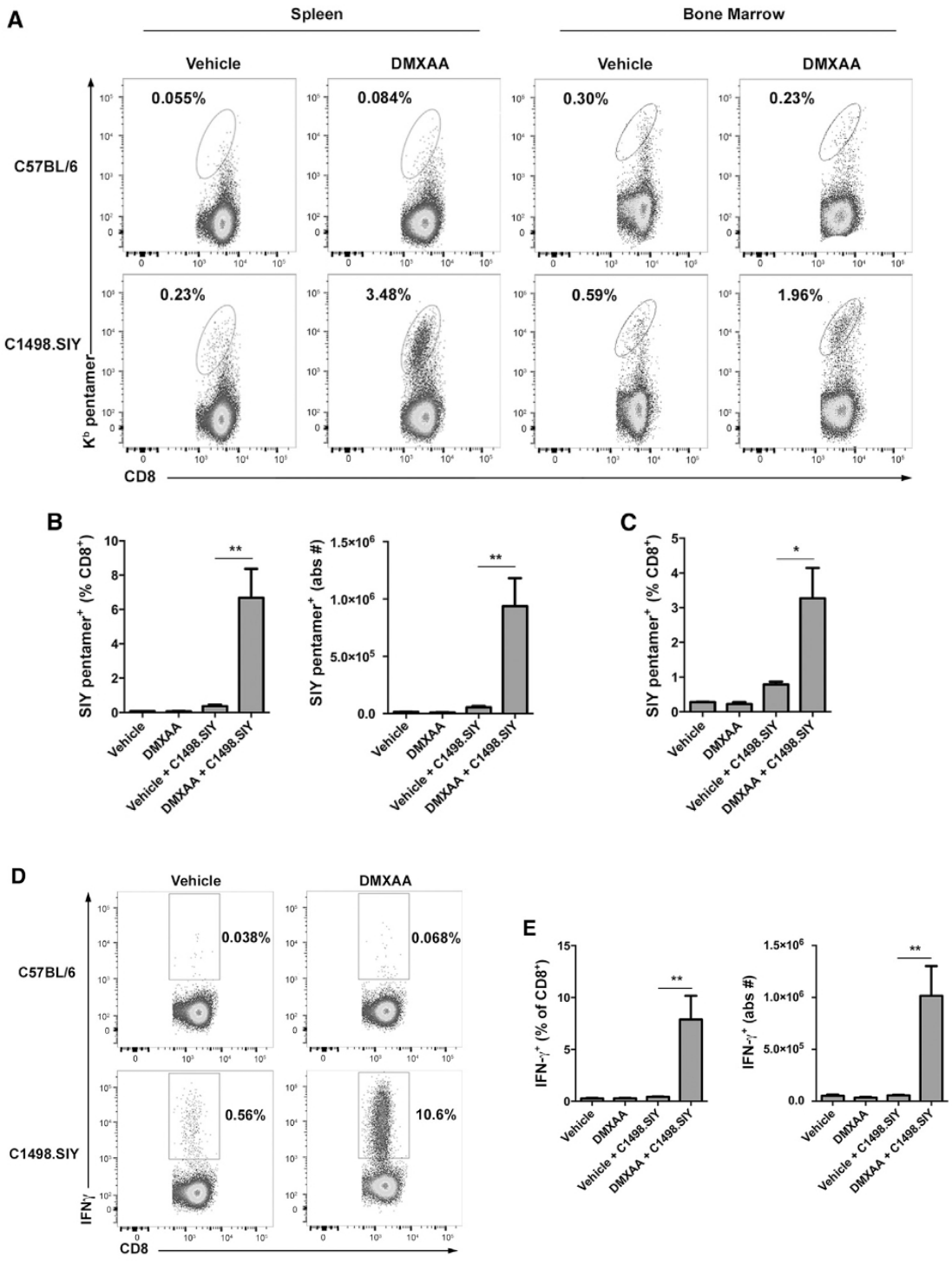

Figure 19. DMXAA-induced STING activation potentiates leukemia-specific CD8$^+$ T cell responses. C57BL/6 mice received 10^6 C1498.SIY cells IV or no C1498.SIY cell inoculation (day 0), were treated with DMXAA 450µg or vehicle control IV (day 5) and analyzed on day 12.

Figure 19 Continued: (A and B) Frequency and absolute number of splenic or bone marrow SIY-specific $CD8^+$ T cells were analyzed following SIY/K^b pentamer staining by flow cytometry. **(A)** Representative plots of SIY/K^b pentamer staining. Numbers indicate frequency of SIY-specific $CD8^+$ T cells. **(B)** Frequency (left), and absolute number (right) of splenic SIY-specific $CD8^+$ T cells. **(C)** Frequency of bone marrow SIY-specific $CD8^+$ T cells. **(D and E)** Spleen cells from indicated mice were re-stimulated in vitro with SIY peptide and production of IFN-γ by $CD8^+$ T cells was analyzed. **(D)** Representative plots demonstrating IFN-γ production by $CD8^+$ T cells are shown. Numbers indicate the frequency of IFN-γ^+ $CD8^+$ T cells. **(E)** Frequency (left) and absolute number (right) of IFN-γ^+ $CD8^+$ T cells in the indicated groups. **(B, C and E)** Data shown are pooled from at least 2 independent experiments each with 3 mice per group and represented as mean ± SEM. *$p < 0.05$ or **$p < 0.01$ for comparison of DMXAA versus vehicle control-treated C1498.SIY-bearing mice.

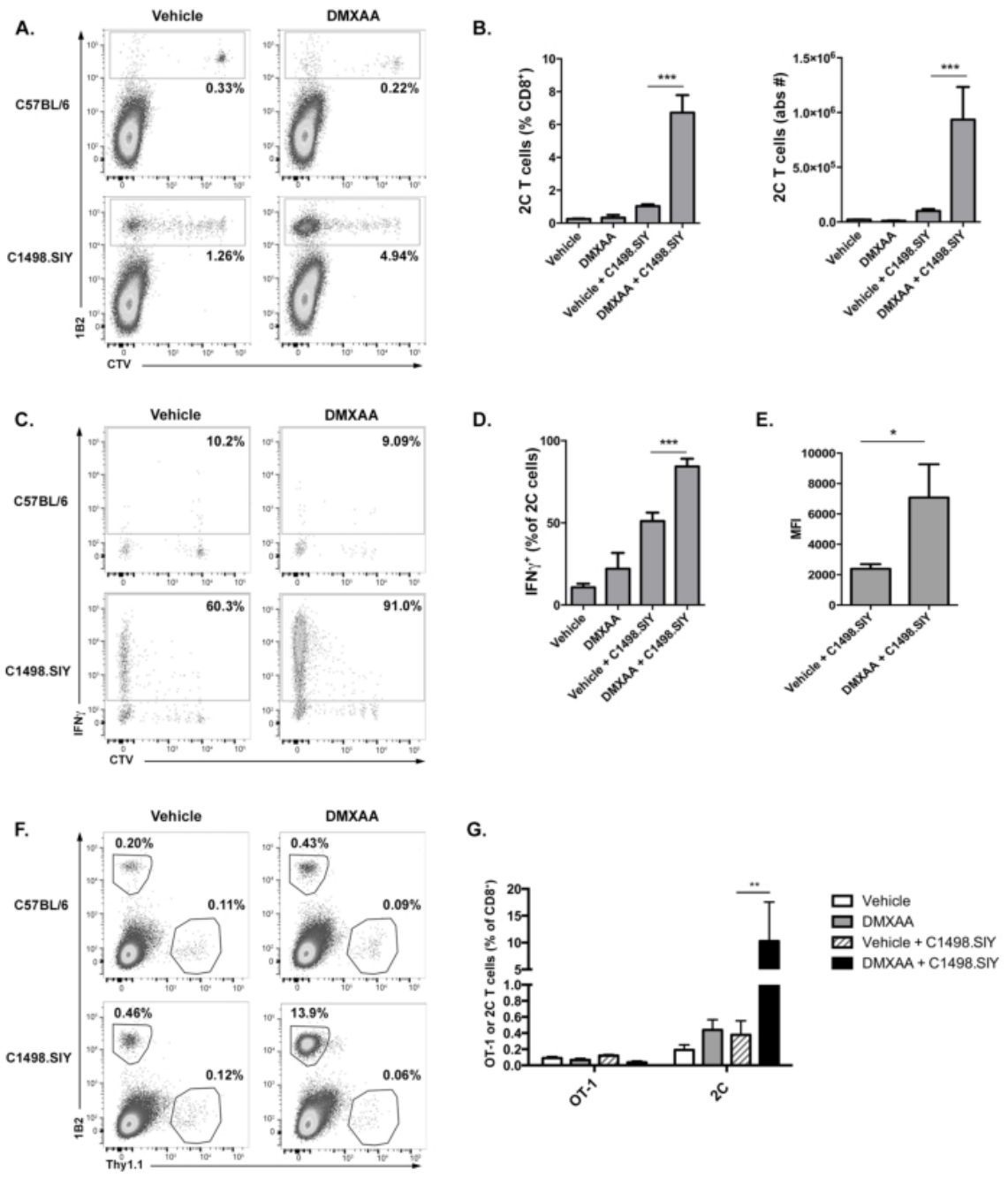

Figure 20. DMXAA leads to expansion of functional leukemia-specific CD8$^+$ T cells following adoptive transfer. 10^6 CTV-labeled CD8 2C T cells **(A-E)** or 2C and OT-I (Thy1.1$^+$) T cells **(F and G)** were adoptively transferred into C57BL/6 mice on day -1, followed by inoculation with 10^6 C1498.SIY cells on day 0. Some C57BL/6 mice received adoptive transfer of 2C T cells, but no C1498.SIY cell challenge. Mice were treated with DMXAA or vehicle control on day 0 **(A-E)** or day 5 **(F and G)** and analyzed 6-7 days later. **(A)** Representative plots depicting CTV dilution of 2C T cells. Numbers indicate the frequency of 2C T cells among the entire CD8$^+$ T cell population.

Figure 20 Continued: B) Frequency (left) and absolute numbers (right) of 2C T cells in the indicated groups. ***$p < 0.001$ for comparison of the frequency and number of 2C T cells present in DMXAA versus vehicle control-treated C1498.SIY-bearing mice. **(C-E)** Spleen cells were restimulated with SIY peptide and production of IFN-γ by 2C T cells was analyzed. **(C)** Representative plots showing IFN-γ production by CTV dilution after gating on 2C T cells. Numbers represent the percentage IFN-γ^+ 2C T cells among the total 2C T cell population. **(D)** Frequency of IFN-γ^+ 2C T cells in each group. ***$p<0.001$ for comparison of the frequency and absolute number of IFN-γ^+ 2C T cells in DMXAA versus vehicle control-treated C1498.SIY-bearing mice. **(E)** MFI of IFN-γ staining in 2C T cells in the indicated groups (*$p < 0.05$). **(F)** Representative plots depicting the relative frequencies of splenic 2C T cells ($1B2^+$) and OT-I T cells ($Thy1.1^+$). Numbers indicate the percentages of $2C^+$ or OT-I T cells among the total $CD8^+$T cell population. **(G)** Quantitated frequencies of OT-I or 2C T cells among total splenic $CD8^+$T cells in each group. **$p<0.01$ for the comparison of 2C T cell frequency between DMXAA and vehicle control-treated C1498.SIY-bearing mice. **(B, D, G)** Data shown are pooled from at least 2 independent experiments each with 3 mice per group and represented as mean ± SEM.

STING activation enhances survival of leukemia-bearing mice

To determine whether the powerful effect of STING activation on T cell priming correlated with improved disease control, animals with established C1498.SIY AML were treated with DMXAA or vehicle, and survival was assessed. As shown in **Figure 21A**, a single dose of DMXAA significantly prolonged survival of leukemia-bearing mice compared to those treated with vehicle control. In fact, approximately 60% of DMXAA-treated mice survived long-term. Because SIY is an immunogenic model antigen, the ability of DMXAA to control the progression of parental C1498 AML was also tested. Here again, DMXAA-induced STING activation as a single therapeutic maneuver significantly prolonged survival, albeit to a lesser extent than in the C1498.SIY model **(Figure 21B)**. Survival following DMXAA or vehicle control treatment was similarly poor in leukemia-bearing *Tmem173*$^{-/-}$ mice, demonstrating that the effect of DMXAA on disease control was STING-dependent **(Figure 21C and D)**.

To investigate the extent to which host type I IFN signaling was important for enhanced survival following STING activation, wild-type and *Ifnar*$^{-/-}$ mice were challenged with C1498 AML cells and treated with DMXAA or vehicle control. Survival was similar in vehicle control-treated wild-type and *Ifnar*$^{-/-}$ mice **(Figure 21E).** As expected, DMXAA treatment led to a significant survival enhancement in wild-type mice, but survival was also extended to some degree in AML-bearing *Ifnar*$^{-/-}$ mice that received DMXAA **(Figure 21E)**. Together, these data demonstrate that STING activation induces both type I IFN-dependent and -independent effects which enhance leukemia-specific immunity and promote survival in AML-bearing mice.

Finally, because DMXAA is a selective agonist of murine, but not human STING, the efficacy of a synthetic CDN STING agonist capable of activating both mouse and human STING was tested. This compound, ML RR-S2 CDA (CDA) has been shown to generate an anti-tumor T cell response and disease regression when administered intra-tumorally in solid tumor models[124]. To assess whether CDA treatment would also extend survival in animals with systemic AML, C57BL/6 mice were challenged with C1498.SIY cells IV, followed by CDA or PBS on day 5. As shown in **Figure 21F**, CDA-treated leukemia-bearing mice survived significantly longer than controls.

DMXAA therapy requires adaptive immunity, and promotes immunologic memory against native AML antigens

To examine the role of the adaptive immune system in regulating the DMXAA effect on survival of AML-bearing mice, C57BL/6 and *Rag2*$^{-/-}$ mice (the later which lack mature B and T cells) were challenged with C1498 or C1498.SIY cells, and treated with DMXAA or vehicle control. As demonstrated previously, DMXAA treatment enhanced survival of C57BL/6 mice following a systemic inoculation of C1498.SIY **(Figure 22A)** or parental C1498 cells **(Figure 22B)** when compared to vehicle control-treated animals. However, the survival of leukemia-bearing *Rag2*$^{-/-}$ mice was identical in DMXAA- and control-treated mice **(Figure 22A and B)**, indicating that the therapeutic effect of STING activation in AML-bearing animals requires adaptive immunity.

It was next determined whether functional memory was generated against native C1498-expressed antigens following DMXAA treatment. C57BL/6 mice that survived a

primary IV C1498.SIY challenge after treatment with DMXAA received a subsequent challenge with parental C1498 cells 100 days later. A second group of C57BL/6 mice received a primary IV C1498 cell challenge simultaneously as a comparator cohort. DMXAA treatment of leukemia-bearing mice promoted a remarkable survival benefit following AML cell re-challenge, clearly demonstrating that effective memory responses are generated against native C1498 antigens following STING activation **(Figure 22C)**.

STING activation is effective in a genetically-engineered AML model

To assess whether STING activation would be effective in a second AML model, the Cbfb-MYH11/Mpl-induced mouse leukemia model (CMM^+) was utilized. This genetically-engineered AML model mimics human inv(16) AML. Mice with established CMM^+ leukemia received DMXAA or vehicle control treatment weekly. STING activation resulted in significant decrease in the frequency of AML cells in spleens of treated mice **(Figure 23A and B)** with a corresponding decrease in splenomegaly (data not shown). These anti-tumor effects translated also into extended survival of DMXAA-treated CMM^+ mice compared to controls **(Figure 23C)**, and demonstrated that the effectiveness of immunotherapy with STING agonists was not limited to a single AML model.

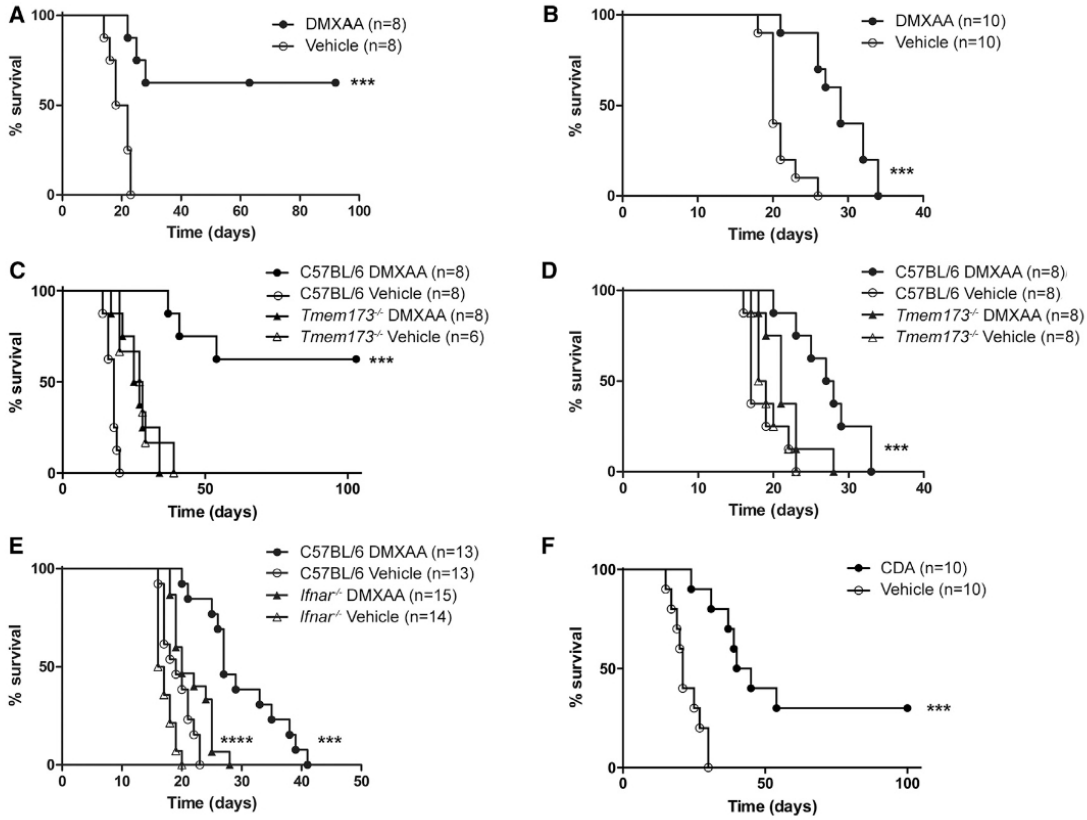

Figure 21. STING activation prolongs survival in mice with AML. C57BL/6 **(A-F),** Tmem173$^{-/-}$ **(C and D)**, or *Ifnar*$^{-/-}$ mice **(E)** were challenged with 10^6 C1498.SIY **(A, C, and F)** or parental C1498 **(B, D, and E)** cells IV. On day 5 **(A, C, and F)**, or on days 3 and 10 **(B, D, and E),** mice were treated with DMXAA **(A-E)** or CDA **(F)** versus vehicle control IV and survival assessed. **(A-F)** Data are pooled from 2-3 independent experiments each with 3-5 mice per group. ***p<0.001 for comparison of survival between DMXAA- and vehicle-treated C57BL/6 mice **(A-E)** or between CDA- and vehicle-treated C57BL/6 mice **(F)**. ****p<0.001 for comparison of survival between DMXAA- and vehicle-treated *Ifnar*$^{-/-}$ mice **(E)**.

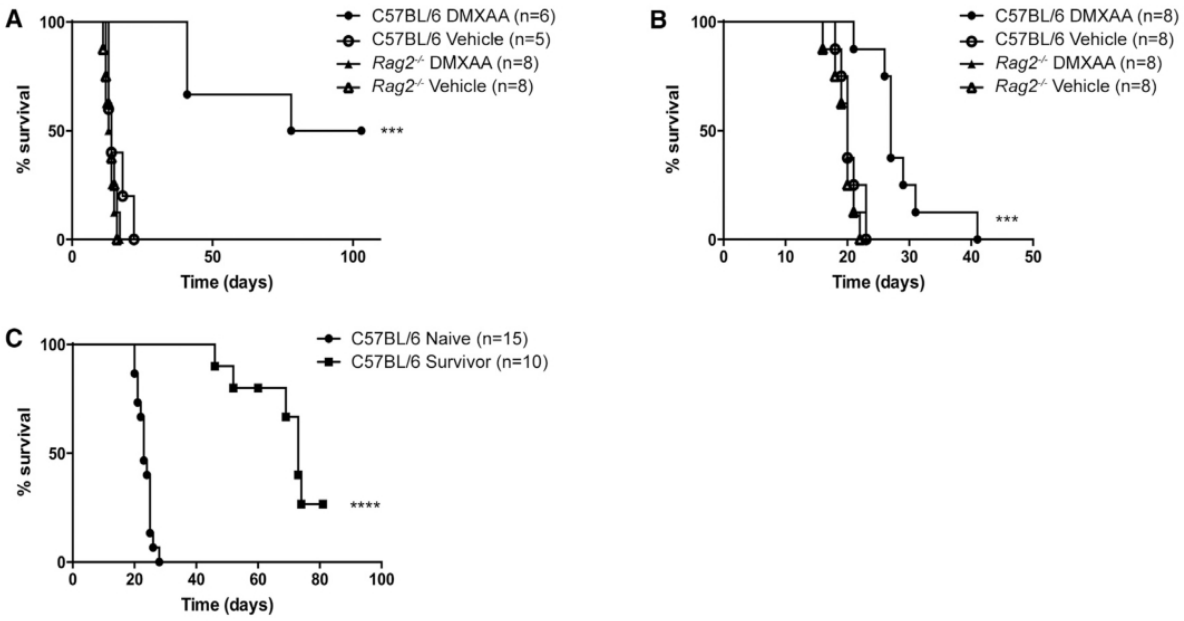

Figure 22. DMXAA therapy requires adaptive immunity and generates effective memory responses to naturally-expressed AML antigens. (A and B) C57BL/6 or *Rag2*$^{-/-}$ mice received 10^6 C1498.SIY **(A)** or C1498 **(B)** cells IV. On day 5 **(A)**, or on days 3 and 10 **(B),** mice were treated with DMXAA or vehicle control IV and survival was assessed. ***p<0.001 for survival in DMXAA- versus vehicle-treated C57BL/6 mice **(C)** DMXAA-treated survivors of a primary C1498.SIY cell challenge received 10^6 parental C1498 cells IV approximately 100 days following the initial C1498.SIY cell inoculation. Naïve C57BL/6 mice inoculated with C1498 cells served as controls. ****p<0.0001 for survival in long-term C1498.SIY survivors versus leukemia-naïve mice following inoculation with C1498 cells.

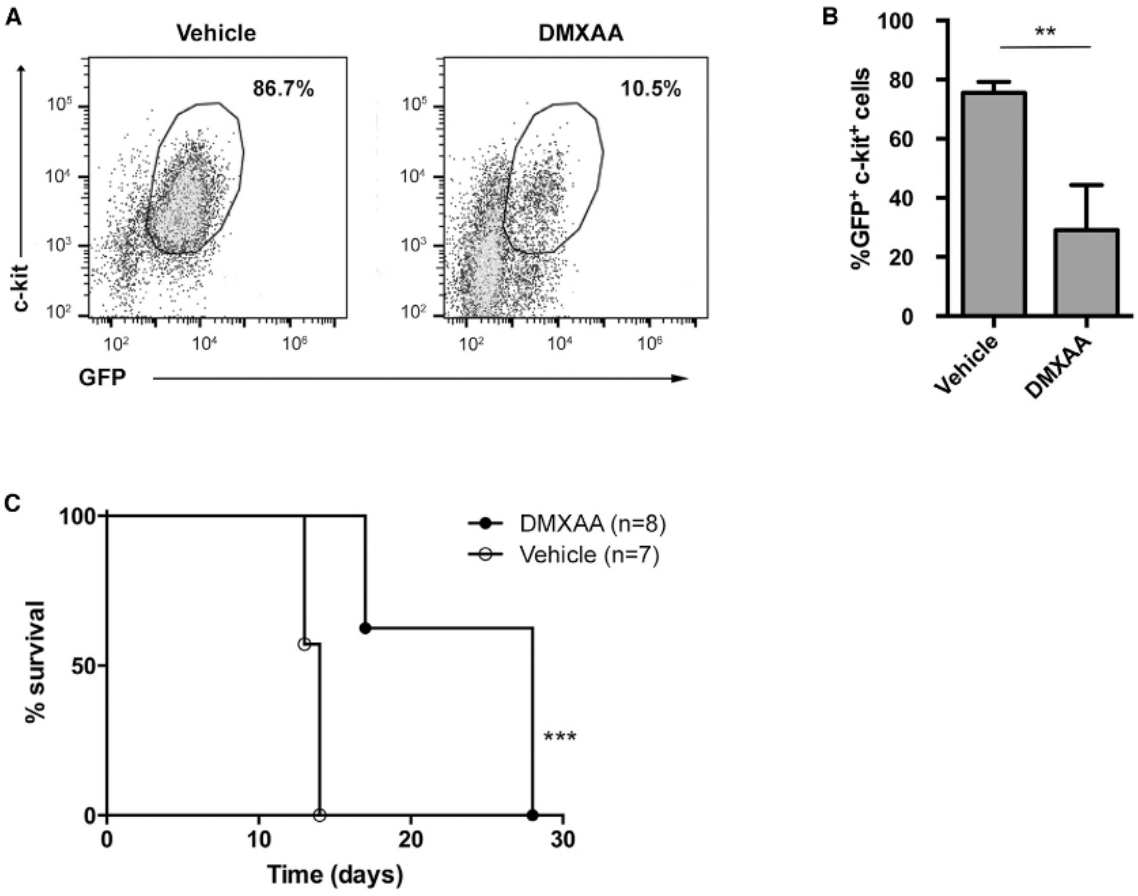

Figure 23. DMXAA decreases tumor burden and prolongs survival in a genetically-engineered AML model. Mice were challenged IV with *Cbfb-MYH11/Mpl*$^+$ (*CMM*$^+$) AML cells on day 0 and were treated with DMXAA or vehicle control on days 7 and 14. **(A and B)** AML burden as measured by the frequency of GFP$^+$ c-kit$^+$ cells in the spleens of DMXAA or vehicle control-treated mice on day 17. **(A)** Representative plots with numbers indicating frequency of GFP$^+$ c-kit$^+$ cells among total spleen cells. **(B)** Quantified frequency of splenic AML cells. Data in **(B)** are pooled from 2 independent experiments each with 3-4 mice per group and represented as mean ± SEM. **p < 0.01 **(C)** Survival of *Cbfb-MYH11/Mpl*$^+$ AML-bearing animals following DMXAA or vehicle control treatment. Data in **(C)** are pooled from 2 independent experiments each with 3-4 mice per group. ***p < 0.001.

Conclusions

Tumor cells growing as a solid mass stimulate the production of host type I IFN that is sensed by Batf3-dependent DCs allowing the subsequent priming of adaptive immunity. With the knowledge that in mice with systemic AML, the same lineage of Batf3-dependent DCs drives T cell tolerance, we first investigated if a type I IFN response was triggered in mice with AML. In fact, at no time point post tumor injection could we detect IFN-β in the spleens of AML bearing mice and there was no survival difference or alteration in T cell priming in IV challenged *Ifnar*$^{-/-}$ mice. Because type I IFN is induced in response to STING activation by solid tumors, we utilized the murine STING agonist, DMXAA, to trigger type I IFN production in mice with systemic AML. DMXAA was able to active host APCs, including CD8α^{+} DCs, resulting in effective priming of AML-specific CD8^{+} T cells and ultimately leading to enhanced survival following leukemia induction.

DISCUSSION AND FUTURE DIRECTIONS[1]

Differential properties of solid tumors versus acute leukemia that may affect interactions with the host immune system

Acute leukemias, including AML, originate in the bone marrow and rapidly disseminate throughout the host as the disease progresses. The short interval between the transformation event and the development of clinical manifestations may prevent the acquisition of somatic mutations in leukemia cells, as well as adequate time for the host immune system to recognize and respond to leukemia-associated antigens. In contrast, solid cancers develop in isolated anatomical locations over a period of months to years, and are drained by local lymph nodes in which anti-tumor T cell priming initially occurs. A long latency between oncogenesis and disease-related symptoms, the genomic instability inherent in malignant cells, and ongoing exposure to carcinogens, may facilitate the generation of large numbers of non-synonymous mutations in solid tumor cells. These mutations can generate tumor-specific antigens which may drive anti-tumor immune responses in patients with select solid tumors, including lung cancer and melanoma[88, 148]. Thus, the pathology of hematopoietic malignancies and solid cancers is quite distinct and, therefore, it is quite conceivable that interactions between the host

[1] Parts of this section are reproduced here, with minimal modification, from Zhang L et al. CD40 ligation reverses T cell tolerance in acute myeloid leukemia. The Journal of clinical investigation 2013, 123(5): 1999-2010 and Curran E et al. STING Pathway Activation Stimulates Potent Immunity against Acute Myeloid Leukemia. Cell reports 2016, 15(11): 2357-2366.

immune system and a disseminated leukemia may diverge from those which occur in the setting of a localized cancer.

Hematological cancers induce a unique state of T cell tolerance

In a straightforward series of experiments, we sought to determine if the adaptive immune system was involved in controlling disease progression in mice with disseminated AML, versus those harboring a localized AML cell tumor. To mimic the development of a localized (solid) tumor, C1498.SIY cells were implanted SC into recipient mice. Conversely, to model systemic leukemia, C1498.SIY cells were inoculated IV. C57BL/6 mice challenged SC with C1498.SIY cells survived significantly longer than those that had received C1498.SIY cells IV. This prolonged survival was due to the activation of the adaptive immune system, as when the same number of C1498.SIY cells were inoculated IV or SC, the survival of *Rag2*$^{-/-}$ mice, which lack T and B cells, was identical. As opposed to a SC tumor challenge, the survival of C57BL/6 and *Rag2*$^{-/-}$ mice following an IV C1498.SIY challenge was equivalent, indicating that adaptive immunity played no role in protecting animals from progression of systemic leukemia. In addition, robust leukemia antigen-specific CD8^{+} T cell responses were detected in C57BL/6 mice challenged SC with C1498.SIY cells, whereas very minimal leukemia-specific CD8^{+} T cell responses were raised in mice with systemic leukemia. Collectively, these results raised questions regarding the ability of the immune system to recognize and respond to antigens derived from circulating leukemia cells **(Figures 1 and 2)**.

To differentiate whether the failure to activate $CD8^+$ T cell responses in mice with systemic leukemia was due to immunological ignorance or active tolerance, a dual IV followed by SC leukemia challenge approach was employed. Here, an IV tumor challenge was able to significantly blunt SIY-specific $CD8^+$ T cell responses raised against a subsequent SC leukemia inoculation. This critical finding conclusively demonstrated that a systemic leukemia cell challenge actively induced a $CD8^+$ T cell tolerant state in the host **(Figure 3)**.

Overall, our results to this point revealed a stark contrast in the nature of $CD8^+$ T cell responses in mice with a localized versus systemic tumor. In the former, robust $CD8^+$ T cell responses were activated, while in the later, a potent state of T cell tolerance was generated. The rapid induction of T cell tolerance in mice with disseminated AML was antigen-specific, and occurred independently of Tregs and MDSCs. We went on to show that leukemia-specific $CD8^+$ T cells underwent abortive proliferation, and were ultimately deleted from mice with systemic AML **(Figures 4 and 5)**.

To our knowledge, this is the first demonstration that a cancer is capable of inducing peripheral tolerance in the form of T cell deletion. Ohlen and colleagues employed Friend murine leukemia virus (FMLV) - transformed leukemia (FBL) in order to study the $CD8^+$ T cell response to an immunodominant epitope (Gag) expressed by FBL cells in a setting where the Gag protein was also transgenically expressed in the liver and to a lesser extent, the thymus (Alb:Gag mice), thus mimicking a self-antigen [149]. In this model, tolerant Gag-specific $CD8^+$ T cells failed to proliferate or produce IL-2 upon restimulation, and demonstrated abnormal calcium flux and Ras/MAPK signaling,

a picture most consistent with T cell anergy, which was later demonstrated to be reversible following IL-15 administration[149, 150]. However, transgenic expression of the target antigen in the liver likely skewed the peripheral tolerance mechanism toward anergy as the dominant outcome; the tolerance in our experiments resulted from antigen derived only from leukemia cells (modeling the $CD8^+$ T cell response to a leukemia-specific antigen). Sotomayor et al. developed a model in which A20 lymphoma cells were engineered to express a model MHC class II-restricted antigen derived from the influenza virus (HA), and showed that naïve HA-specific $CD4^+$ TCR transgenic T cells harbored an anergic phenotype following adoptive transfer into hosts that had received systemic challenge with A20-HA cells[151]. In a subsequent publication, the same group found that induction of lymphoma-specific $CD4^+$ T cell anergy required antigen presentation by a bone marrow-derived cell[133].

It is interesting to speculate that T cell deletion and anergy might represent a continuum of dysfunctional T cell activation. Whether a T cell becomes functionally activated, is anergized, or is deleted likely depends upon the affinity of the TCR for its antigen and the context in which the antigen is encountered. For example, Sherman et al have demonstrated that TCR transgenic $CD8^+$ T cells were instructed to undergo an abortive proliferative response and to become tolerant upon transfer into mice in which the cognate antigen was cross-presented by quiescent APC in a non-inflamed lymph node environment[152, 153]. By administering the antigenic peptide systemically into mice, it was determined that higher doses of antigen led to T cell anergy, while repeated low doses of antigen promoted T cell deletion[154]. While T cell deletion appeared to be a major mechanism of T cell tolerance in mice with disseminated C1498 leukemia,

experimental evidence also demonstrated that the small number of antigen-specific $CD8^+$ T cells that escaped deletion were rendered anergic, indicated by their inability to produce effector cytokines following ex vivo restimulation. Although the discussion here is centered around $CD8^+$ T cell responses against the model SIY antigen in leukemia-bearing mice, it is important to note that an identical $CD8^+$ T cell tolerant state developed in mice challenged with parental C1498 cells, and thus, was not dependent on the SIY antigen itself.

Evidence supporting host APCs as the mediators of T cell tolerance in leukemia-bearing mice

It was initially speculated that the T cell tolerant phenotype in leukemia-bearing mice might have arisen following interactions between leukemia-specific $CD8^+$ T cells and AML cells presenting antigen in the context of MHC class I molecules. This hypothesis was formed based on two observations. First, circulating AML cells have access to a secondary lymphoid organ - namely the spleen. During solid tumor development, tumor-derived antigens reach the draining lymph node via the lymphatics, but tumor cells themselves are often restricted to peripheral locations. Therefore, tumor-specific $CD8^+$ T cells in hosts with solid malignancies are initially primed on professional APCs in lymph nodes. In contrast, AML cells could be found in the spleen where they had the opportunity to present antigen to naïve, leukemia-specific $CD8^+$ T cells. Secondly, C1498 cells do not express the costimulatory markers CD80, CD86 or CD40 **(data not shown)**. Therefore, direct antigen presentation by leukemia cells to $CD8^+$ T

cells would be expected to occur in the absence of costimulation, resulting in T cell tolerance. Interestingly, forced expression of the B7.1 costimulatory molecule on C1498.SIY cells prior to their IV inoculation into mice did not prevent the induction of $CD8^+$ T cell tolerance (**data not shown**), suggesting that direct antigen presentation by AML cells may not be driving T cell tolerance. Additionally, the fact that agonistic anti-CD40 antibody treatment was able to prevent, and partially reverse, T cell tolerance in leukemia-bearing mice implicated host APCs as the cellular mediators of T cell tolerance **(Figure 6)**.

<u>$CD8\alpha^+$ DCs engulf and cross-present leukemia-derived antigens</u>

In an attempt to characterize the APCs that were possibly driving T cell tolerance in AML-bearing mice, we took an unbiased approach aimed at identifying the APCs capable of engulfing circulating AML cells. Through both classical flow cytometry and ImageStream analysis, we identified that splenic $CD8\alpha^+$ DCs were exclusive in their ability to acquire proteins derived from circulating AML cells and to cross-present leukemia-derived antigens to $CD8^+$ T cells in a TAP-1-dependent manner **(Figures 9 and 11)**. Years ago, it was discovered that $CD8\alpha^+$ DCs could acquire cellular material from circulating apoptotic cells and subsequently induced T cell tolerance[155, 156]. We therefore hypothesized that dead or dying leukemia cells (or their cellular proteins) were being selectively phagocytosed by $CD8\alpha^+$ DCs in vivo. However, when C1498 cells were chemotherapy-treated **(Figure 8)** or irradiated **(data not shown)** prior to their IV inoculation, we did not observe a higher frequency of $CD8\alpha^+$ DCs that contained AML

cell-derived protein compared to an IV inoculation with live C1498 cells. Fascinatingly, proteins from dead or dying C1498 cells were much more likely to be engulfed by $CD11b^{+}F4/80^{+}$ macrophages suggesting that AML cell phagocytosis may be occurring in the absence of substantial cell death.

Possible mechanisms of AML cell uptake by $CD8\alpha^{+}$ DCs

The discovery that $CD8\alpha^{+}$ DCs were uniquely capable of acquiring leukemia cellular material, despite the wide variety of phagocytic cells that reside in the murine spleen, was remarkable. At present, however, the mechanism(s) utilized by $CD8\alpha^{+}$ DCs to specifically engulf AML cells is unclear. The fact that these DCs also exclusively acquired proteins derived from a second AML line, as well as from IV-inoculated B16 melanoma cells, suggests that this is a general property of $CD8\alpha^{+}$ DCs **(Figure 10)**. One possibility is that phagocytosis of leukemia cell-derived material by $CD8\alpha^{+}$ DCs could be due to their anatomical localization in the spleen which permitted primary access to leukemia cells entering the spleen from the vasculature. This hypothesis is supported from work by Steinman and colleges who identified a population of $CD8\alpha^{+}$ DCs in the splenic marginal zone which migrated into the T cell zone upon maturation[157]. $CD8\alpha^{+}$ DCs in the marginal zone would be perfectly positioned to encounter blood-borne leukemia cells entering the spleen.

Alternatively, $CD8\alpha^{+}$ DCs may express a unique repertoire of PRRs which allow their “sensing” of circulating leukemia cells. Favoring this hypothesis, we have begun to examine known endocytic receptors differentially or uniquely expressed on $CD8\alpha^{+}$ DCs

as candidates for regulating the engulfment and/or cross-presentation of AML antigens. DNGR-1 (encoded by *Clec9a*) is a c-type lectin most highly expressed by CD8α$^+$ DCs which recognizes polymerized actin filaments exposed on necrotic cells, and which mediates the cross-presentation of dead cell-associated antigens[120, 158, 159]. Although DNGR-1 has a hemi-ITAM motif with a Syk binding site important for its function, rigorous studies have demonstrated that DNGR-1 ligation does not activate DCs, at least at the level of costimulatory marker expression and cytokine production[160]. Further, DNGR-1 does not appear to affect the uptake of necrotic cargo, but rather supports antigen trafficking to recycling vesicles which prevent antigen degradation and promote cross-presentation[160]. Consistent with this, *Clec9a*$^{-/-}$ CD8α$^+$ DCs were not defective in their ability to engulf circulating leukemia cells **(Figure 24A)**. Additionally, the abortive proliferation of leukemia-specific CD8$^+$ T cells still occurred in *Clec9a*$^{-/-}$ mice **(Figures 24B)**. These data demonstrate that DNGR-1 is dispensable for the cross-presentation of antigens derived from circulating leukemia cells. While the mechanism as to how CD8α$^+$ DCs detect and acquire leukemia-derived antigens remains elusive, it is possible that other endocytic receptors expressed on CD8α$^+$ DCs are involved[19].

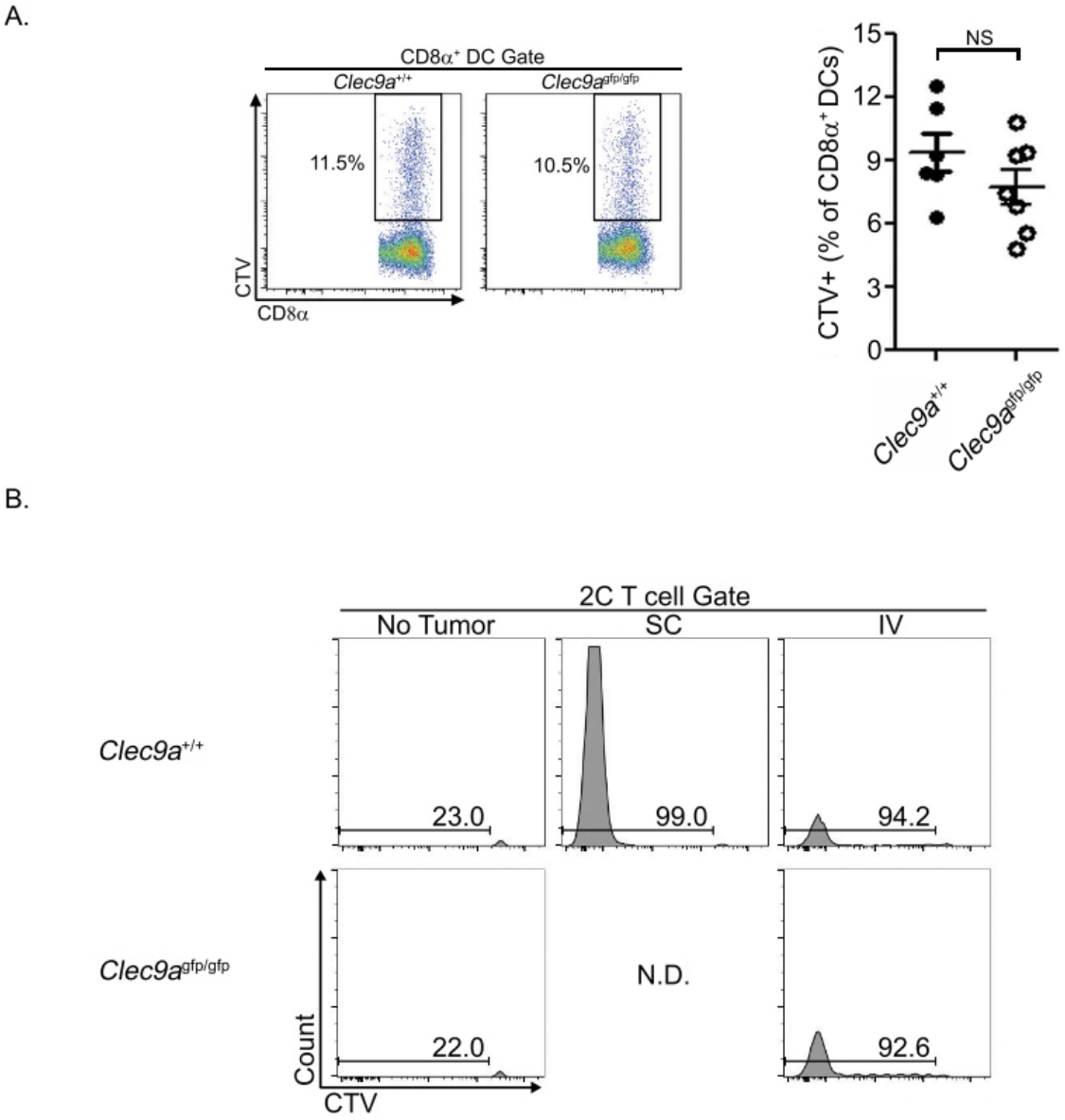

Figure 24. *Clec9a* is dispensible for the cross-presentation of AML-derived antigens in vivo. A) 4 x 10^6 C1498 AML cells were labeled with CTV and injected into *Clec9a*$^{+/+}$ or *Clec9a*$^{gfp/gfp}$ (*Clec9a*$^{-/-}$) mice. 3 hours later, spleen suspensions were stained with antibodies against CD11c and CD8α and analyzed via flow cytometry for CTV flouresence. **B)** 10^6 CTV-labeled CD8$^+$ 2C cells were transferred into *Clec9a*$^{+/+}$ or *Clec9a*$^{gfp/gfp}$ mice which received 10^6 C1498.SIY cells IV or SC the next day. Six days later, spleen cells were harvested and stained with antibodies against TCRβ, CD8α, CD45.1 and 1B2 to identify transferred 2C T cells. Plots shown are gated on 2C T cells. Data represent 2 independent experiments. NS- not signifigant, N.D. – not determined

CD8α$^+$ DCs are critical for leukemia-antigen recognition by CD8$^+$ T cells

Another remarkable finding from our experiments was that CD8α$^+$ DCs were essential in the generation of AML-specific immune responses. In leukemia-bearing *Batf3*$^{-/-}$ hosts, antigen-specific CD8$^+$ T cells were much less likely to proliferate or to upregulate CD69 and Nur77, which indicated that: 1) CD8α$^+$ DCs were necessary for leukemia antigen cross-presentation to CD8$^+$ T cells in vivo, and 2) in the absence of CD8α$^+$ DCs, host CD8$^+$ T cells appeared largely ignorant of leukemia-derived antigens **(Figure 12)**. Moreover, the frequency and number of leukemia-specific CD8$^+$ T cells recovered from AML-bearing *Batf3*$^{-/-}$ mice was similar to naïve mice, further supporting the conclusion that leukemia-specific T cells rarely encountered antigen in the absence of CD8α$^+$ DCs. The finding of immunological ignorance to leukemia-derived antigens in the absence of CD8α$^+$ DCs also argued that AML cells were not efficient at direct antigen presentation to CD8$^+$ T cells in vivo. Additionally, this data demonstrated that no other host APC can compensate and cross-present leukemia-derived antigens, even in the absence of CD8α$^+$ DCs.

CD8α$^+$ DCs induce leukemia-specific T cell tolerance

The observation that leukemia-specific CD8$^+$ T cells in *Batf3*$^{-/-}$ mice failed to encounter antigen strongly suggested that immature CD8α$^+$ DCs were actively driving T cell tolerance. That CD8α$^+$ DCs induce leukemia-specific T cell tolerance was subsequently shown in experiments in which “ignorant” leukemia specific CD8$^+$ T cells from IV challenged *Batf3*$^{-/-}$ mice responded to an immunogenic vaccine, but tolerized T

cells in C57BL/6 mice did not **(Figure 14)**. This result is consistent with a published report in a systemic lymphoma model which demonstrated that the induction of lymphoma-specific $CD4^+$ T cell anergy required presentation of lymphoma antigens by host bone marrow-derived cells[133]. However, the host APC that regulated lymphoma antigen-specific tolerance was not identified. Together, these data indicate that host APCs, and specifically $CD8\alpha^+$ DCs, play a critical role in inducing T cell tolerance against disseminated hematopoietic malignancies.

Our data highlights how the same DC subset can program different T cell fates in mice with solid versus hematopoietic malignancies. In response to a growing solid tumor, $CD8^+$ T cells become primed by mature Batf3-dependent DCs and impede disease progression, at least temporarily. In contrast, in animals with disseminated AML, our data suggest that leukemia-specific $CD8^+$ T cells are never functionally primed, but rather are rapidly tolerized by immature $CD8\alpha^+$ DCs early in the disease process. That $CD8\alpha^+$ DCs actively promoted T cell tolerance in mice with disseminated leukemia, but supported T cell priming in mice with solid tumors, hinted that the maturation status of this APC subset may drive T cell tolerance.

Immunotherapy against C1498 requires $CD8\alpha^+$ DCs

Consistent with the observation that $CD8^+$ T cells are never functionally primed in AML bearing mice, the most effective immunotherapies, at least in our hands, which activate host APCs, rather than those focused on re-activating dysfunctional $CD8^+$ T cells. For example, interrupting PD-1/PD-L1 interactions in leukemia-bearing mice

yielded a mild survival benefit but no mice were ever "cured" with this approach[110]. In contrast, anti-CD40, poly(I:C), and DMXAA all promoted impressive prolongation of survival of AML-bearing mice and, in some cases, these therapies led to disease cure. Alternatively, the data presented here suggest that AML may be an attractive disease target for adoptive cell therapy. Here, leukemia-specific $CD8^+$ T cells could be activated and expanded in vitro prior to autologous transfusion, bypassing tolerogenic antigen presentation by immature $CD8\alpha^+$ DCs.

Recently, two groups have indicated that the presence of Batf3-dependent DCs correlates with responses to PD-1/PD-L1 blockade therapy in pre-clinical solid cancer models[68, 69]. Similarly, we found that both anti-CD40 and poly(I:C) immunotherapies were ineffective in leukemia-bearing *Batf3*$^{-/-}$ mice **(Figure 15)**. However, these results need to be interpreted with caution, as Batf3-dependend DCs were required for antigen presentation in our AML model. Therefore, we cannot conclude with absolute certainty that the efficacy of these treatments relies on their ability to directly activate $CD8\alpha^+$ DCs. Regardless, it has become clear that the presence of Batf3-lineage DCs is critical for successful immunotherapy against both solid and blood cancers, and the presence of Batf3-dependent DCs in the tumor microenvironment has been correlated with improved survival in several types of solid cancer[67].

Disseminated AML fails to induce type I IFN

A growing body of evidence has defined a prominent role for Batf3-dependent $CD8\alpha^+$ and $CD103^+$ DCs in generating functional immune responses against solid

cancers. In contrast, our results demonstrate that the same DC subset which primes productive T cell responses against solid tumors actively promotes immune tolerance to systemic leukemia. These observations indicate that environmental cues perceived by $CD8\alpha^+$ DCs may regulate their ability to either prime or tolerize cancer-specific $CD8^+$ T cells. Keeping in mind the essential role of type I IFN in enabling $CD8\alpha^+$ DCs to activate $CD8^+$ T cell responses in hosts with solid tumors, we speculated that a lack of type I IFN induction in mice with AML may underlie the tolerogenic phenotype of $CD8\alpha^+$ DCs in this setting[64]. Whereas *ifnb* mRNA was readily detectable in the tumor-draining LNs of mice challenged SC with C1498 cells, it was not detectable in spleen cells of mice following an IV C1498 challenge. Moreover, the proliferation of leukemia-specific $CD8^+$ T cells occurred independently of type I IFN signaling in hosts with disseminated AML but was, as expected, severely affected in *Ifnar*$^{-/-}$ mice following a localized AML cell inoculation. Finally, wild-type and *Ifnar*$^{-/-}$ mice succumbed similarly following an IV C1498 inoculation **(Figure 16)**. Overall, these data suggest that a type I IFN response is not induced in AML-bearing mice. The observation that AML failed to induce a host type I IFN response is indicative of an impaired capacity of the innate immune system to respond to a disseminated leukemia.

Leukemia derived DNA can be detected in splenic $CD8\alpha^+$ DCs

We hypothesized that the lack of type I IFN production in AML bearing mice may be due to the failed activation of the STING pathway, which is required for IFN-β production in mice with solid tumors[63]. By labeling leukemia cells with CTV, we have

demonstrated that leukemia-derived proteins are actively acquired by splenic $CD8\alpha^+$ DCs. To determine if leukemia-derived DNA could also be detected in $CD8\alpha^+$ DCs, and thus trigger STING activation, C1498 cells were labeled with EDU overnight and subsequently labeled with CTV immediately prior to IV injection into recipient mice. As with CTV fluorescence, EDU was detected exclusively within $CD8\alpha^+$ DCs **(Figure 25A)**. When $CD8\alpha^+$ DCs were simultaneously analyzed for their uptake of tumor-derived DNA and protein via flow cytometry, three distinct populations of $CD8\alpha^+$ DCs were present: EDU^-CTV^-, EDU^-CTV^+ and EDU^+CTV^+ **(Figure 25B)**. Internalization of leukemia-derived DNA was confirmed via Image Stream analysis **(Figure 25C)**. Together, these data demonstrate that type I IFN is not induced following IV C1498 cell challenge, although leukemia-derived DNA can be detected within a small population of $CD8\alpha^+$ DCs. It is thus possible that: 1) leukemia cell-derived DNA fails to gain access to the cytosol of $CD8\alpha^+$ DCs in order to activate STING, 2) leukemia-derived DNA is transferred to the cytosol of $CD8\alpha^+$ DCs and activates STING, but negative regulatory pathways prevent downstream activation of type I IFN expression or 3) proper STING activation is occurring, but the small fraction of DNA-containing $CD8\alpha^+$ DCs is insufficient to generate an effective type I IFN response. Future work will focus on determining if the STING pathway is activated in $CD8\alpha^+$ DCs which contain AML-derived DNA.

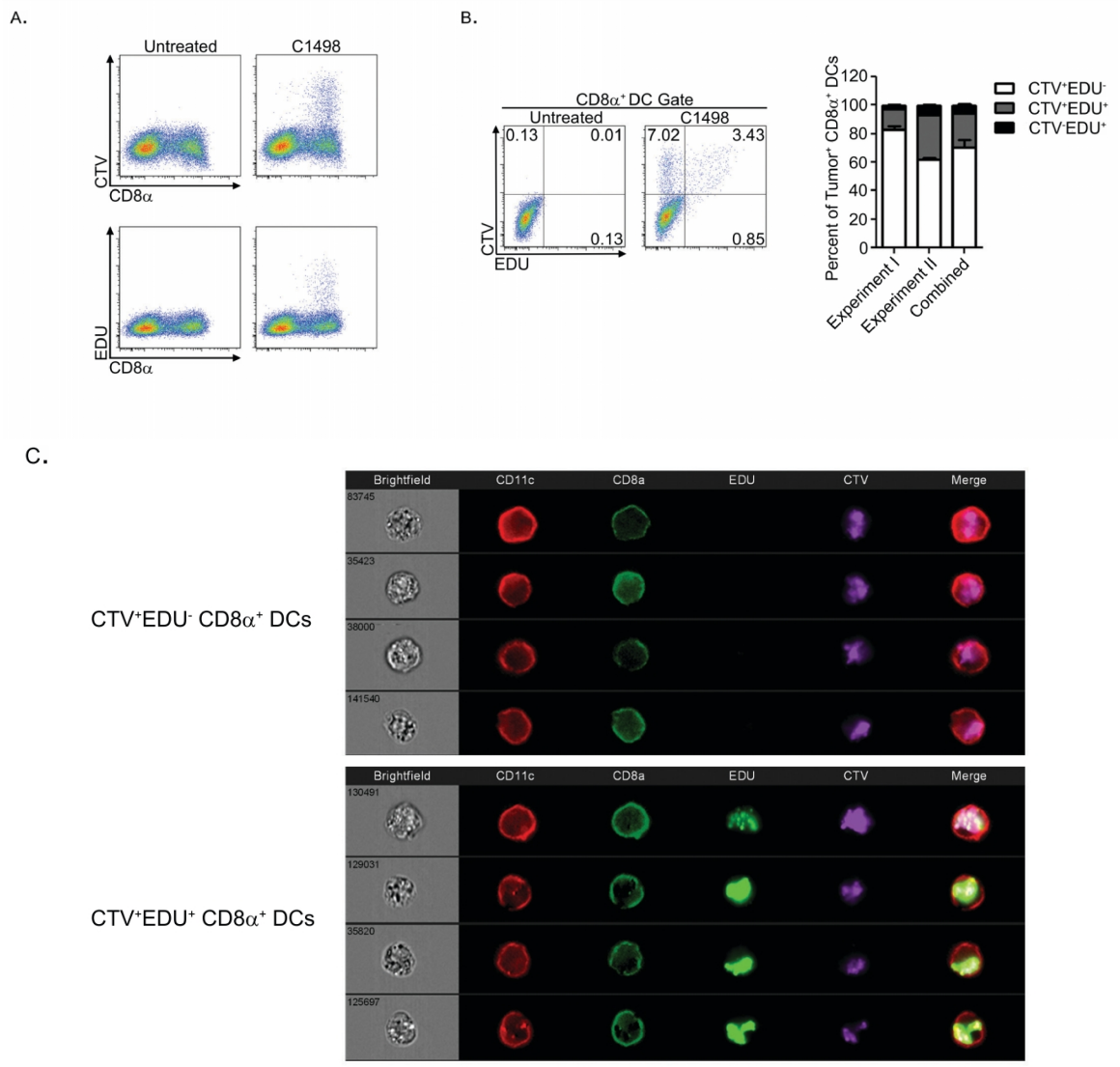

Figure 25. Tumor-engulfing CD8α$^+$ DCs contain leukemia-derived DNA. A-C) 4 x 10^6 C1498 AML cells were co-labeled with EDU and CTV as described in the materials and methods and injected into C57BL/6 mice. 3 hours later, spleen suspensions were stained with antibodies against CD11c and CD8α and analyzed via flow cytometry A) or ImageStream B) for leukemia derived protein (CTV) and DNA (EDU) within DCs. Data represent 2 independent experiments.

Activating STING to induce anti-leukemia immunity

Although we do not currently have a thorough understanding as to why type I IFN is not induced in mice with systemic AML, its critical role in bridging innate and adaptive tumor immune responses is clear. Thus, we postulated that purposeful induction of type I IFN in leukemia-bearing mice by activating the STING pathway would result in the generation of potent anti-leukemia immunity. We chose the synthetic murine STING agonist for this purpose. A single dose of DMXAA was sufficient to induce the production of type I IFN, as well as other pro-inflammatory cytokines, resulting in the maturation of host APCs, which culminated in markedly enhanced AML-specific $CD8^+$ T cell responses and prolonged survival **(Figures 17-21)**. The therapeutic effect of DMXAA was STING-dependent, and partially required type I IFN responsiveness in host cells. The latter finding is interesting, and suggests that additional cytokines, such as TNF-α, may be functioning downstream of STING activation to promote anti-leukemia immunity. In fact, DMXAA was initially shown to mediate cancer regression through an effect on tumor vasculature, largely through TNF-α[161, 162]. That STING activation stimulates production of a variety of cytokines aside from type I IFN argues that this approach might be superior to treatment with type I IFN alone, which has demonstrated limited clinical efficacy in AML[163, 164]. Furthermore, STING activation is more effective in controlling established leukemia than approaches targeting other nucleic acid sensing receptors, including toll-like receptor 3 (TLR3) and TLR9[122]. Another important observation was the requirement for adaptive immunity following STING activation, as DMXAA treatment was completely ineffective when administered to leukemia-bearing *Rag2*$^{-/-}$ mice **(Figure 22)**. This contrasts to what has been reported in some solid tumor

models, in which a partial T cell-independent effect of STING agonists has been described[124]. The T cell-independent effect of STING activation in solid tumors may be related to the well-known anti-angiogenic properties of IFN-β and vascular destructive effects of TNF-α[162, 165], which are perhaps more relevant in neo-vascularized solid cancers compared to acute leukemia.

Because DMXAA binds murine, but not human STING, we also tested cyclic-di-nucleotides (CDNs) as an alternative approach with translational potential for human cancers. Modified CDNs have been developed which bind both murine and all human STING alleles, and like DMXAA, potently induce activation of the STING axis. ML RR-S2 CDA (CDA), a lead CDN, has shown potent anti-tumor activity in several studies[124]. Systemic delivery of CDA to AML-bearing mice in our study was also effective at improving survival of AML-bearing animals, similar to DMXAA treatment **(Figure 21)**.

Combination therapy for AML

Although STING activation appears to be broadly effective as cancer immunotherapy, DMXAA was only able to cure ~50% of mice with established AML. Therefore, we speculate that counter-regulatory immune evasion pathways, including IFN-γ-induced PD-L1 upregulation in the tumor environment[73, 146], enhanced production of the immunosuppressive indolamine-2,3-dioxygenase (IDO) enzyme, and the influx of regulatory T cells into the tumor environment may limit its use as a single agent. Thus, defining immune escape pathways that are activated following STING agonist therapy,

and developing therapeutic combination strategies to override them will be important to consider in future studies.

The nature of antigens recognized by leukemia-specific T cells

Leukemia and other blood cancers typically harbor relatively low numbers of somatic mutations compared to most solid tumors. Therefore, the nature of the antigens derived from leukemia cells may be in the form of tumor associated antigens, rather than neoantigens derived from mutated cancer proteins. That being said, even cancers with small numbers of mutations can harbor immunogenic neoepitopes. Additionally, the nature of the antigens recognized by $CD8^+$ T cells could vary based on the type of leukemia. For example, in chronic myeloid leukemia (CML), a disease-defining chromosomal translocation creates a fusion protein of the breakpoint cluster region (BCR) and ABL1 proteins, which results in constitutive kinase activity and drives tumorigenesis. Here, the BCR-Abl breakpoint is a potential neo-antigen which would be shared among all patients with CML, although peptides derived from partial processing of this fusion protein may not be able to bind all human HLA haplotypes. Interesting, $CD4^+$ T cell responses can be generated against the BCR-ABL fusion protein; however, a substantial portion of BCR-ABL specific cells are $Foxp3^+$ regulatory T cells which have been suggested to promote disease progression[166].

The generation of a T cell clone which recognizes an endogenous AML-antigen

Because no naturally occurring AML-derived antigen is currently known, we utilized the model SIY peptide as a leukemia specific antigen in these studies. It is likely that the mechanisms of tolerance in AML bearing mice are shaped by the nature of the antigens recognized by $CD8^+$ T cells (TSA vs TAA). Several years ago a $CD8^+$ T cell clone (termed T15) was found to be reactive against parental C1498 cells, and promoted prolonged survival of leukemia-bearing mice following adoptive transfer[167]. Spectratyping cDNA of this C1498 reactive T cell clone, revealed its expression of TCR Vβ1 and Vα10 **(data not shown).** When the T15 TCRα and β were retrovirally transduced into $CD8^+$ T cells, we found that this TCR was uniquely reactive against C1498, and did not recognize other tumor cells lines, including the virally induced leukemia cell line FBL **(Figure 26).** Importantly, the recognition of C1498 was MHC I restricted and could be blocked with the addition of a blocking anti-H-$2K^bD^b$ antibody. We are currently in the process of generating a transgenic mouse expressing the TCR from this C1498-specific clone in order to be able to study its homeostasis and functionality in leukemia-bearing mice, as well as to define the antigen it naturally recognizes.

A.

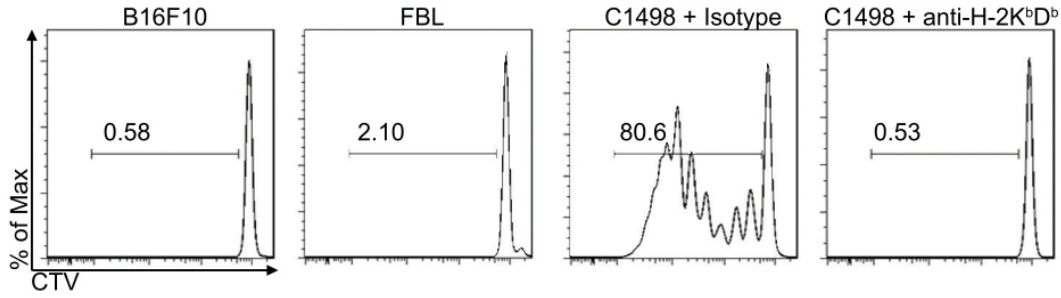

Figure 26. A T cell clone which specifically recognizes a natural C1498-derived antigen. A) The TCRβ and α chains of a T cell clone (termed T15) reported to recognize C1498 were cloned and retrovirally introduced into *Rag2*$^{-/-}$ bone marrow. This bone marrow was used to reconstitute a lethally irradiated C57BL/6 mouse and splenocytes from this chimera were CTV-labeled and cultured with irradiated B16F10 (melanoma), FBL (a virally induced AML) or C1498 cells in vitro for 4 days. CTV dilution was then analyzed via FACs. In some cultures, T15 cells were cultured in the presence of 1μg/ml anti-H2-K^{b}D^b or isotype control antibody. Data represent 2 independent experiments.

Summary

While much is known about how solid tumors can evade immune detection and destruction, relatively little is known about immune evasion mechanisms employed by hematopoietic malignancies, like AML. Using a transplantable mouse model of AML, we discovered a unique immune evasion mechanism in mice with systemic leukemia. In AML-bearing mice, we found that leukemia-specific $CD8^+$ T cells were subjected to abortive proliferation, and subsequent deletion, leading to a dense state of T cell tolerance. We later went onto show that $CD8\alpha^+$ DCs were acquiring and cross-presenting leukemia-derived antigens and ultimately promoting the T cell tolerant state. The discovery that $CD8\alpha^+$ DCs mediate AML-specific T cell tolerance is critical in that it highlights a stark difference in the regulation of immune responses to solid versus hematological cancers, and also because it may facilitate the development of immunotherapeutic strategies to specifically target the activation of these DCs. For example, we have shown that $CD8\alpha^+$ DCs, which express high levels of TLR-3, can be successfully targeted with a synthetic agonist, resulting in enhanced leukemia-specific T cell responses and protection from AML progression. Additionally, the STING agonist DMXAA was able to activate host APCs, including $CD8\alpha^+$ DCs, and ultimately reverse T cell tolerance in AML-bearing mice. These results provide the framework to incorporate approaches aimed at activating $CD8\alpha^+$ DCs into immunotherapies for patients with systemic hematological cancers.

REFERENCES

1. Bassing CH, Swat W, Alt FW. The Mechanism and Regulation of Chromosomal V(D)J Recombination. *Cell* 2002, **109**(2, Supplement 1)**:** S45-S55.

2. Nikolich-Zugich J, Slifka MK, Messaoudi I. The many important facets of T-cell repertoire diversity. *Nature reviews Immunology* 2004, **4**(2)**:** 123-132.

3. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature reviews Immunology* 2014, **14**(6)**:** 377-391.

4. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *The Journal of Immunology* 1995, **155**(3)**:** 1151-1164.

5. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nature immunology* 2007, **8**(2)**:** 191-197.

6. Steinman RM, Hawiger D, Nussenzweig MC. TOLEROGENIC DENDRITIC CELLS. *Annual review of immunology* 2003, **21**(1)**:** 685-711.

7. Kurts C, Kosaka H, Carbone FR, Miller JF, Heath WR. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *The Journal of experimental medicine* 1997, **186**(2)**:** 239-245.

8. Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *The Journal of experimental medicine* 2002, **196**(12)**:** 1627-1638.

9. Luckashenak N, Schroeder S, Endt K, Schmidt D, Mahnke K, Bachmann MF*, et al.* Constitutive crosspresentation of tissue antigens by dendritic cells controls CD8+ T cell tolerance in vivo. *Immunity* 2008, **28**(4)**:** 521-532.

10. Iwasaki A, Medzhitov R. Control of adaptive immunity by the innate immune system. *Nature immunology* 2015, **16**(4)**:** 343-353.

11. Medzhitov R, Janeway CA. Decoding the Patterns of Self and Nonself by the Innate Immune System. *Science* 2002, **296**(5566)**:** 298-300.

12. Matzinger P. The danger model: a renewed sense of self. *Science* 2002, **296**(5566)**:** 301-305.

13. Guermonprez P, Valladeau J, Zitvogel L, Théry C, Amigorena S. ANTIGEN PRESENTATION AND T CELL STIMULATION BY DENDRITIC CELLS. *Annual review of immunology* 2002, **20**(1)**:** 621-667.

14. Blum JS, Wearsch PA, Cresswell P. Pathways of Antigen Processing. *Annual review of immunology* 2013, **31:** 443-473.

15. Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *The Journal of experimental medicine* 1976, **143**(5)**:** 1283-1288.

16. Bevan MJ. Minor H Antigens Introduced on H-2 Different Stimulating Cells Cross-React at the Cytotoxic T Cell Level during in Vivo Priming. *The Journal of Immunology* 1976, **117**(6)**:** 2233-2238.

17. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. *Nature reviews Immunology* 2012, **12**(8)**:** 557-569.

18. Cresswell P, Ackerman AL, Giodini A, Peaper DR, Wearsch PA. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunological reviews* 2005, **207**(1)**:** 145-157.

19. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annual review of immunology* 2013, **31:** 563-604.

20. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nature reviews Immunology* 2015, **15**(8)**:** 471-485.

21. Vremec D, Pooley J, Hochrein H, Wu L, Shortman K. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *Journal of immunology* 2000, **164**(6)**:** 2978-2986.

22. Shortman K, Heath WR. The CD8+ dendritic cell subset. *Immunological reviews* 2010, **234**(1)**:** 18-31.

23. Edelson BT, KC W, Juang R, Kohyama M, Benoit LA, Klekotka PA*, et al.* Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8α+ conventional dendritic cells. *The Journal of experimental medicine* 2010, **207**(4)**:** 823-836.

24. den Haan JMM, Lehar SM, Bevan MJ. Cd8+ but Not Cd8− Dendritic Cells Cross-Prime Cytotoxic T Cells in Vivo. *The Journal of experimental medicine* 2000, **192**(12)**:** 1685-1696.

25. den Haan JMM, Bevan MJ. Constitutive versus Activation-dependent Cross-Presentation of Immune Complexes by CD8+ and CD8- Dendritic Cells In Vivo. *Journal of Experimental Medicine* 2002, **196**(6)**:** 817-827.

26. Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S*, et al.* Differential antigen processing by dendritic cell subsets in vivo. *Science* 2007, **315**(5808)**:** 107-111.

27. Schnorrer P, Behrens GM, Wilson NS, Pooley JL, Smith CM, El-Sukkari D*, et al.* The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**(28)**:** 10729-10734.

28. Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 2005, **307**(5715)**:** 1630-1634.

29. Savina A, Jancic C, Hugues S, Guermonprez P, Vargas P, Moura IC*, et al.* NOX2 Controls Phagosomal pH to Regulate Antigen Processing during Crosspresentation by Dendritic Cells. *Cell* 2006, **126**(1)**:** 205-218.

30. Savina A, Peres A, Cebrian I, Carmo N, Moita C, Hacohen N*, et al.* The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. *Immunity* 2009, **30**(4)**:** 544-555.

31. Vander Lugt B, Khan AA, Hackney JA, Agrawal S, Lesch J, Zhou M*, et al.* Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nature immunology* 2014, **15**(2)**:** 161-167.

32. Satpathy AT, Wu X, Albring JC, Murphy KM. Re(de)fining the dendritic cell lineage. *Nature immunology* 2012, **13**(12)**:** 1145-1154.

33. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M*, et al.* Batf3 Deficiency Reveals a Critical Role for CD8α+ Dendritic Cells in Cytotoxic T Cell Immunity. *Science* 2008, **322**(5904)**:** 1097-1100.

34. Mouriès J, Moron G, Schlecht G, Escriou N, Dadaglio G, Leclerc C. Plasmacytoid dendritic cells efficiently cross-prime naive T cells in vivo after TLR activation. *Blood* 2008, **112**(9)**:** 3713-3722.

35. Parish CR. Cancer immunotherapy: The past, the present and the future. *Immunology and cell biology* 2003, **81**(2)**:** 106-113.

36. Gross L. Intradermal Immunization of C3H Mice against a Sarcoma That Originated in an Animal of the Same Line. *Cancer research* 1943, **3**(5)**:** 326-333.

37. Foley EJ. Antigenic Properties of Methylcholanthrene-induced Tumors in Mice of the Strain of Origin. *Cancer research* 1953, **13**(12)**:** 835-837.

38. Klein G, Sjögren HO, Klein E, Hellström KE. Demonstration of Resistance against Methylcholanthreneinduced Sarcomas in the Primary Autochthonous Host. *Cancer research* 1960, **20**(11)**:** 1561-1572.

39. Coulie PG, Van den Eynde BJ, van der Bruggen P, Boon T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nature reviews Cancer* 2014, **14**(2)**:** 135-146.

40. Boon T, Kellermann O. Rejection by syngeneic mice of cell variants obtained by mutagenesis of a malignant teratocarcinoma cell line. *Proceedings of the National Academy of Sciences of the United States of America* 1977, **74**(1)**:** 272-275.

41. Boon T, Van Pel A. Teratocarcinoma cell variants rejected by syngeneic mice: protection of mice immunized with these variants against other variants and against the original malignant cell line. *Proceedings of the National Academy of Sciences of the United States of America* 1978, **75**(3)**:** 1519-1523.

42. Fratta E, Coral S, Covre A, Parisi G, Colizzi F, Danielli R*, et al.* The biology of cancer testis antigens: Putative function, regulation and therapeutic potential. *Molecular oncology* 2011, **5**(2)**:** 164-182.

43. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B*, et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991, **254**(5038)**:** 1643-1647.

44. Weon JL, Potts PR. The MAGE protein family and cancer. *Current Opinion in Cell Biology* 2015, **37:** 1-8.

45. Smith HA, McNeel DG. The SSX Family of Cancer-Testis Antigens as Target Proteins for Tumor Therapy. *Clinical and Developmental Immunology* 2010, **2010:** 18.

46. Chen Y-T, Scanlan MJ, Sahin U, Türeci Ö, Gure AO, Tsang S*, et al.* A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proceedings of the National Academy of Sciences* 1997, **94**(5)**:** 1914-1918.

47. Gnjatic S, Nishikawa H, Jungbluth AA, Güre AO, Ritter G, Jäger E*, et al.* NY-ESO-1: Review of an Immunogenic Tumor Antigen. *Advances in Cancer Research*, vol. Volume 95. Academic Press, 2006, pp 1-30.

48. Ruiz R, Hunis B, Raez LE. Immunotherapeutic Agents in Non-small-cell Lung Cancer Finally Coming to the Front Lines. *Current Oncology Reports* 2014, **16**(9)**:** 1-10.

49. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science* 2015, **348**(6230)**:** 69-74.

50. Robbins PF, Lu Y-C, El-Gamil M, Li YF, Gross C, Gartner J*, et al.* Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nature medicine* 2013, **19**(6)**:** 747-752.

51. Castle JC, Kreiter S, Diekmann J, Löwer M, van de Roemer N, de Graaf J*, et al.* Exploiting the Mutanome for Tumor Vaccination. *Cancer research* 2012, **72**(5)**:** 1081-1091.

52. Tran E, Turcotte S, Gros A, Robbins PF, Lu Y-C, Dudley ME*, et al.* Cancer Immunotherapy Based on Mutation-Specific CD4+ T Cells in a Patient with Epithelial Cancer. *Science* 2014, **344**(6184)**:** 641-645.

53. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin AV*, et al.* Signatures of mutational processes in human cancer. *Nature* 2013, **500**(7463)**:** 415-421.

54. Tran E, Ahmadzadeh M, Lu Y-C, Gros A, Turcotte S, Robbins PF*, et al.* Immunogenicity of somatic mutations in human gastrointestinal cancers. *Science* 2015, **350**(6266)**:** 1387-1390.

55. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. *Immunity* 2013, **39**(1)**:** 1-10.

56. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. *Nature reviews Immunology* 2009, **9**(5)**:** 353-363.

57. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nature immunology* 2013, **14**(10)**:** 1014-1022.

58. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C*, et al.* Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. *Science* 2006, **313**(5795)**:** 1960-1964.

59. Diamond MS, Kinder M, Matsushita H, Mashayekhi M, Dunn GP, Archambault JM*, et al.* Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *The Journal of experimental medicine* 2011, **208**(10)**:** 1989-2003.

60. Fuertes MB, Kacha AK, Kline J, Woo SR, Kranz DM, Murphy KM*, et al.* Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. *The Journal of experimental medicine* 2011, **208**(10)**:** 2005-2016.

61. Gajewski TF, Louahed J, Brichard VG. Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. *Cancer journal* 2010, **16**(4)**:** 399-403.

62. Snijders AM, Langley S, Mao JH, Bhatnagar S, Bjornstad KA, Rosen CJ*, et al.* An interferon signature identified by RNA-sequencing of mammary tissues varies across the estrous cycle and is predictive of metastasis-free survival. *Oncotarget* 2014, **5**(12)**:** 4011-4025.

63. Woo SR, Fuertes MB, Corrales L, Spranger S, Furdyna MJ, Leung MY*, et al.* STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity* 2014, **41**(5)**:** 830-842.

64. Woo SR, Corrales L, Gajewski TF. Innate Immune Recognition of Cancer. *Annual review of immunology* 2015.

65. Asano K, Nabeyama A, Miyake Y, Qiu CH, Kurita A, Tomura M*, et al.* CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. *Immunity* 2011, **34**(1)**:** 85-95.

66. Backer R, Schwandt T, Greuter M, Oosting M, Jungerkes F, Tuting T*, et al.* Effective collaboration between marginal metallophilic macrophages and CD8+ dendritic cells in the generation of cytotoxic T cells. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(1)**:** 216-221.

67. Broz ML, Binnewies M, Boldajipour B, Nelson AE, Pollack JL, Erle DJ*, et al.* Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer cell* 2014, **26**(5)**:** 638-652.

68. Salmon H, Idoyaga J, Rahman A, Leboeuf M, Remark R, Jordan S*, et al.* Expansion and Activation of CD103(+) Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity* 2016, **44**(4)**:** 924-938.

69. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic [bgr]-catenin signalling prevents anti-tumour immunity. *Nature* 2015, **523**(7559)**:** 231-235.

70. Driessens G, Kline J, Gajewski TF. Costimulatory and coinhibitory receptors in anti-tumor immunity. *Immunological reviews* 2009, **229**(1)**:** 126-144.

71. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and Its Ligands in Tolerance and Immunity. *Annual review of immunology* 2008, **26**(1)**:** 677-704.

72. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nature reviews Immunology* 2004, **4**(5)**:** 336-347.

73. Spranger S, Spaapen RM, Zha Y, Williams J, Meng Y, Ha TT*, et al.* Up-Regulation of PD-L1, IDO, and Tregs in the Melanoma Tumor Microenvironment Is Driven by CD8+ T Cells. *Science translational medicine* 2013, **5**(200)**:** 200ra116-200ra116.

74. Cynthia A. Chambers, Michael S. Kuhns, Jackson G. Egen a, Allison*2 JP. CTLA-4-MEDIATED INHIBITION IN REGULATION OF T CELL RESPONSES: Mechanisms and Manipulation in Tumor Immunotherapy. *Annual review of immunology* 2001, **19**(1)**:** 565-594.

75. Collins AV, Brodie DW, Gilbert RJC, Iaboni A, Manso-Sancho R, Walse B*, et al.* The Interaction Properties of Costimulatory Molecules Revisited. *Immunity* 2002, **17**(2)**:** 201-210.

76. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM*, et al.* Trans-Endocytosis of CD80 and CD86: A Molecular Basis for the Cell-Extrinsic Function of CTLA-4. *Science* 2011, **332**(6029)**:** 600-603.

77. Blank C, Brown I, Peterson AC, Spiotto M, Iwai Y, Honjo T*, et al.* PD-L1/B7H-1 Inhibits the Effector Phase of Tumor Rejection by T Cell Receptor (TCR) Transgenic CD8+ T Cells. *Cancer research* 2004, **64**(3)**:** 1140-1145.

78. deLeeuw RJ, Kost SE, Kakal JA, Nelson BH. The Prognostic Value of FoxP3+ Tumor-Infiltrating Lymphocytes in Cancer: A Critical Review of the Literature. *American Association for Cancer Research* 2012, **18**(11)**:** 3022-3029.

79. Hsieh C-S, Lee H-M, Lio C-WJ. Selection of regulatory T cells in the thymus. *Nature reviews Immunology* 2012, **12**(3)**:** 157-167.

80. Chen W, Jin W, Hardegen N, Lei K-j, Li L, Marinos N*, et al.* Conversion of Peripheral CD4+CD25− Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3. *The Journal of experimental medicine* 2003, **198**(12)**:** 1875-1886.

81. Moo-Young TA, Larson JW, Belt BA, Tan MC, Hawkins WG, Eberlein TJ*, et al.* Tumor derived TGF-Beta mediates conversion of CD4+Foxp3+ regulatory T cells in a murine model of pancreas cancer. *Journal of immunotherapy (Hagerstown, Md : 1997)* 2009, **32**(1)**:** 10.1097/CJI.1090b1013e318189f318113c.

82. Malchow S, Leventhal DS, Nishi S, Fischer BI, Shen L, Paner GP*, et al.* Aire-Dependent Thymic Development of Tumor-Associated Regulatory T Cells. *Science* 2013, **339**(6124)**:** 1219-1224.

83. Savage PA, Malchow S, Leventhal DS. Basic principles of tumor-associated regulatory T cell biology. *Trends in Immunology* 2013, **34**(1)**:** 33-40.

84. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang L-P*, et al.* Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and Treg cells. *Nature* 2011, **475**(7355)**:** 226-230.

85. Dunn GP, Old LJ, Schreiber RD. The Three Es of Cancer Immunoediting. *Annual review of immunology* 2004, **22**(1)**:** 329-360.

86. Schreiber RD, Old LJ, Smyth MJ. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. *Science* 2011, **331**(6024)**:** 1565-1570.

87. Cabrera CM, Jiménez P, Cabrera T, Esparza C, Ruiz-Cabello F, Garrido F. Total loss of MHC class I in colorectal tumors can be explained by two molecular pathways: β2-microglobulin inactivation in MSI-positive tumors and LMP7/TAP2 downregulation in MSI-negative tumors. *Tissue antigens* 2003, **61**(3)**:** 211-219.

88. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* 2015, **160**(1-2)**:** 48-61.

89. Korman AJ, Peggs KS, Allison JP. Checkpoint Blockade in Cancer Immunotherapy. *Advances in immunology* 2006, **90:** 297-339.

90. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB*, et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *The New England journal of medicine* 2010, **363**(8)**:** 711-723.

91. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF*, et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *The New England journal of medicine* 2012, **366**(26)**:** 2443-2454.

92. Chen L, Han X. Anti–PD-1/PD-L1 therapy of human cancer: past, present, and future. *The Journal of clinical investigation*, **125**(9)**:** 3384-3391.

93. Herbst RS, Soria J-C, Kowanetz M, Fine GD, Hamid O, Gordon MS*, et al.* Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* 2014, **515**(7528)**:** 563-567.

94. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJM, Robert L*, et al.* PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 2014, **515**(7528)**:** 568-571.

95. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* 2015, **348**(6230)**:** 62-68.

96. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ*, et al.* Cancer Regression and Autoimmunity in Patients After Clonal Repopulation with Antitumor Lymphocytes. *Science* 2002, **298**(5594)**:** 850-854.

97. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer research* 1999, **59**(13)**:** 3128-3133.

98. Bos PD, Plitas G, Rudra D, Lee SY, Rudensky AY. Transient regulatory T cell ablation deters oncogene-driven breast cancer and enhances radiotherapy. *The Journal of experimental medicine* 2013, **210**(11)**:** 2435-2466.

99. Attia P, Maker AV, Haworth LR, Rogers-Freezer L, Rosenberg SA. Inability of a Fusion Protein of IL-2 and Diphtheria Toxin (Denileukin Diftitox, DAB(389)IL-2, ONTAK) to Eliminate Regulatory T Lymphocytes in Patients With Melanoma. *Journal of immunotherapy (Hagerstown, Md : 1997)* 2005, **28**(6)**:** 582-592.

100. Barrett DM, Grupp SA, June CH. Chimeric Antigen Receptor– and TCR-Modified T Cells Enter Main Street and Wall Street. *The Journal of Immunology* 2015, **195**(3)**:** 755-761.

101. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR*, et al.* Chimeric Antigen Receptor–Modified T Cells for Acute Lymphoid Leukemia. *New England Journal of Medicine* 2013, **368**(16)**:** 1509-1518.

102. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. *New England Journal of Medicine* 2015, **373**(12)**:** 1136-1152.

103. Teague RM, Kline J. Immune evasion in acute myeloid leukemia: current concepts and future directions. *Journal for immunotherapy of cancer* 2013, **1**(13).

104. Greiner J, Bullinger L, Guinn B-a, Döhner H, Schmitt M. Leukemia-Associated Antigens Are Critical for the Proliferation of Acute Myeloid Leukemia Cells. *American Association for Cancer Research* 2008, **14**(22)**:** 7161-7166.

105. Ohminami H, Yasukawa M, Fujita S. HLA class I-restricted lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte clone specific for WT1 peptide. *Blood* 2000, **95**(1)**:** 286-293.

106. Di Stasi A, Jimenez AM, Minagawa K, Al-Obaidi M, Rezvani K. Review of the results of WT1 peptide vaccination strategies for myelodysplastic syndromes and acute myeloid leukemia from 9 different studies. *Frontiers in immunology* 2015, **6**.

107. Szczepanski MJ, Szajnik M, Czystowska M, Mandapathil M, Strauss L, Welsh A*, et al.* Increased Frequency and Suppression by Regulatory T Cells in Patients with Acute Myelogenous Leukemia. *American Association for Cancer Research* 2009, **15**(10)**:** 3325-3332.

108. Zhou Q, Bucher C, Munger ME, Highfill SL, Tolar J, Munn DH*, et al.* Depletion of endogenous tumor-associated regulatory T cells improves the efficacy of adoptive cytotoxic T-cell immunotherapy in murine acute myeloid leukemia. *Blood* 2009, **114**(18)**:** 3793-3802.

109. Zhou Q, Munger ME, Highfill SL, Tolar J, Weigel BJ, Riddle M*, et al.* Program death-1 signaling and regulatory T cells collaborate to resist the function of adoptively transferred cytotoxic T lymphocytes in advanced acute myeloid leukemia. *Blood* 2010, **116**(14)**:** 2484-2493.

110. Zhang L, Gajewski TF, Kline J. PD-1/PD-L1 interactions inhibit antitumor immune responses in a murine acute myeloid leukemia model. *Blood* 2009, **114**(8)**:** 1545-1552.

111. Zhang L, Chen X, Liu X, Kline DE, Teague RM, Gajewski TF*, et al.* CD40 ligation reverses T cell tolerance in acute myeloid leukemia. *The Journal of clinical investigation* 2013, **123**(5)**:** 1999-2010.

112. Curran E, Chen X, Corrales L, Kline DE, Dubensky TW, Jr., Duttagupta P*, et al.* STING Pathway Activation Stimulates Potent Immunity against Acute Myeloid Leukemia. *Cell reports* 2016, **15**(11)**:** 2357-2366.

113. Reinhardt RL, Hong S, Kang S-J, Wang Z-e, Locksley RM. Visualization of IL-12/23p40 In Vivo Reveals Immunostimulatory Dendritic Cell Migrants that Promote Th1 Differentiation. *The Journal of Immunology* 2006, **177**(3)**:** 1618-1627.

114. Kaer LV, Ashton-Rickardt PG, Ploegh HL, Tonegawa S. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4−8+ T cells. *Cell* 1992, **71**(7)**:** 1205-1214.

115. Spiotto MT, Yu P, Rowley DA, Nishimura MI, Meredith SC, Gajewski TF*, et al.* Increasing tumor antigen expression overcomes "ignorance" to solid tumors via crosspresentation by bone marrow-derived stromal cells. *Immunity* 2002, **17**(6)**:** 737-747.

116. Chao DT LG, Boise LH, White LS, Thompson GB et al. Bcl-XL and Bcl-2 repress a common pathway of cell death. *The Journal of experimental medicine* 1995, **182**(3)**:** 821-828.

117. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J*, et al.* T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *The Journal of experimental medicine* 2011, **208**(6)**:** 1279-1289.

118. Muller U, Steinhoff U, Reis L, Hemmi S, Pavlovic J, Zinkernagel R*, et al.* Functional role of type I and type II interferons in antiviral defense. *Science* 1994, **264**(5167)**:** 1918-1921.

119. Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* 2008, **455**(7213)**:** 674-678.

120. Sancho D, Joffre OP, Keller AM, Rogers NC, Martinez D, Hernanz-Falcon P*, et al.* Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 2009, **458**(7240)**:** 899-903.

121. Kuo Y-H, Landrette SF, Heilman SA, Perrat PN, Garrett L, Liu PP*, et al.* Cbfβ-SMMHC induces distinct abnormal myeloid progenitors able to develop acute myeloid leukemia. *Cancer cell* 2006, **9**(1)**:** 57-68.

122. Hossain DMS, Dos Santos C, Zhang Q, Kozlowska A, Liu H, Gao C*, et al.* Leukemia cell–targeted STAT3 silencing and TLR9 triggering generate systemic antitumor immunity. *Blood* 2014, **123**(1)**:** 15-25.

123. Flies DB, Chen L. A simple and rapid vortex method for preparing antigen/adjuvant emulsions for immunization. *Journal of immunological methods* 2003, **276**(1-2)**:** 239-242.

124. Corrales L, Glickman LH, McWhirter SM, Kanne DB, Sivick KE, Katibah GE*, et al.* Direct Activation of STING in the Tumor Microenvironment Leads to Potent and Systemic Tumor Regression and Immunity. *Cell reports* 2015, **11**(7)**:** 1018-1030.

125. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL*, et al.* Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nature medicine* 2007, **13**(1)**:** 54-61.

126. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A*, et al.* Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nature medicine* 2007, **13**(9)**:** 1050-1059.

127. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nature reviews Immunology* 2006, **6**(4)**:** 295-307.

128. Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. *Cancer immunology, immunotherapy : CII* 2010, **59**(10)**:** 1593-1600.

129. Wekerle T, Kurtz J, Bigenzahn S, Takeuchi Y, Sykes M. Mechanisms of transplant tolerance induction using costimulatory blockade. *Current opinion in immunology* 2002, **14**(5)**:** 592-600.

130. Diehl L, den Boer AT, Schoenberger SP, van der Voort EI, Schumacher TN, Melief CJ*, et al.* CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nature medicine* 1999, **5**(7)**:** 774-779.

131. Sotomayor EM, Borrello I, Tubb E, Rattis F-M, Bien H, Lu Z*, et al.* Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through in vivo ligation of CD40. *Nature medicine* 1999, **5**(7)**:** 780-787.

132. French RR, Chan HTC, Tutt AL, Glennie MJ. CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. *Nature medicine* 1999, **5**(5)**:** 548-553.

133. Sotomayor EM, Borrello I, Rattis F-M, Cuenca AG, Abrams J, Staveley-O'Carroll K*, et al.* Cross-presentation of tumor antigens by bone marrow–derived antigen-presenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression. *Blood* 2001, **98**(4)**:** 1070-1077.

134. Staveley-O'Carroll K, Sotomayor E, Montgomery J, Borrello I, Hwang L, Fein S*, et al.* Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proceedings of the National Academy of Sciences* 1998, **95**(3)**:** 1178-1183.

135. Cebrian I, Visentin G, Blanchard N, Jouve M, Bobard A, Moita C*, et al.* Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. *Cell* 2011, **147**(6)**:** 1355-1368.

136. Jelinek I, Leonard JN, Price GE, Brown KN, Meyer-Manlapat A, Goldsmith PK*, et al.* TLR3-Specific Double-Stranded RNA Oligonucleotide Adjuvants Induce Dendritic Cell Cross-Presentation, CTL Responses, and Antiviral Protection. *The Journal of Immunology* 2011, **186**(4)**:** 2422-2429.

137. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R*, et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *The New England journal of medicine* 2005, **353**(25)**:** 2654-2666.

138. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH*, et al.* Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2011, **29**(15)**:** 1949-1955.

139. Hwang WT, Adams SF, Tahirovic E, Hagemann IS, Coukos G. Prognostic significance of tumor-infiltrating T cells in ovarian cancer: a meta-analysis. *Gynecologic oncology* 2012, **124**(2)**:** 192-198.

140. Gajewski TF. Failure at the Effector Phase: Immune Barriers at the Level of the Melanoma Tumor Microenvironment. *Clinical Cancer Research* 2007, **13**(18)**:** 5256-5261.

141. Dunn GP, Bruce AT, Sheehan KC, Shankaran V, Uppaluri R, Bui JD*, et al.* A critical function for type I interferons in cancer immunoediting. *Nature immunology* 2005, **6**(7)**:** 722-729.

142. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. *Science* 2013, **339**(6121)**:** 786-791.

143. Wu J, Sun L, Chen X, Du F, Shi H, Chen C*, et al.* Cyclic GMP-AMP Is an Endogenous Second Messenger in Innate Immune Signaling by Cytosolic DNA. *Science* 2013, **339**(6121)**:** 826-830.

144. Ishikawa H, Ma Z, Barber GN. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* 2009, **461**(7265)**:** 788-792.

145. Chen H, Sun H, You F, Sun W, Zhou X, Chen L*, et al.* Activation of STAT6 by STING Is Critical for Antiviral Innate Immunity. *Cell*, **147**(2)**:** 436-446.

146. Fu J, Kanne DB, Leong M, Glickman LH, McWhirter SM, Lemmens E*, et al.* STING agonist formulated cancer vaccines can cure established tumors resistant to PD-1 blockade. *Science translational medicine* 2015, **7**(283)**:** 283ra252-283ra252.

147. Montoya M, Schiavoni G, Mattei F, Gresser I, Belardelli F, Borrow P*, et al.* Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* 2002, **99**(9)**:** 3263-3271.

148. Brown SD, Warren RL, Gibb EA, Martin SD, Spinelli JJ, Nelson BH*, et al.* Neo-antigens predicted by tumor genome meta-analysis correlate with increased patient survival. *Genome Research* 2014, **24**(5)**:** 743-750.

149. Ohlen C, Kalos M, Cheng LE, Shur AC, Hong DJ, Carson BD*, et al.* CD8(+) T cell tolerance to a tumor-associated antigen is maintained at the level of expansion rather than effector function. *J Exp Med* 2002, **195**(11)**:** 1407-1418.

150. Teague RM, Sather BD, Sacks JA, Huang MZ, Dossett ML, Morimoto J*, et al.* Interleukin-15 rescues tolerant CD8+ T cells for use in adoptive immunotherapy of established tumors. *Nature medicine* 2006, **12**(3)**:** 335-341.

151. Staveley-O'Carroll K, Sotomayor E, Montgomery J, Borrello I, Hwang L, Fein S*, et al.* Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc Natl Acad Sci U S A* 1998, **95**(3)**:** 1178-1183.

152. Hernandez J, Aung S, Redmond WL, Sherman LA. Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J Exp Med* 2001, **194**(6)**:** 707-717.

153. Hernandez J, Aung S, Marquardt K, Sherman LA. Uncoupling of proliferative potential and gain of effector function by CD8(+) T cells responding to self-antigens. *J Exp Med* 2002, **196**(3)**:** 323-333.

154. Redmond WL, Marincek BC, Sherman LA. Distinct requirements for deletion versus anergy during CD8 T cell peripheral tolerance in vivo. *J Immunol* 2005, **174**(4)**:** 2046-2053.

155. Iyoda T, Shimoyama S, Liu K, Omatsu Y, Akiyama Y, Maeda Y*, et al.* The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *The Journal of experimental medicine* 2002, **195**(10)**:** 1289-1302.

156. Steinman RM, Turley S, Mellman I, Inaba K. The Induction of Tolerance by Dendritic Cells That Have Captured Apoptotic Cells. *The Journal of experimental medicine* 2000, **191**(3)**:** 411-416.

157. Idoyaga J, Suda N, Suda K, Park CG, Steinman RM. Antibody to Langerin/CD207 localizes large numbers of CD8alpha+ dendritic cells to the

marginal zone of mouse spleen. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**(5)**:** 1524-1529.

158. Zhang JG, Czabotar PE, Policheni AN, Caminschi I, Wan SS, Kitsoulis S*, et al.* The dendritic cell receptor Clec9A binds damaged cells via exposed actin filaments. *Immunity* 2012, **36**(4)**:** 646-657.

159. Ahrens S, Zelenay S, Sancho D, Hanč P, Kjær S, Feest C*, et al.* F-Actin Is an Evolutionarily Conserved Damage-Associated Molecular Pattern Recognized by DNGR-1, a Receptor for Dead Cells. *Immunity* 2012, **36**(4)**:** 635-645.

160. Zelenay S, Keller AM, Whitney PG, Schraml BU, Deddouche S, Rogers NC*, et al.* The dendritic cell receptor DNGR-1 controls endocytic handling of necrotic cell antigens to favor cross-priming of CTLs in virus-infected mice. *The Journal of clinical investigation* 2012, **122**(5)**:** 1615-1627.

161. Joseph WR, Cao Z, Mountjoy KG, Marshall ES, Baguley BC, Ching L-M. Stimulation of Tumors to Synthesize Tumor Necrosis Factor-α in Situ Using 5,6-Dimethylxanthenone-4-acetic Acid. *A Novel Approach to Cancer Therapy* 1999, **59**(3)**:** 633-638.

162. Zhao L, Ching LM, Kestell P, Baguley BC. The antitumour activity of 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in TNF receptor-1 knockout mice. *British journal of cancer* 2002, **87**(4)**:** 465-470.

163. Smits ELJM, Anguille S, Berneman ZN. Interferon α may be back on track to treat acute myeloid leukemia. *Oncoimmunology* 2013, **2**(4)**:** e23619.

164. Anguille S, Lion E, Willemen Y, Van Tendeloo VFI, Berneman ZN, Smits ELJM. Interferon-[alpha] in acute myeloid leukemia: an old drug revisited. *Leukemia* 2011, **25**(5)**:** 739-748.

165. Spaapen RM, Leung MYK, Fuertes MB, Kline JP, Zhang L, Zheng Y*, et al.* Therapeutic Activity of High-Dose Intratumoral IFN-β Requires Direct Effect on the Tumor Vasculature. *The Journal of Immunology* 2014, **193**(8)**:** 4254-4260.

166. Manlove LS, Berquam-Vrieze KE, Pauken KE, Williams RT, Jenkins MK, Farrar MA. Adaptive Immunity to Leukemia Is Inhibited by Cross-Reactive Induced Regulatory T Cells. *The Journal of Immunology* 2015, **195**(8)**:** 4028-4037.

167. Boyer MW, Vallera DA, Taylor PA, Gray GS, Katsanis E, Gorden K*, et al.* The Role of B7 Costimulation by Murine Acute Myeloid Leukemia in the Generation and Function of a CD8+ T-Cell Line With Potent In Vivo Graft-Versus-Leukemia Properties. *Blood* 1997, **89**(9)**:** 3477-3485.