T CELL RECEPTOR-INDEPENDENT FACTORS THAT CONTROL SECONDARY EFFECTOR $CD4^+$ T CELL FUNCTION

by

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ABSTRACT

The activation of $CD4^+$ T cells is controlled via three distinct signals: TCR recognition of the peptide:MHCII complex, coactivation via ligands and receptors expressed on antigen-presenting cells and the T cell, and Signal 3 cytokine signaling. It is this third signal that leads to initial differentiation into the multiple T helper subsets such as Th1, Th2, Th17, and Treg. While the role of Signal 3 cytokines is well-defined in programming differentiation after activation of naïve T cells, their effects on memory $CD4^+$ T cell responses have not been extensively studied. Here we show that interruption of cytokine signaling during secondary $CD4^+$ T cell responses alters their effector function. These effects are independent of TCR affinity for antigen, demonstrating a critical role for appropriate cytokine signaling in the successful production of robust secondary $CD4^+$ T cell responses.

During secondary challenge with *Listeria monocytogenes*, $CD4^+$ T cell responses are differentially regulated by Type I IFN (IFN-I) and IL-12. Effector function is depressed in the presence of IFN-I signaling, while IL-12 promotes the differentiation of highly functional secondary effector cells. Additionally, the mechanisms of regulation by both cytokines may intersect, as IFN-I inhibits the production of IL-12 as well as IFNγ, a critical cytokine for Th1 responses and bacterial clearance. Expansion kinetics are also controlled via these cytokines, with IL-12 promoting robust initial expansion and IFN-I inhibiting expansion but required for limiting contraction during memory formation.

Importantly, memory $CD4^+$ T cells alone are able to mediate significant protection from heterologous secondary challenge with *Listeria*. This protection is entirely dependent upon TNF, as neutralization of this cytokine results in complete loss of $CD4^+$ memory T cell-mediated protection. Surprisingly, IFNγ is not required for protection mediated by secondary effector $CD4^+$ T cells in this setting, though it is required for protection from primary challenge with *Listeria*. Rather, TNF-dependent differentiation of secondary effector Th1 cells drives increased classical macrophage activation, leading to more rapid bacterial clearance. Together, we demonstrate an important role for cytokine signaling in determining the strength of the secondary response of $CD4^+$ T cells, which can directly influence the protective capacity of these memory cells depending on the type of infection.

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CHAPTER 1

INTRODUCTION

Primary T Cell Responses

Upon recognition of cognate antigen by the T cell receptor (TCR), T cells undergo rapid activation, differentiation, and expansion into effector cells. These activated T cells can then perform a variety of functions to facilitate pathogen clearance, such as direct cell killing in the case of $CD8^+$ killer T cells (CTLs) or coordination of immune responses of other cell types in the case of $CD4^+$ helper T cells (1–4). $CD4^+$ T cells can differentiate into a variety of subsets with distinct functionalities including T helper 1 (Th1), Th2, Th17, and T regulatory cells (Tregs). Th1 cells produce IFNγ, IL-2, and TNF and are induced by intracellular pathogens such as viruses and intracellular bacteria. Th2 cells produce IL-4 and IL-5 and are induced by extracellular parasites and during allergic reactions. Th17 cells are primarily found at mucosal surfaces in response to extracellular bacteria and fungi and produce IL-17 and IL-21. Tregs are induced in response to inflammation to prevent uncontrolled inflammation and resolve immune responses via production of IL-10 (1). Importantly, $CD8^+$ T cells require $CD4^+$ T cells, particularly Th1 cells, in order to produce optimal primary responses that result in a memory population capable of producing a robust secondary effector response (5, 6).

During an acute infection antigen presenting cells (APCs) process and present antigen to $CD8^+$ and $CD4^+$ T cells via the major histocompatibility complex (MHC) I or II, respectively. Cells with TCR specificity to the presented antigen are activated and expand upon recognition of peptide presented by MHC (peptide:MHC), producing a polyclonal population of T cells with varying affinities for antigen. After pathogen clearance, the population size of these primary effector cells decreases by up to 95%, leaving a small population of long-lived memory cells that are able to rapidly respond to

subsequent antigen encounter (7, 8). The memory population responds more rapidly and robustly to secondary challenge with the same pathogen, resulting in faster clearance of successive infections. However, the processes that regulate primary immune responses may be distinct from those that regulate subsequent rechallenges.

Signals of T Cell Activation

During a primary immune response, T cells are activated via three distinct signals. Signal 1 is the initial TCR recognition of peptide:MHC on the surface of antigen presenting cells (9–11). Signal 2 is comprised of costimulatory molecules on the surfaces of both the APC and the T cell, such as CD28 on naïve T cells binding to CD80/86 (B7-1/2) on the surface of APCs. Signal 3 is comprised of inflammatory molecules known as cytokines, typically produced by cells of the innate immune system, which help to differentiate and activate both CTLs and CD4+ helper cells. For example, CTL activation requires Th1 help via production of cytokines such as IFNγ and IL-2. Activation of $CD4^+$ T helper cells is complex, as specific cytokine signaling leads to lineage commitment into helper subsets with defined function (e.g., IL-12 promotes Th1 differentiation, IL-4 promotes Th2, etc.) (12). While Signal 3 cytokines are well studied in the context of naïve T helper cell activation and differentiation, the role of cytokine signaling in secondary effector responses is poorly understood. The production of Signal 3 cytokines is dramatically altered when comparing primary and secondary T cell responses, as contribution of memory responses alters the cytokine milieu early in a secondary response. Our work and that of other groups suggests that activation of secondary effector T cells may have different Signal 3 requirements than that of primary effectors (8, 13, 14).

Signal 3 Cytokines for T Cell Activation

Type I IFNs (IFN-I), comprised of 14 IFN-α genes and one IFN-β in mice (15), are key Signal 3 cytokines. While IFN-I signaling is required for protection from viral infections, loss of IFN-I signaling proves to be protective during some bacterial infections, including *Listeria monocytogenes* (Lm) (16, 17). Significant work has been done to determine the role of IFN-I signaling in $CD8^+$ T cell activation in both viral and bacterial settings. Interestingly, while there are opposite roles for IFN-I in overall protection from viral and bacterial infections, in both settings there is a requirement for IFN-I signaling in $CD8^+$ T cell activation (11, 18–20). During the first week of a primary viral infection with lymphocytic choriomeningitis virus (LCMV) antigen-specific $CD8^+$ T cells undergo up to 10,000-fold expansion. Expansion is dependent upon IFN-I signaling, as loss of the IFN-I receptor on $CD8^+$ T cells causes an almost complete loss of clonal expansion during infection (18). The enhancement of T cell responses only occurs when T cells encounter IFN-I during or after T cell priming, as preincubation of T cells with IFN-I leads to dampening of their responsiveness (7, 21–23). Additionally, the role of IFN-I appears to be primarily in reducing contraction or promoting expansion during the later phase of the acute response. Consistent with this, there is no defect observed in the expansion of $CD8^+$ T cells for the first few days of a viral infection in the absence of IFN-I signaling, but there is a defect observed in the later phase of the primary immune response (24). Others have shown that IFN-I induces resistance to apoptosis, including through upregulation of the antiapoptotic protein Bcl-2 and sustained expression of IL-2Rα on T cells (20, 25). The role of IFN-I signaling to T cells is dependent upon the pathogen, as IFN-I is required for expansion of $CD8^+$ T cells during LCMV infection, but loss of direct IFN-I signaling alone

is not sufficient to reduce CTL numbers during infection with *Listeria* (11).

Further understanding of the role IFN-I plays in shaping the inflammatory environment of different immune responses is required to elucidate the mechanisms by which IFN-I signaling mediates protection or susceptibility, as well and how it controls T cell responses in different settings. Due to the ubiquitous expression of IFNAR, most cell types can be influenced by IFN-I signaling. Dendritic cells (DCs) are a source of IFN-I during infection, with plasmacytoid DCs (pDCs) producing IFN-I in response to viral infection and conventional DCs producing IFN-I in response to bacteria present in the phagosome but not in the cytosol (26, 27). IFN-I signaling is subsequently induced in T cells and other cells, particularly macrophages in the case of Lm and other intracellular infections. It has been observed that IFN-I signaling can reduce surface expression of the type II interferon receptor (IFNγR) on macrophages rapidly after infection (28, 29). Macrophages that are refractory to IFNγ signaling are unable to properly upregulate proinflammatory signaling pathways and cytokine production, reducing the overall inflammatory environment and dampening the protective immune response in the case of acute infection. While much of the published literature would suggest an apparently simple model wherein IFN-I signaling is detrimental to bacterial clearance and IFNγ signaling is beneficial, the situation is not quite so straightforward. Both IFN-I and IFNγ signal through Stat1, causing some signaling redundancies that may have unforeseen implications in long-term immunity (30).

Type II IFN, comprised of only a single IFNγ gene, is well studied in innate and adaptive immune responses. As previously mentioned, macrophages require IFNγ responsiveness to promote protection from bacterial infections. Whole animal knockout of

the IFNγR leads to early death by normally sublethal amounts of Lm (31). Cell-specific receptor knockouts show that macrophages and $CD8^+$ DCs are particularly responsible for increased susceptibility when lacking the IFNγR during a primary response to Lm (32–34). This is partially due to a reduction of MHCII on macrophages and reduced listericidal activity, as well as a loss of the initial burst of IL-12 traditionally provided by $CD8^+$ DCs. In addition to roles in innate cell function, IFNγ has a very important pathogen-dependent role in the development of T cell responses. In general, Th1 cells are required for formation of optimal $CD8^+$ T cell responses, with reduced numbers and functional capacity for $CD8^+$ memory T cells generated in the absence of $CD4^+$ T cells (5, 35). IFNγ is centrally important for this T cell help, as Th1 cells produce significant levels of IFNγ, and a lack of $CD4^+$ T cell help during CTL responses results in reduced IFNγ production by $CD8^+$ T cells. Others have also observed that the secondary response to a *Listeria* infection requires IFNγ, and increased systemic levels of this cytokine provide increased protection during the secondary response (36). However, we observe that this is not always the case, as we show that memory $CD4^+$ T cell-mediated protection from *Listeria* infection is not altered by the loss of IFNγ through cytokine neutralization (Chapter 3). While IFNγ generally promotes protection and clearance, exceptions to this rule exist (37). Taken together, we can conclude that the interferon family of cytokines has a complex role in promoting immunity that is dependent on the infectious and inflammatory environment.

Interferons alone are not responsible for regulating protective immunity. Much of the work that has examined IFN-I as a Signal 3 cytokine has also shown that IL-12 is important in defining the dominant cytokine profile of the immune response. IFN-I has a regulatory effect on the production of IL-12, and *Ifnar*$^{-/-}$ mice infected with Lm show much

higher systemic levels of IL-12 than their WT counterparts (19). Both IFN-I and IL-12 have important roles in the production of effective $CD8^+$ T cell effector cells. Specifically, IL-12 is required in addition to IFN-I during the later phase of the adaptive immune response to prolong expression of the IL-2Rα and promote sustained division (11, 20). During *Listeria* infection, IL-12 signaling is required to drive the differentiation of short-lived effector cells (SLECs), a critical component of protective immunity to this pathogen. T cells lacking the IL-12 receptor showed no defect in SLEC differentiation in the setting of a viral infection, indicating that cell fate is determined independent of IL-12 signaling in an antiviral response (10). While the immune response to a viral infection is dominated by IFN-I signaling, IL-12 is more critical for protective responses to bacterial pathogens, as evidenced by normal $CD8^+$ T cell expansion and function in response to lymphocytic choriomeningitis virus (LCMV) and vaccinia virus (VV) when these cells lack the IL-12 receptor. This is not as simple as differences caused by viral and bacterial infections, as vesicular stomatitis virus (VSV) infection of mice with IL-12RKO $CD8^+$ T cells induces less robust antigen-specific T cell responses in the absence of both IFN-I and IL-12 signaling, indicating both cooperative and distinct roles for these signaling pathways in modulating T cell responses (11).

Production of IL-12 can be induced in three ways. First, IL-12 can be produced in a T cell-independent manner through direct stimulation of innate cells such as macrophages and DCs. Second, T cell-dependent production of IL-12 is initiated when CD40 on the surface of macrophages or DCs binds CD40L expressed on T cells (38). Finally, there is a third method of IL-12 induction that involves macrophages recognizing components of the extracellular matrix and inducing production, though this is a much less important source

of IL-12 in the setting of an acute infection (39). IL-12 is known to induce a number of important proinflammatory cytokines, such as IFNγ, tumor necrosis factor (TNF), IL-6 and IL-1β (36, 40, 41). Previous work implies that protection during a secondary infection is IL-12 independent, as IL-12 depletion during secondary challenge with *Listeria* exhibited no increase in bacterial burden (41, 42). There appears to be a feedback loop involving IFN-I, IFNγ, and IL-12 signaling, particularly in a primary *Listeria* infection, but the role of these cytokines specifically concerning $CD4^+$ T cells and secondary responses is not completely understood.

While much of the focus on the impact of Signal 3 cytokine signaling to T cells has focused on the aforementioned interferons and IL-12, there are additional cytokines that may play important roles in T cell-mediated immunity. TNF is a pleotropic cytokine that can lead to activation-induced cell death (AICD) and thereby control the magnitude of the T cell response (43). In addition to the death domain, TNF-activated NF-κB signaling induces a variety of proinflammatory cytokines. Similarly, IFNγ signaling through Stat1 can also be increased by the presence of TNF, resulting in stronger IFNγ signaling (44). Importantly, *Listeria* infection of TNFR-/- mice resulted in an IL-12 deficiency, indicating an important role for TNF in the induction of IL-12 and downstream cytokine signaling (45). Though there is significant literature studying the role of TNF, the pleotropic nature of this cytokine makes it difficult to fully determine the specific role of TNF signaling in T cell-mediated protection. More targeted study of the role of this cytokine and its various signaling pathways will elucidate individual aspects of the function of TNF.

Induction of IL-6 is one hallmark of *Listeria* infection (46). Classical signaling through the IL-6 pathway occurs via the IL-6 receptor that is present on the surface of

hepatocytes and some leukocytes. More ubiquitously, IL-6 is also able to bind a soluble version of the IL-6Rα, which can subsequently bind and dimerize with the ubiquitously expressed gp130 chain of the receptor (47). Termed trans-signaling, this pathway is dispensable for normal immunity, but loss of classical signaling causes impaired control of infection and large changes to the overall inflammatory environment. IL-6 is induced by both IFNγ and TNF, and overexpression of IL-6 increases protection from primary *Listeria* infection (48, 49). Production of IL-6 can occur in both T cell-dependent and -independent manners, with macrophages being implicated as a major source of the cytokine (50, 51). Interestingly, IL-6 has differential effects on naïve and memory $CD4^+$ T cells, with a requirement during primary responses for the formation of productive memory populations but proving expendable during the secondary response (52). However, loss of protection due to IL-6 knockout during a primary infection has been shown to be reversible through administration of recombinant IL-12, indicating that these cytokines signal through the same or similar pathways to mediate protection (53). As with all examples reviewed here, it is likely that these are not hard and fast rules for every type of infection, but rather will change based on the inflammatory environment formed by individual pathogens. Rather, what should be noted is the relationship between all of the cytokines enumerated so far, and the direct and sometimes opposing roles they have been shown to play in T cell immune responses.

All of the aforementioned cytokines are broadly inflammatory, though some, such as TNF, have been shown to perform immunoregulatory functions as well. Importantly, all inflammatory immune responses also induce expression of immune dampening factors to allow for proper resolution of inflammation after pathogen clearance. IL-10 is an

antiinflammatory cytokine produced by Tregs and monocytes that downregulates the expression of costimulatory molecules on macrophages as well as the production of Th1 cytokines (54). It is therefore unsurprising that treatment with IL-10 neutralizing antibody leads to increased resistance from infection with *Listeria*. However, without IL-10 signaling, complete clearance may be inhibited, likely due to anergy and/or death of T cells caused by prolonged exposure to high levels of inflammation (55). Consistent with this, memory cells generated in *il10*$^{-/-}$ mice were lower in number and protective capacity. As with IL-6, signaling from IL-10 to T cells was only required during primary memory formation, and memory cells generated in a WT host and subsequently transferred to an *il10*$^{-/-}$ host could provide protection from a subsequent challenge (56). As the evidence presented here for Signal 3 cytokines demonstrates, requirements for individual cytokines are dependent on a number of factors, including type of pathogen, inflammatory environment, and phase of the response. It is one of the goals of our lab to determine the specific roles of these and other cytokines in the induction of both primary and secondary CD4+ T cell responses.

Models for Study of Secondary Responses

The study of secondary responses in individual cell types is challenging, as secondary challenge of a host results in stimulation of all specific memory cells, including CD8^{+} T cells, CD4^{+} T cells, B cells, and even some innate memory (57). Unfortunately, this makes it impossible to determine the contribution of individual cell types in protection from secondary challenge. Many groups, including ours, have utilized an adoptive transfer model wherein specific cell subsets are isolated and then transferred to a secondary, naïve host (8, 10, 19). It is then possible to study antigen-specific secondary responses in an

individual cell type, as we can control which memory populations are present. We have utilized adoptive transfer of SMARTA T cells, a monoclonal TCR transgenic line with specificity to the MHCII-restricted GP_{61-80} epitope of LCMV. This approach has allowed us to study TCR-independent mechanisms of T cell activation due to the presence of a single TCR with a specific affinity for antigen (58). However, the adoptive transfer model may fail to accurately replicate an actual secondary challenge due to high numbers of antigen-specific cells involved in the transfer. For this reason, we also utilized a prime-boost model of rechallenge in the same host. In our prime-boost model, termed heterologous rechallenge, animals are first infected with LCMV. After memory formation (>30 days), animals are given a secondary challenge with *Listeria* expressing the MHCII-restricted GP_{61-80} epitope of LCMV (Lm-gp61) (2, 8). Using this method, we can specifically restimulate memory $CD4^+$ T cells produced by the primary LCMV infection without rapid clearance mediated by $CD8^+$ memory T cells or antibodies. More importantly, this allows us to study the protective role of endogenous $CD4^+$ T cell memory without the confounding effects of cell transfer. The following dissertation utilizes both models, often in conjunction, to study the role of the inflammatory environment on secondary effector CD4+ T cell responses and to assess the specific inflammatory cytokines involved.

Clinical Relevance of CD4+ T Cell Memory

For the development of vaccines and immunotherapuetics, understanding of the mechanisms that regulate immune responses is invaluable for producing treatments that appropriately manipulate the immune system to the benefit of the patient. This is most commonly apparent in the use of vaccines and adjuvants. Vaccinations are performed with

the purpose of producing long-lived immune memory to prevent severe infection from encounter of the pathogen (59). Adjuvants are provided in conjunction to stimulate the innate immune system and allow for a complete T cell response to the vaccine antigen. Successful vaccines result in limited bystander activation, targeted stimulation of antigen-specific cells, and subsequent formation of long-lived memory cells (60). Single vaccinations are not always sufficient to form immunity for the life of the individual, and both homologous and heterologous prime-boost strategies have been used in vaccine therapies. Differences in $CD4^+$ and $CD8^+$ T cell activation are one reason for the use of prime-boost, as in certain cases low doses of antigen are sufficient to stimulate one cell type and not the other, while high doses can impede memory formation in some cases (61). Heterologous prime-boost vaccination may be sufficient to engage appropriate responses from both $CD4^+$ T cells and CTLs and result in long-lasting immune memory. Due to the vast amount of literature indicating the important role for cytokine signaling throughout the entire T cell response, understanding the signaling molecules that are produced during vaccination and their roles in T cell responses will allow for the generation of both more robust vaccines and potentially new adjuvants.

Personalized therapies revolving around the genetic and immune profiles of tumors are becoming more common in cancer immunotherapy, wherein clinicians manipulate a patient's own immune system to more robustly fight the cancer based on specific markers present in their immune systems and tumors (62). Clinical trials are underway for a variety of combination therapies involving oncolytic viruses (Takara), and chimeric antigen receptor (CAR)-T cell therapy has also been used to target cancer cells (63). Difficulties arise when it comes to manipulating the immune system of a patient with cancer, as $CD4^+$

T cells in particular can have both antitumor and immunosuppressive properties. While Th1 cells are beneficial to the immune response against cancer cells, Treg cells dampen the immune response and lead to suppression of both $CD4^+$ T cell and CTL responses, allowing for tumor immune evasion and growth (64–66). The presence of active tumor infiltrating lymphocytes (TILs) in the tumor has been shown to positively correlate to patient outcome (62, 67), supporting a therapeutic approach of limiting Treg responses while promoting recruitment and antitumor activity of other T cells. Unfortunately, simple transient depletion of the $CD4^+$ T cell compartment would likely not be effective, as it would eliminate proinflammatory $CD4^+$ T cell help along with Tregs, reducing CTL efficacy. Mouse studies show that $CD4^+$ T cell vaccines can cause tumor rejection, so improved antitumor vaccines may just be the next step in cancer immunotherapy. In order to one day develop therapeutics such as these for use in human subjects, we must first understand the mechanisms that produce optimal T cell recall responses.

Summary of Dissertation

While a significant amount of T cell research focuses on $CD8^+$ T cell responses, I argue that understanding of $CD4^+$ T cells and the mechanisms controlling their responses is equally important. This is due to the broadly immunoregulatory and immunostimulatory roles of T helper cells, in addition to some more recently discovered direct roles in cell killing (68, 69). The following dissertation focuses primarily on secondary effector responses of antigen-specific $CD4^+$ T cells. With these and future studies, we hope to understand the protective role of memory $CD4^+$ T cells alone and the signaling mechanisms that control them. Understanding differences between primary and secondary effector responses, specifically environmental cues that dictate strong secondary responses, will

allow for the development of more robust prime-boost vaccinations and immunotherapeutics.

To determine the role of Signal 3 cytokines in reactivation of memory $CD4^+$ T cells, we utilized the previously described TCR transgenic, adoptive transfer, and heterologous secondary challenge models to study TCR-independent mechanisms of T cell activation. In Chapter 2, we examine the role of IL-12 and IFN-I in secondary $CD4^+$ T cell effector function during bacterial challenge. We observe a TCR-independent effect on T cell functional avidity, a measure of T cell responsiveness to cognate antigen, with IL-12 and IFN-I having opposite effects on secondary effector function. Specifically, we note that in the presence of IL-12 signaling there are elevated levels of IFNγ and increased functional avidity of secondary effector T cells at peak response compared to a primary effector population. IFN-I, on the other hand, inhibits circulating IFNγ levels as well as T cell functional avidity. IFNγ inhibition by IFN-I may be through direct signaling to IFNAR on T cells, as levels were not elevated when all cells except for memory T cells lacked this receptor. Otherwise, IFN-I appears to play an indirect role in controlling $CD4^+$ T cell function. We observe similar roles for IL-10 signaling and IFN-I signaling, though the mechanisms each cytokine uses to impact effector T cell function appear to be different due to the downstream cytokines each regulates.

Other cytokines may also be important for secondary effector Th1 cells, most importantly IFNγ and TNF. Previous work by other groups has shown a key role for TNF in $CD8^+$ T cell effector responses, and IFNγ is a known important mediator of protection from primary infection with *Listeria*. In Chapter 3, we explore the roles of IFNγ and TNF in a secondary *Listeria* infection where protection is mediated by CD4+ memory T cells.

Surprisingly, IFNγ is expendable for memory $CD4^+$ T cell-mediated protection from secondary infection, as evidenced by little to no loss of protection when IFNγ is neutralized. Rather, TNF appears to be necessary for $CD4^+$ T cell mediated protection, with complete loss of protection observed in the absence of TNF signaling. This effect correlates with a decrease in classically activated macrophages, particularly macrophages expressing IFNγR1. While IFNγ neutralization does not greatly affect protection, expression of its receptor appears to be important for macrophages nonetheless, indicating that there is still a role for IFNγ signaling in secondary protection mediated by $CD4^+$ T cells. Interestingly, in data presented in both Chapters 2 and 3, we observe significant changes in IL-6 concentrations when we manipulate the cytokine milieu via neutralizing or blocking antibodies. This appears to be secondary to the effects on protection, as neutralization of IL-6 has no significant effect on $CD4^+$ T cell-mediated protection. Other work has indicated a role for IL-6 in primary immune responses, but in our hands there appears to be no significant role for IL-6 in secondary immune responses. Overall, the data presented in this dissertation describe mechanisms of programming secondary $CD4^+$ T cell responses by cytokine signaling in a TCR and possibly antigen independent manner.

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CHAPTER 2

TCR-INDEPENDENT SIGNAL 3 CYTOKINE CONTROL OF SECONDARY EFFECTOR CD4$^+$ T CELL RESPONSES VIA IL-12, IFN-I, AND IL-10

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Abstract

Monoclonal memory $CD4^+$ T cell populations are not functionally static, as the inflammatory environment can alter T cell function independent of TCR affinity. Two of the most prominent Signal 3 cytokines for $CD8^+$ T cell activation, IL-12 and IFN-I, can also program $CD4^+$ T cell responses during secondary challenge. IL-12 is required for IFNγ production and promotes the formation of highly functional secondary effector cells as measured by functional avidity. Conversely, IFN-I signaling inhibits secondary effector $CD4^+$ T cell functional avidity, as well as diminishes IL-12 and IFNγ production. Interestingly, while IL-12 and IFN-I signaling are both requisite for the formation of primary memory $CD4^+$ T cells after viral infection, secondary effector responses and subsequent memory formation do not require direct IL-12 or IFN-I during heterologous bacterial challenge. IFN-I can induce IL-10 in certain situations, and we observe that loss of IL-10 results in increased T cell functional avidity. However, changes in systemic inflammation induced by loss of IL-10 are different compared to loss of IFN-I signaling, implying that IL-10 and IFN-I influence T cell responses via distinct mechanisms. Overall, we show that IL-12 and IFN-I, acting as Signal 3 cytokines, differentially regulate IFNγ expression and secondary effector $CD4^+$ T cell responses in a TCR and antigen-independent manner. Additionally, we show that other cytokines may also have similar roles concerning T cell function, as IL-10 can influence these responses likely via different mechanisms than IFN-I.

Introduction

T cells are critical mediators of immunity, with both $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T helper cells performing crucial and indispensable roles in pathogen clearance. $CD4^+$ T cells are particularly important, as they provide help to a variety of immune cell subsets including CTLs and B cells via stimulatory signals (1, 2). Upon initial activation, naïve $CD4^+$ T cells differentiate into a variety of subsets, including T helper 1 (Th1), Th2, Th17, and T regulatory cells (Treg) (3, 4). This initial differentiation requires three separate signals: antigen recognition, costimulation, and a third signal provided by soluble immune signaling molecules known as cytokines (5, 6). Signal 1, antigen recognition, occurs when antigen presented in the major histocompatibility complex (MHCII in the case of $CD4^+$ T cells) is recognized by the T cell receptor (TCR). Costimulatory molecules on the T cell then recognize their counterpart on the antigen-presenting cell (APC), providing the second signal. Finally, cytokine signaling induced by innate cells can signal to these $CD4^+$ T cells and skew them to different T helper subsets (IL-12 promotes Th1 differentiation, etc.) (7). These cytokines are generally referred to as Signal 3 cytokines and have roles in both $CD4^+$ and $CD8^+$ T cell activation. While literature concerning their role in the primary differentiation of T helper cells is abundant, Signal 3 cytokines in the context of secondary effector differentiation and function is less well defined.

In the context of $CD8^+$ T cells, two of the most studied Signal 3 cytokines are IL-12 and Type I IFN family, comprised of 13 IFN-α genes and a single IFN-β in mice (8). Both cytokines have been shown to be necessary for the formation of robust antigen-specific $CD8^+$ T cell responses, with loss of either cytokine resulting in impaired responses

in a pathogen-dependent manner. During viral infections, IFN-I signaling has a dominant role as evidenced by decreased expansion of T cells lacking the IFN-α/β receptor (IFNAR) (9). IFN-I and IL-12 are both important for $CD8^+$ T cell responses in immune responses to bacteria, as loss of signaling by either cytokine to $CD8^+$ T cells during an infection to *Listeria monocytogenes* (Lm) reduces expansion, and loss of both cytokines in concert results in almost complete loss of expansion (10, 11). As with most biological rules there are exceptions to the pathogen-dependent role of these cytokines; for example, loss of IL-12 during the CD8+ T cell priming phase of *Chlamydia trachomatis* infection actually improves the expansion and memory formation of these cells (12). The differences in the requirement of these cytokines is likely due to the distinct inflammatory environments formed during these different infections. We hypothesized that the inflammatory milieu could also augment secondary CD4+ effector responses, despite lineage commitment by previously differentiated primary memory cells. Differences in cytokine requirements for primary and secondary $CD4^+$ T cell-mediated protection have been documented, supporting our hypothesis (13).

Previous work from our lab has shown that IL-12 and IFN-I differentially regulate secondary $CD4^+$ T cell responses to *Listeria* infection (14). T cell sensitivity, a measure of T cell function, is calculated using functional avidity curves. Cells with high functional avidity are sensitive to low concentrations of peptide presented by MHC, while those with low functional avidity require higher peptide concentrations to induce a response. The effective concentration to elicit a half-maximal response of cytokine production (EC_{50}), typically reported using IFNγ for both $CD4^+$ and $CD8^+$ T cells, is lower the higher the functional avidity. We observe that depletion of IL-12 reduces the functional avidity,

whereas blockade of IFN-I signaling increases the functional avidity of these T cells. This effect is independent of TCR affinity, as we observe this effect in a monoclonal population of memory cells. However, this effect is dependent upon the inflammatory environment, as differences in secondary effector T cell function are only observed after transfer to a naïve host prior to secondary challenge. We set forth to determine which changes to the overall inflammatory environment IL-12 neutralization and IFNAR blockade were producing, and how these changes mediate effects on T cell function. We show overall changes in levels of systemic IFNγ with either antibody treatment that are directly correlated to T cell function, in consensus with previously published work in *Listeria* infections (15–18). Additionally, we observe a complicated role for IFN-I signaling in $CD4^+$ T cell expansion that appears to be phase-dependent, with IFN-I signaling being required during contraction to maintain the size of the secondary memory population, though it inhibits early IFNγ production. Utilizing knock-out mice, we also show that loss of IL-10, another cytokine important to *Listeria* infections (19–21), can increase T cell functional avidity. Overall, we observe that while secondary effector $CD4^+$ T cells have already been programmed with their helper subset, they still require Signal 3 cytokine signaling to form optimal secondary effector responses and memory populations.

Materials and Methods

Mice and adoptive transfers

Four to six week old C57Bl/6J mice were ordered from Jackson Laboratories (Bar Harbor, ME) for immediate use. Additionally, a small colony of these mice was maintained in our mouse facility at the University of Utah. $Thy1.1^+$ SMARTA transgenic mice were bred and maintained in our mouse facility (22–24). CD45.1 (Ly5.1) mice were ordered

from Jackson laboratories (stock no. 002014) for use in adoptive transfer studies. SMARTA mice were bred to *ifnγ*$^{-/-}$ (stock no. 002287), *il12rβ2*$^{-/-}$ (stock no. 003248), and *Ifnar*$^{-/-}$ (stock no. 32045-JAX, kindly provided by Janis Weis from their colony at the University of Utah) to generate gene knock-out SMARTA mice for use in adoptive transfer experiments. *Ifnar*$^{-/-}$ and *il10*$^{-/-}$ (stock no. 002251) mice were also supplied by the Janis Weis lab for use as hosts in adoptive transfer studies (25). For adoptive transfers, untouched or memory CD4^{+} SMARTA cells were isolated from the spleens of naïve or memory mice, respectively, and isolated using MACS CD4^{+} T cell isolation kits, with the addition of biotinylated anti-CD44 for the isolation of untouched CD4^{+} T cells for a primary infection (14). One day prior to infection, 10^4 to 10^5 cells were injected intravenously (i.v.).

Infections and antibody treatments

LCMV-Armstrong and Lm-gp61 were stored and propagated as previously described (24). For primary infections with LCMV-Arm, mice were injected intraperitoneally (i.p.) with 2 x10^5 plaque forming units (PFU). For heterologous secondary infections with Lm-gp61, bacteria were first grown to log phase in BHI media as determined by the O.D. at 600 nm (0.3-0.7). Mice were then injected i.v. with 2x10^5 colony forming units (CFU), unless otherwise indicated. Primary infections with LCMV were done between 5-10 weeks of age, while primary and secondary infections with Lm-gp61 were done 6 weeks later, when mice were 10-15 weeks old. All experiments using animals were performed under a protocol approved by the University of Utah IACUC (Protocol #15-09004, approved 9/23/2015). All antibody treatments were performed i.p. one day prior to infection. For anti-IL-12 antibody treatments, mice received a 0.5 mg injection of anti-IL-12 antibody (BioXCell, clone C17.8) one day prior to challenge, as previously. For

IFN-I blockade, mice received a 1.25 mg injection of anti-IFNAR1 antibody (BioXCell, clone MAR1-5A3) i.p. one day prior to infection as previous (14).

Serum cytokine analysis

Mice were bled on days 1 and 3 postchallenge. Blood was allowed to clot at room temperature then spun at max speed in a microcentrifuge for 20 minutes. Serum was collected and stored at -20C. Serum cytokine concentrations were measured using a custom 6-plex LEGENDplex bead-based cytokine assay (Biolegend; IFNγ 740153, IL-1B 740157, IL-6 740159, IL-10 740158, IL-12 (p70) 740156, TNF-α 740154, Standard 740371, Detection antibodies 740165, buffer set B 740373) or a ProcartaPlex panel (eBioscience; IFNα and IFNβ panel EPX020-22187-901, custom panel for IL-18, IL-6, IFNγ, TNF, IL-12p70, GM-CSF, IL-1β).

Tissue and cell preparations

Whole spleens and liver portions were collected in the tissue culture hood in 2 mL sterile PBS for clearance assays. Other tissue collections were done at the bench in nonsterile conditions. Livers were weighed, and all organs were dissociated using frosted microscope slides. For assessment of bacterial load, serial 1:10 dilutions were performed in sterile PBS and aliquots were plated on brain heart infusion agar (BHI) agar plates. Plates were incubated at 37°C overnight. Colony counts were reported as CFU/spleen or CFU/g of liver. For all other cell preparations, dissociated tissues were places in single cell suspension in DMEM containing 10% fetal bovine serum (FBS), L-glutamine and Pen/Strep. For detection of intracellular cytokines, splenocytes were incubated with GP_{61-80} peptide (GLKGPDIYKGVYQFKSVEFD) for 4 hours in the presence of Brefeldin A

(Golgi Plug), followed by fixation and permeabilization, according to the manufacturer's instructions (BD Biosciences).

Flow cytometry

Cells were suspended in PBS + 1% FBS, then stained with fluorescent dye-conjugated antibodies as indicated (Biolegend, San Diego, CA; BD Biosciences, San Jose, CA; eBioscience, San Diego, CA) for 20-40 minutes. Samples were collected on a LSRFortessa flow cytometer or FACSCanto (BD Biosciences) and analyzed using FlowJo (TreeStar).

Statistical analysis

Statistical significance was determined using the student's t-test for two groups and ANOVA for more than two groups using GraphPad Prism 7 software. Graphs depict mean ± SEM, with a p value of less than 0.05 being considered significant. p values are indicated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Results

IFN-I and IL-12 differentially regulate systemic inflammation

Our lab has previously produced data showing that, when transferred to naïve hosts prior to infection, secondary effector CD4+ T cell function can be augmented by manipulation of the IL-12 or IFN-I signaling pathways. To determine how disrupting these pathways influenced the systemic cytokine milieu, we adoptively transferred transgenic SMARTA $CD4^+$ T cells to naïve WT B6 hosts and infected with LCMV. After memory formation, we performed a secondary adoptive transfer of these memory SMARTA cells to new, naïve hosts and then challenged with *Listeria* expressing the MHCII-restricted

GP_{61-80} epitope of LCMV (Lm-gp61). Cytokine pathways were disrupted with i.p. injection of neutralizing or blocking antibodies to IL-12 or IFNAR, respectively, 1 day prior to infection. As previously stated, we observe increased T cell functional avidity at day 7 postchallenge in the absence of IFN-I, but a decrease in T cell function in the absence of IL-12 (14). This is only true when these signaling pathways are inhibited at the start of infection, as neutralization of either IL-12 or IFNAR signaling postinfection but prior to peak effector responses results in no difference when compared to T cells from PBS control animals (Chulwoo Kim, data not shown).

To determine which early inflammatory signals might be controlling peak $CD4^+$ T cell effector function, we examined changes to the systemic inflammatory environment caused by interruption of either IL-12 or IFN-I signaling 24 hours postchallenge with Lm-gp61. We observed an increase in systemic IL-12p70 in the absence of IFN-I signaling, supporting previously published work (Fig. 2.1A). Additionally, we observe a similar increase in IFNγ in the absence of IFN-I. However, when IL-12 is neutralized, IFNγ levels are decreased, showing that IFNγ levels are controlled by IL-12 levels (Fig. 2.1B). This relative decrease in IFNγ is also observed when SMARTA cells are rechallenged in immune hosts, but the amount of total IFNγ is much higher, possibly explaining why we do not observe downstream effects on T cell function (data not shown). Similarly, we observe increased IL-6 in the absence of IFN-I signaling, though no change in IL-6 levels were observed in the absence of IL-12 signaling (Fig. 2.1C). IL-18 has been shown to synergize with IL-12 to induce IFNγ production in Th1 cells (26). However, while levels of IL-18 were increased with either antibody treatment, the lower limit of quantification (LOQ) was so high as to make these results uninterpretable based on data from only 3 mice

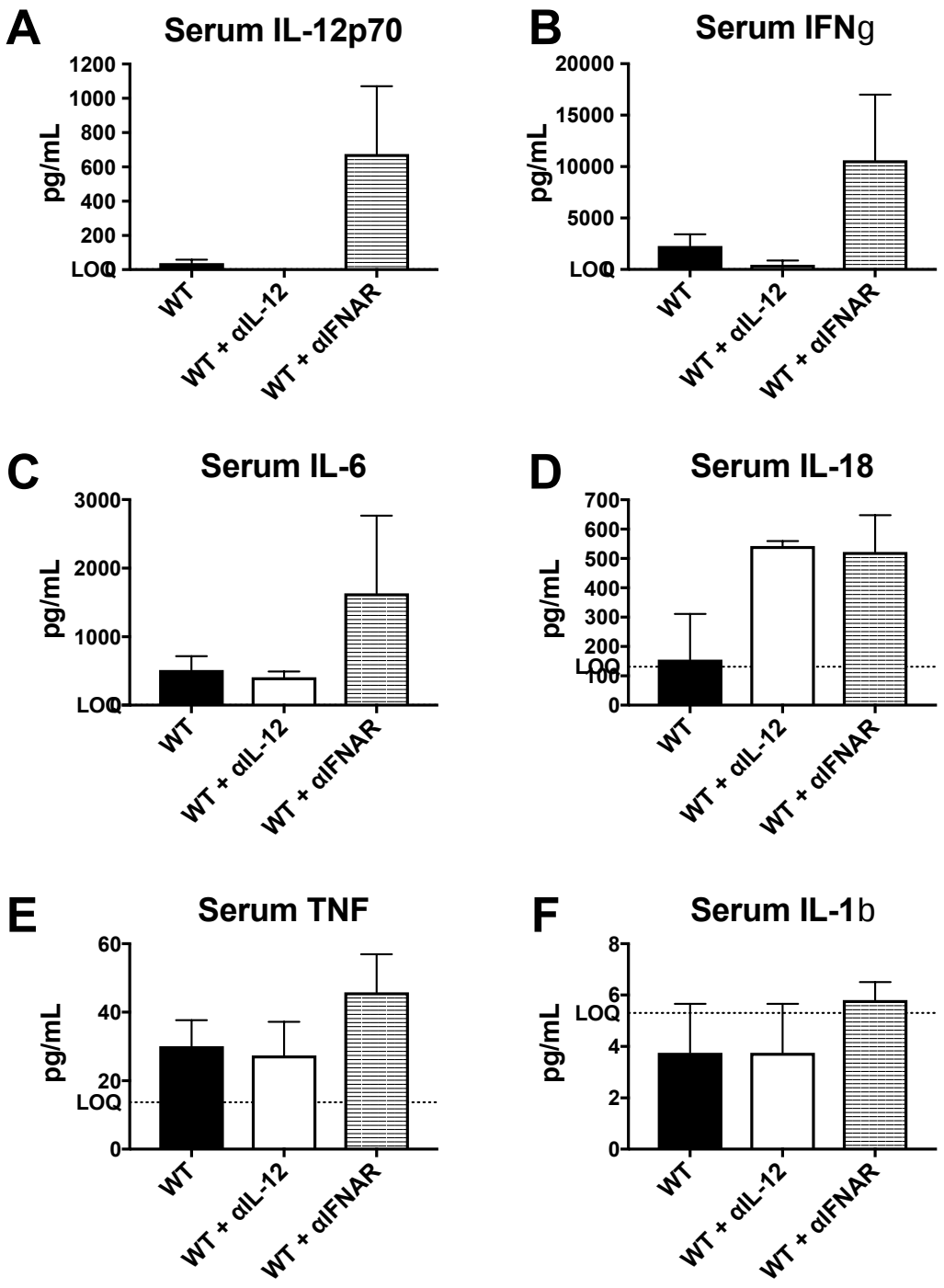

Figure 2.1. IFN-I and IL-12 differentially regulate systemic inflammation. Serum cytokines were analyzed by ProcartaPlex kit for concentrations 1 day after infection. Striking differences were detected for IL-12p70 (A), IFNγ (B), and IL-6 (C). Nonsignificant or uninterpretable data were detected for IL-18 (D), TNF (E), IL-1β (F), and others (data not shown). n=3 mice/group. Dotted line indicates LLOQ.

(Fig. 2.1D). No other noteworthy differences in serum cytokines were observed in our selected cytokine panel (Fig. 2.1E, 2.1F, data not shown).

Early TCR-independent signaling events control CD4$^+$ secondary effector function

As mentioned, we observe defects in T cell effector function at peak effector time points (day 7 postchallenge) in the absence of IL-12, while mice lacking IFN-I signaling produce more functionally adept effector cells. This corresponds to changes to IL-12 and IFNγ levels in the serum 1 day postchallenge. Levels of systemic inflammation throughout the first 7 days leading to peak effector response shows that the bulk of systemic inflammation occurs early after infection (IL-12, IFNγ, and IL-6), with levels dropping precipitously by day 3 and being nearly undetectable by day 5, days before the observed changes in T cell effector function (Fig. 2.2A-C). These levels drop regardless of antibody treatment or control treatment, demonstrating that changes to very early levels of these cytokines is sufficient to produce functional changes in secondary effector T cells in this heterologous secondary challenge model. Levels of TNF and IL-1β remain low in the serum throughout the effector response (Fig. 2.2D, E). IL-18 levels appear to remain high when either IL-12 or IFN-I signaling are disrupted, though again the high LLOQ results in data that is difficult to draw conclusions from (Fig. 2.2F).

Another measure of T cell function is maintenance of T cells in the memory pool. It is well understood that the memory pool selects for highly functional T cells, so increased memory population size would indicate increased T cell function throughout the effector phase (27). We observe decreased expansion of secondary effector cells in the absence of IL-12, but a significant increase in expansion by day 7 postchallenge in the absence of

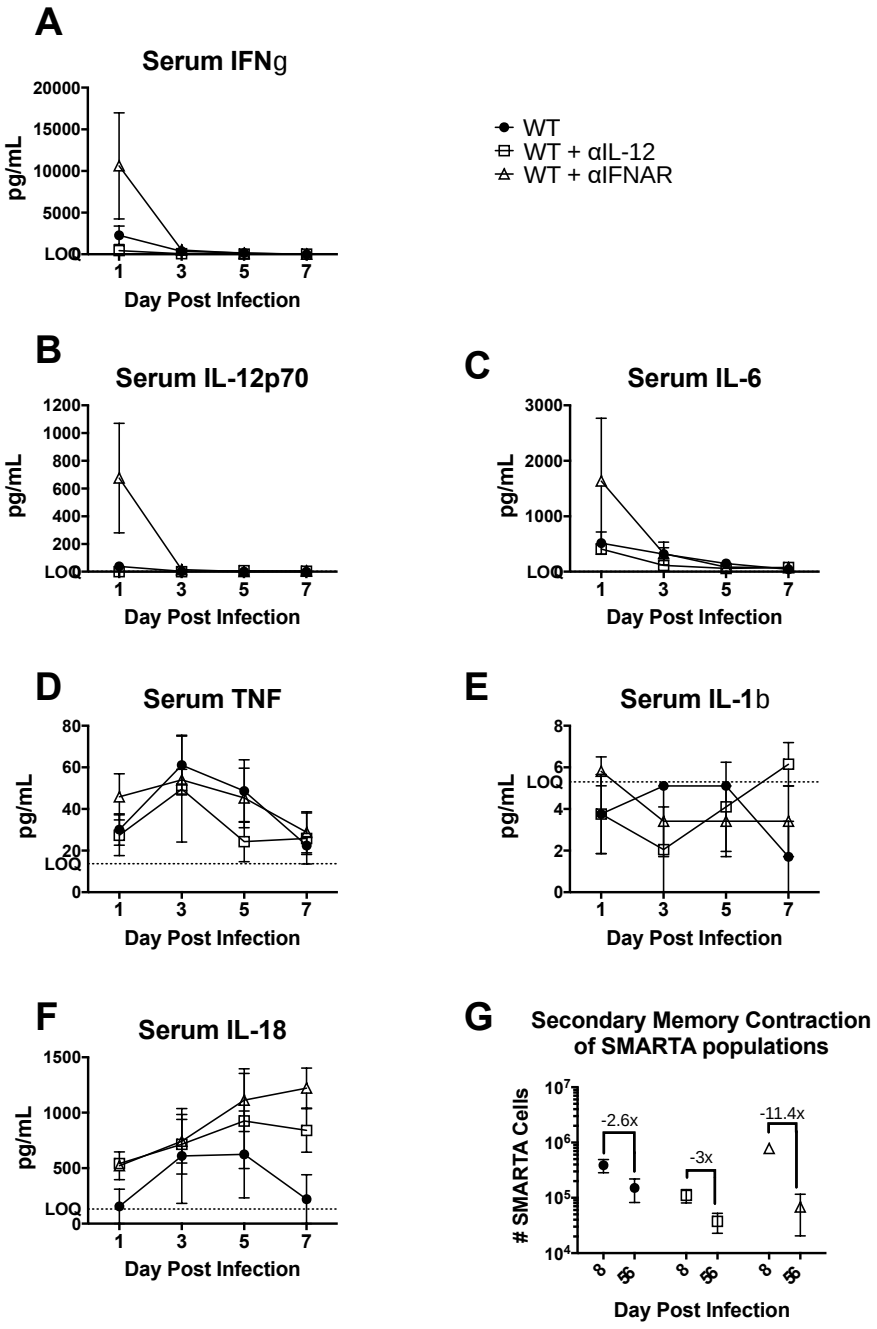

Figure 2.2. Early cytokine signaling controls secondary CD4+ T cell responses. (A-F) Serum collected as in Fig. 2.1 on various days postchallenge with Lm-gp61 were analyzed for systemic concentrations of various cytokines by ProcartaPlex. High levels are detected only at very early timepoints for IFNγ (A), IL-12p70 (B), and IL-6 (C). TNF (D), IL-1β (E), and IL-18 (F) levels showed no interesting patterns. (G) Numbers of Thy1.1^{+} SMARTA cells were calculated based on staining by flow cytometry and whole spleen numbers at days 8 and 56 postchallenge with Lm-gp61. Numbers reflect fold contraction between those timepoints. n=3 mice/group. Dotted lines indicate LLOQ.

IFN-I. Contraction of secondary effector cells, measured as a fold decrease in the number of antigen-specific SMARTA cells, showed a requirement for IFN-I signaling later in infection, however. While the relative contraction was similar between control and anti-IL-12 treated animals, regardless of peak effector population size, animals lacking early IFN-I signaling had substantially greater contraction to the memory population (Fig. 2.2G). This indicates an early programming of effector cells for retention in memory that is dependent on IFN-I signaling. This is interesting, as early initial expansion and effector function are benefitted in the absence of this signaling. This mirrors other work published on the role of IFN-I and IL-12 in $CD8^+$ T cell function and expansion, where IFN-I in particular has a complicated role dependent on the phase of T cell response.

T cells require direct IL-12 and IFN-I signaling to form memory after viral infection

While treatment with anti-IL-12 and anti-IFNAR antibodies causes changes in T cell function, this does not indicate whether the role of these cytokines mediate these effects by directly signaling to T cells or inducing signaling cascades in other cells that subsequently induce changes in T cell function. To answer this question, we bred SMARTA mice deficient in either the IFN-I receptor IFNAR1 or the inducible IL-12Rβ2 subunit of the IL-12 receptor. We initially established whether these cells could form primary memory, which is a critical component of the heterologous secondary challenge model. To test this, we cotransferred equal numbers of WT ($Thy1.1^+$) and receptor knock-out SMARTA ($Thy1.1^+Thy1.2^+$) cells into WT B6 hosts and challenged with LCMV. By 8 days postchallenge, we saw defects in expansion of both receptor knock-out cell types compared to WT SMARTA cells, which were detectable by flow cytometry

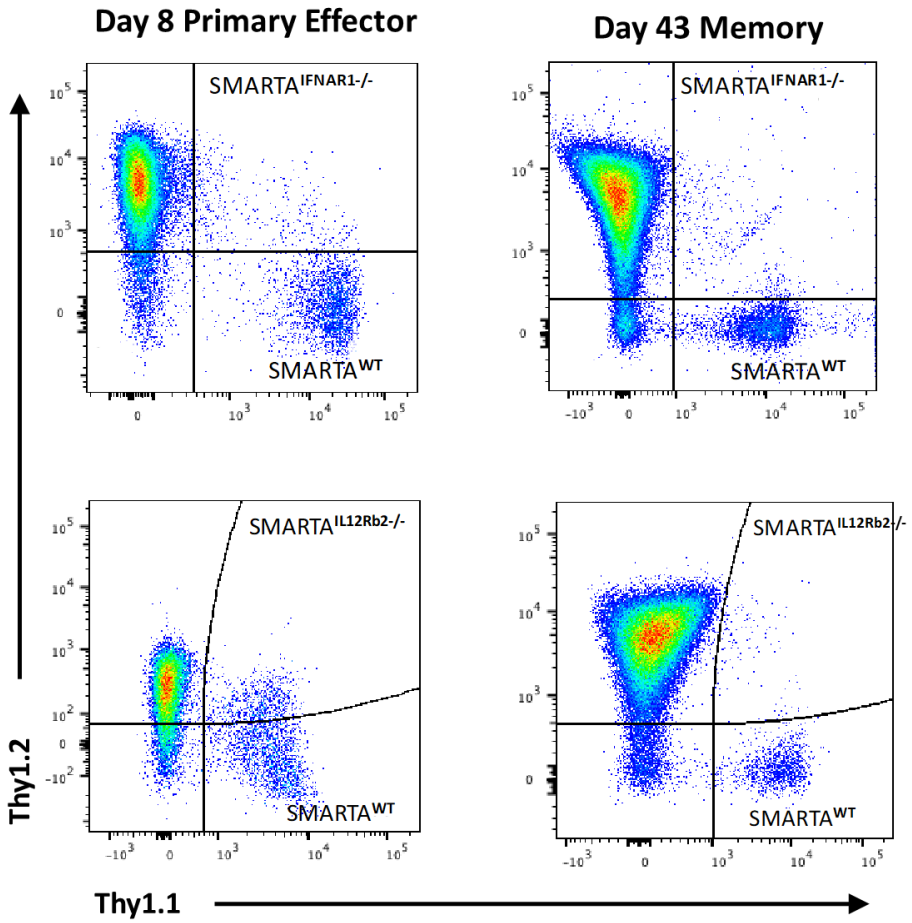

Figure 2.3. T cells require IL-12 and IFN-I signaling to form memory after viral infection. Representative flow plots of $CD4^+$ cells showing WT SMARTA ($Thy1.1^+$) and receptor knock-out SMARTA ($Thy1.1^+Thy1.2^+$) cells transferred to a WT ($Thy1.2^+$) host at days 8 and 43 postchallenge with LCMV.

staining (Fig. 2.3). This was greater in IFNAR1 knock-out SMARTA cells, as we could not detect these cells by day 8. Other groups have published similar data, evidencing a requirement for IFN- I signaling directly to Th1 primary effector cells during a viral infection (28). SMARTA cells lacking the IL-12Rβ2 subunit appear to be present at day 8 postchallenge as measured by flow cytometry, but none of these cells are detectable in the memory population. This is not a surprising result, as IL-12 signaling is required for initial $CD4^+$ T cell differentiation to the Th1 effector subset. Together, these data show that $CD4^+$

Th1 effector cells require both direct IL-12 and IFN-I signaling to properly respond to viral infections and form memory. Unfortunately, this renders these mice useless as tools to study the requirement of direct IL-12 and IFN-I signaling on T cells during a secondary effector response due to the lack of memory formation.

Endogenous IFNγ expression by CD4$^+$ effector cells is required for optimal response

For our previously published work examining the role of Signal 3 cytokines on T cell function, we show changes in functional avidity differentially mediated by IL-12 and IFN-I signaling. We were curious as to how critical this endogenous IFNγ production was for controlling overall T cell function, and to test this we bred IFNγ-deficient SMARTA cells (Thy1.1$^+$Thy1.2$^+$) for use in our heterologous secondary challenge model. Somewhat surprisingly, these cells were able to form a memory population that we could then cotransfer to naïve B6 hosts for heterologous secondary challenge with Lm-gp61. While initial adoptive transfer was performed at a 1:1 ratio between WT and *ifnγ*$^{-/-}$ SMARTA, by 50 days postchallenge the memory population was skewed towards WT 3:1 (data not shown). We observe no defect in functional avidity in the absence of endogenous IFNγ production as measured by TNF at memory or secondary effector timepoints (Fig. 2.4A, data not shown). While somewhat surprising, due to these cells being Th1 secondary effector cells, this result can likely be explained by the presence of a large variety of other cells that produce IFNγ (e.g., WT SMARTA, endogenous CD4$^+$ T cells, macrophages). We do however notice a significant decrease in the total numbers of SMARTA cells, and the number of these cells producing multiple cytokines (TNF$^+$IL-2$^+$) (Fig. 2.4B, C). The latter is an artifact of the total population size, as there are no differences in the proportion

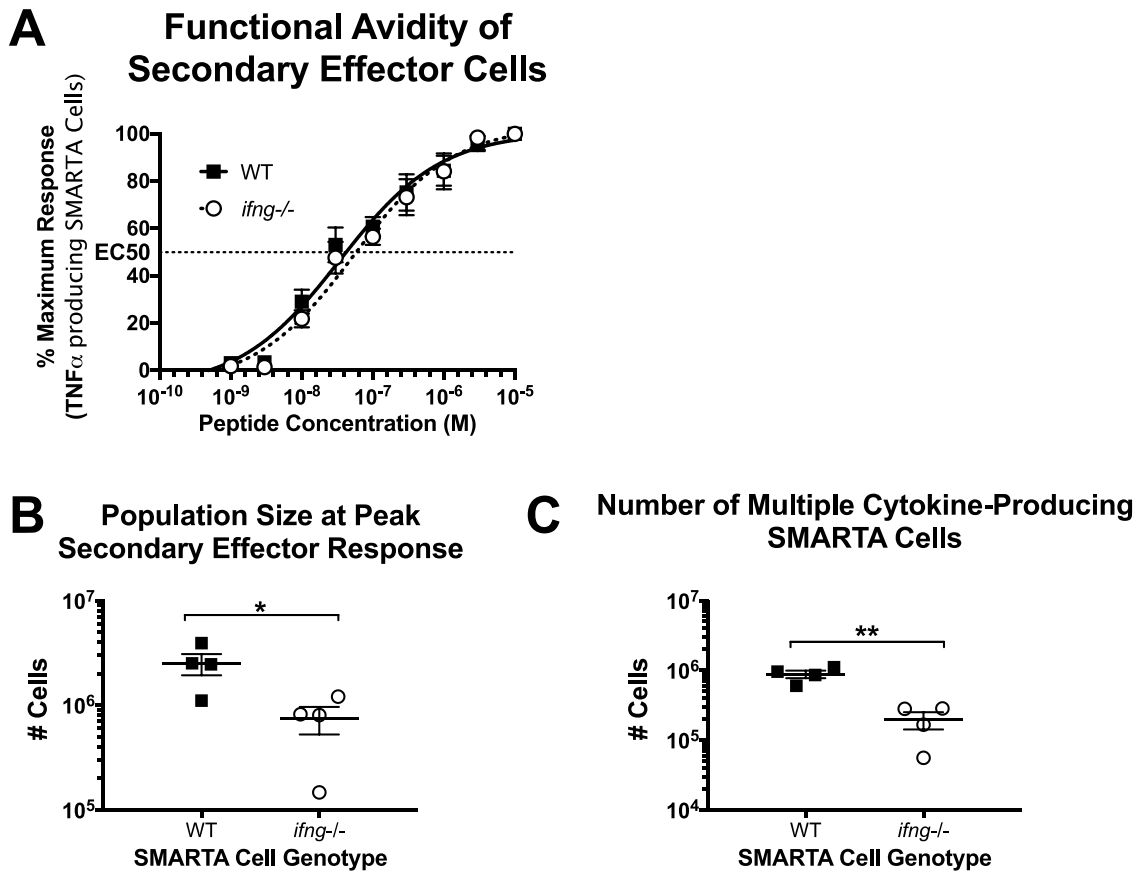

Figure 2.4. CD4+ T cells require endogenous IFNγ production for maximum expansion. (A) WT and IFNγ$^{-/-}$ SMARTA cells in the same hosts were stimulated ex vivo with decreasing concentrations of gp61-80 peptide and stained intracellularly for production of TNF. Functional avidity curves were calculated, and the dotted line indicated the EC_{50}. (B) Total numbers of SMARTA cells in the spleen from each type were calculated using flow cytometry staining and whole spleen counts. (C) Total numbers of TNF^{+}IL-2^{+} SMARTA cells were calculated using flow cytometry staining and the total numbers of SMARTA cells. n=4 mice, cells were cotransferred into each mouse.

of WT and *ifnγ*$^{-/-}$ SMARTA cells with multi-cytokine potential. These data support a role for IFNγ production in effective expansion of CD4^{+} effector T cells, potentially via an autocrine signaling pathway, though they also indicate that effector function is at least partially independent of endogenous IFNγ production.

Loss of IFN-I signaling positively impacts T cell function in a T cell-indirect manner

As previously mentioned, antibody-mediated IFNAR blockade experiments do not show if IFN-I is signaling directly to T cells to dampen T cell functionality. While *Ifnar*$^{-/-}$ SMARTA cells are not a viable option for our secondary challenge model (Fig. 2.3), we are able to address whether indirect loss of IFN-I signaling on all cell types except the SMARTA T cells is sufficient to induce changes in T cell function by using *Ifnar*$^{-/-}$ secondary hosts. To accomplish this, we adoptively transferred memory SMARTA cells as before, but this time using either WT controls or *Ifnar*$^{-/-}$ mice as secondary hosts, and subsequently heterologously challenged with Lm-gp61. It is important to note that, as with previous work with *Listeria* challenge of *Ifnar*$^{-/-}$ hosts, the pathogen is cleared more rapidly with the loss of IFN-I signaling (Fig. 2.5A). We also observe a similar increase in T cell functional avidity when all host cells lack IFNAR, indicating that these functional changes are produced via indirect signaling by IFN-I (Fig. 2.5B). Unlike with our IFNAR-blockade experiments, there are no significant differences in total numbers of SMARTA cells 7 days postinfection with the loss of IFN-I responsiveness in the host, indicating a partial role for IFN-I signaling directly to T cells that is specific to controlling cell expansion (Fig. 2.5C).

The fact that these hosts more rapidly clear pathogen could be complicating the interpretation of these results, as the memory pool is populated by T cells with higher

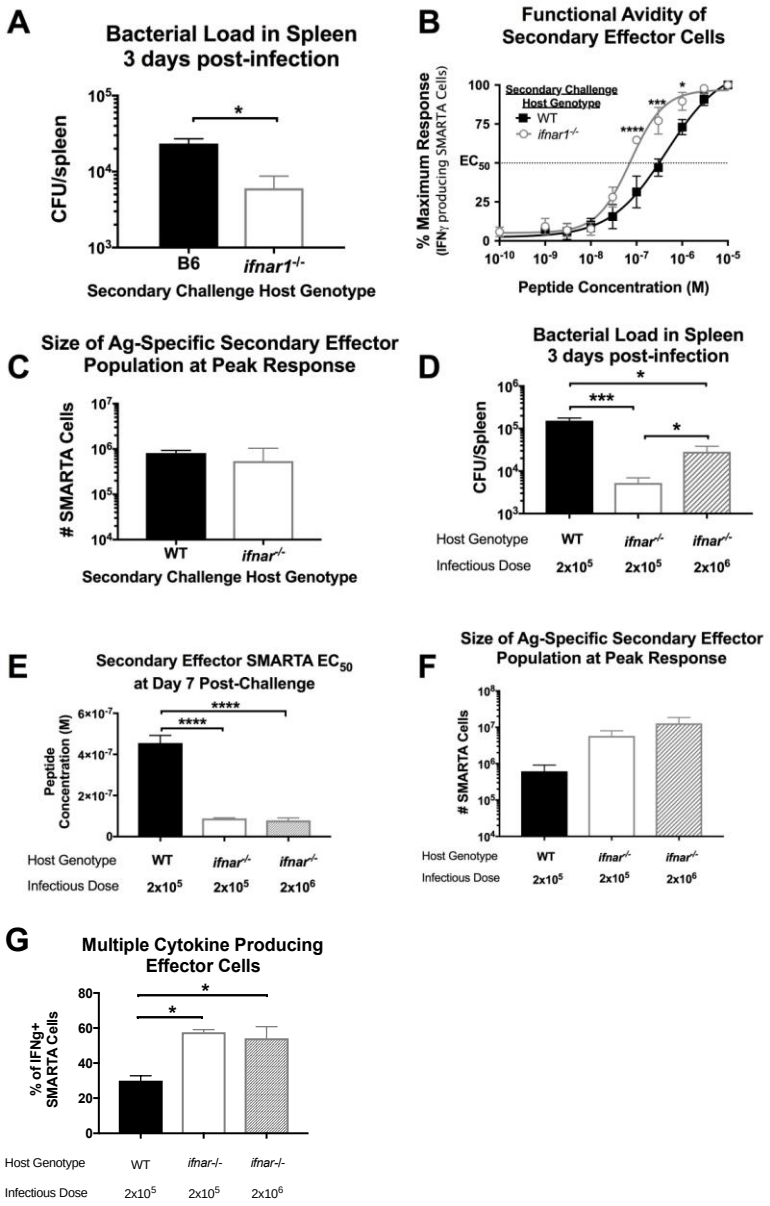

Figure 2.5. Effects of IFN-I are CD4+ T cell-indirect and antigen-independent. Memory SMARTA cells from LCMV-immune mice were transferred to naïve WT or *Ifnar*$^{-/-}$ hosts and infected with Lm-gp61. (A) Bacterial burden at 3 days postchallenge were measured by CFU of Lm-gp61 per spleen. (B) The functional avidity as measured by IFNγ production by SMARTA cells in responses to ex vivo stimulation with decreasing concentrations of gp61-80 peptide. (C) The number of SMARTA cells in the spleen as calculated from flow cytometry staining and total splenocyte numbers at day 7 postchallenge. (D) Bacterial burden 3 days postchallenge with Lm-gp61 at the initial infectious dose indicated. (E) The functional avidity of the SMARTA population in response to different infectious doses, as noted in the figure, reported as the EC_{50}. (F) Effector population size at day 7 postchallenge. (G) Proportion of IFNγ-producing SMARTA cells that are also able to make TNF and IL-2, as determined by flow cytometry staining, at day 7 postchallenge. n=2-3 mice/group.

functional avidity and faster clearance may correlate to more rapid formation of the memory population (14, 23, 27, 29). To rectify this, we increased the infectious dose to produce similar levels of bacterial in the spleen as the WT host 3 days postchallenge. While 10-fold increase of the infectious dose in an *Ifnar*$^{-/-}$ host still results in significantly fewer bacteria in the spleen by day 3 postchallenge compared to a WT animal, the difference between clearance in WT and *Ifnar*$^{-/-}$ animals is smaller than when both genotypes are given equivalent doses (Fig. 2.5D). Importantly, changing early the bacterial burden in the spleen has no effect on later T cell functional avidity, as the EC_{50} at day 7 postchallenge is effectively identical regardless of infectious dose in the *Ifnar*$^{-/-}$ host (Fig. 2.5E). Similarly, infectious dose has no significant effect on the number of SMARTA secondary effector cells (Fig. 2.5F). Both infectious doses result in increased proportions of IFNγ-producing cells also producing TNF and IL-2 compared to SMARTA cells in WT hosts, but increasing the infectious dose does not induce a difference when compared to the lower dose (Fig. 2.5G). Taken together, we have shown a T cell indirect and antigen-independent role for IFN-I signaling in controlling the extent of $CD4^+$ effector responses during a heterologous *Listeria* challenge.

Challenge of Ifnar$^{-/-}$ *mice results in a distinct inflammatory profile*

With use of neutralizing and blocking antibodies, as in Figures 2.1 and 2.2, we expected and observed broad changes to the cytokine milieu. Similarly, challenge of *Ifnar*$^{-/-}$ hosts with lm-gp61 should induce an altered inflammatory state due to the lack of productive IFN-I signaling in most cells. We observe that WT mice actually have higher serum levels of IFNγ 1 day postchallenge (Fig. 2.6A). This is the opposite of what we observe with IFNAR-blockade experiments, and further supports a direct role for IFN-I

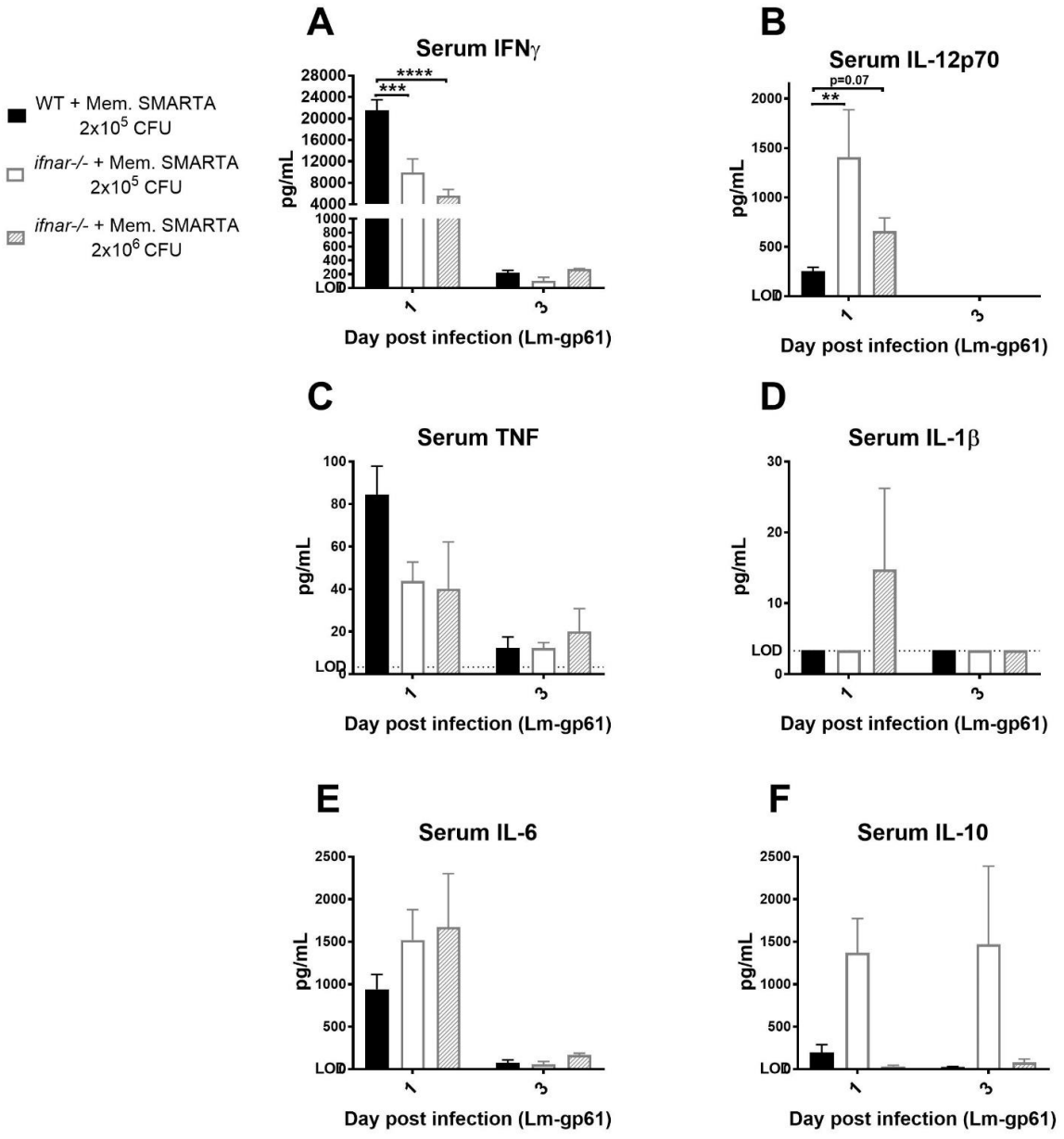

Figure 2.6. Systemic inflammation in WT and *Ifnar*-/- animals after Lm-gp61 challenge. Serum was obtained on days 1 and 3 postchallenge and analyzed via LegendPlex multiplex kits (A-F). n=2-3 mice/group.

signaling to $CD4^+$ T cells. Specifically, it appears that IFN-I may reduce T cell production of IFNγ through IFNAR on the T cells themselves. While this is sufficient to reduce systemic levels of IFNγ early in infection, we still observe functional changes to T cells in these hosts at later timepoints. As with IFNAR-blockade, we do observe an increase in IL-12 levels by day 1 postchallenge when *Ifnar*$^{-/-}$ hosts are infected with a normal or high infectious dose (Fig. 2.6B). While the higher infectious dose does result in lower levels of IL-12 compared to the normal infectious dose, this difference is not significant and it is still higher than in WT animals. While none of the other cytokines observed showed truly significant differences when all groups were compared (Fig. 2.6C-E), IL-10 did show a confusing profile. When *Ifnar*$^{-/-}$ hosts were infected with comparable levels of bacteria as WT hosts, there was a significant increase in IL-10, but not when they were infected with 10-fold more bacteria (Fig. 2.6F). While these results are statistically significant, we fail to draw any real conclusions from this due to the large error range and low number of mice per group. Repetition of this experiment will show if this is accurate or just an artifact due to an outlier animal.

Loss of IL-10 exerts similar effects on T cell function as loss of IFN-I signaling

As previously mentioned, one of the downstream effects of IFN-I signaling is the induction of IL-10 (30–32). IL-10, produced by Tregs and innate cells such as macrophages, is a potent antiinflammatory molecule that can mediate downregulation of proinflammatory cytokines such as IFNγ and IL-12. We hypothesized that one way IFN-I signaling may impede the formation of secondary effector cells with high functional avidity was via induction of IL-10, a cytokine known to inhibit production of IL-12. To test this,

we repeated earlier adoptive transfer experiments, but this time using *il10*$^{-/-}$ hosts rather than *Ifnar*$^{-/-}$ hosts. Similar to what we observe in *Ifnar*$^{-/-}$ animals and consistent with previously published work, *il10*$^{-/-}$ animals clear *Listeria* infections more rapidly (Fig. 2.7A). SMARTA cells in *il10*$^{-/-}$ animals exhibited increased functional avidity but no significant differences in expansion by day 7 postchallenge (Fig. 2.7B, C), but as with challenge in *Ifnar*$^{-/-}$ hosts this could be due to more rapid clearance and contraction to memory populations. To rectify this, we again increased the infectious dose by 10-fold and examined whether these changes to T cell function were independent of the bacterial load (i.e., antigen-independent). Increasing the initial infectious dose results in day 3 bacterial loads in the spleen similar to those of WT animals infected with the normal infectious dose (Fig. 2.7D). Interestingly, while the clearance is approximately the same between normally and highly infected *il10*$^{-/-}$ mice, there in a further significant increase in functional avidity of these cells, compared to both WT and *il10*$^{-/-}$ animals infected with normal bacteria (Fig. 2.7E). As this is an initial study, repetition of this experiment will be required to determine if this is an antigen-dependent increase in effector function or just a slight difference due to low group numbers. While there could be an antigen-dependent effect on the functional avidity of these cells, there is no difference in expansion due to differences in bacterial burden (Fig. 2.7F).

IL-10 and IFN-I have different effects on systemic inflammation

We examined serum cytokine concentrations during early response to Lm-gp61 challenge and, while there are some similarities to the cytokine profile produced by challenge of *Ifnar*$^{-/-}$ mice, the overall systemic cytokine milieu is distinct for the *il10*$^{-/-}$ and *Ifnar*$^{-/-}$ hosts (Fig. 2.8). Specifically, IFNγ levels are similarly lowered in *il10*$^{-/-}$ hosts

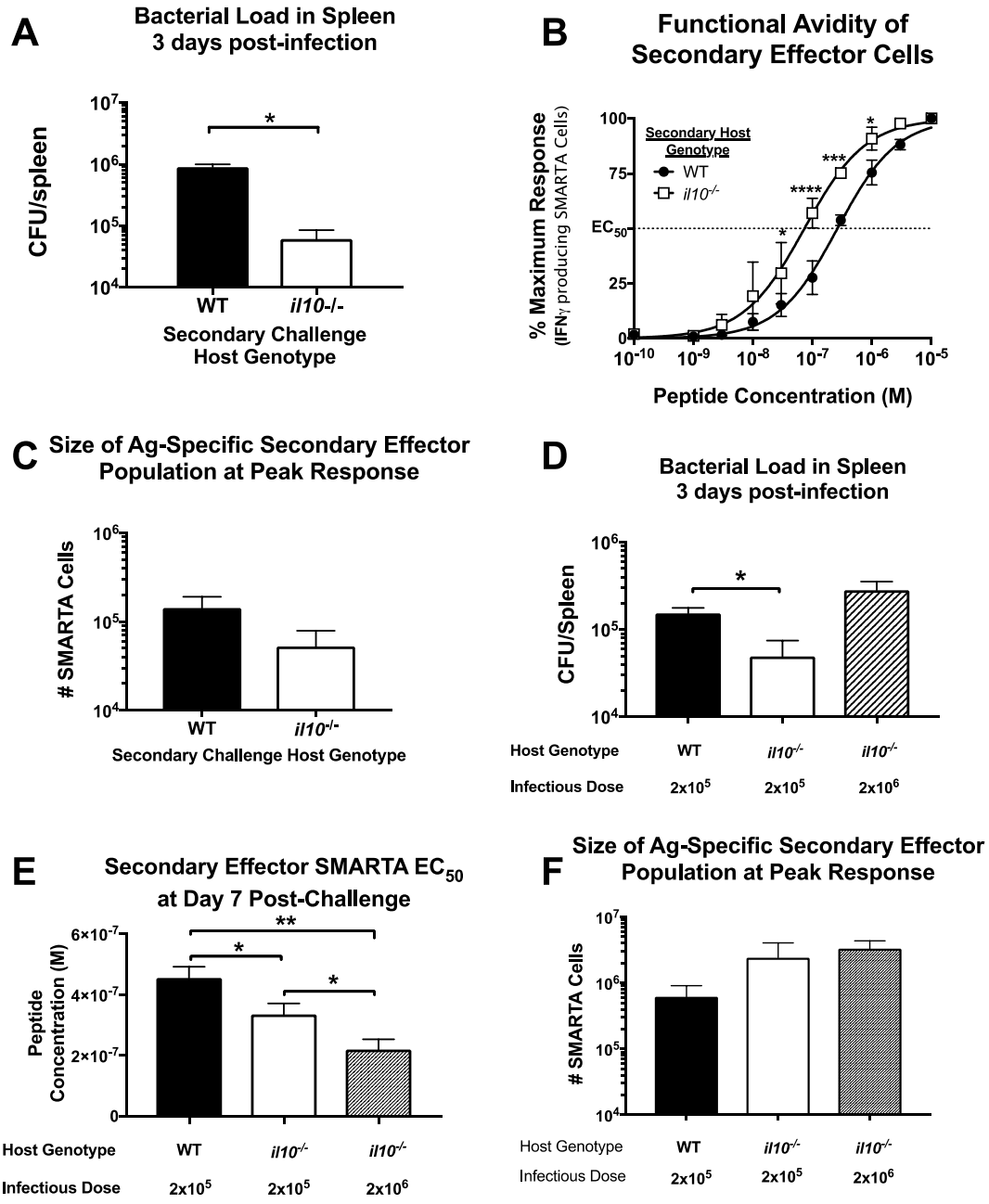

Figure 2.7. Loss of IL-10 exerts similar effects on T cell function as loss of IFN-I signaling. Memory SMARTA cells were transferred to naïve WT or *il10*$^{-/-}$ hosts and infected with Lm-gp61. (A) Bacterial burden at 3 days postchallenge were measured by CFU of Lm-gp61 per spleen. (B) The functional avidity as measured by IFNγ production by SMARTA cells in responses to ex vivo stimulation with decreasing concentrations of gp61-80 peptide. (C) The number of SMARTA cells in the spleen as calculated from flow cytometry staining and total splenocyte numbers at day 7 postchallenge. (D) Bacterial burden 3 days postchallenge at the initial infectious dose indicated. (E) The functional avidity of the SMARTA population in response to different infectious doses, as noted in the figure, reported as the EC_{50}. (F) Effector population size at day 7 postchallenge. n=3 mice/group.

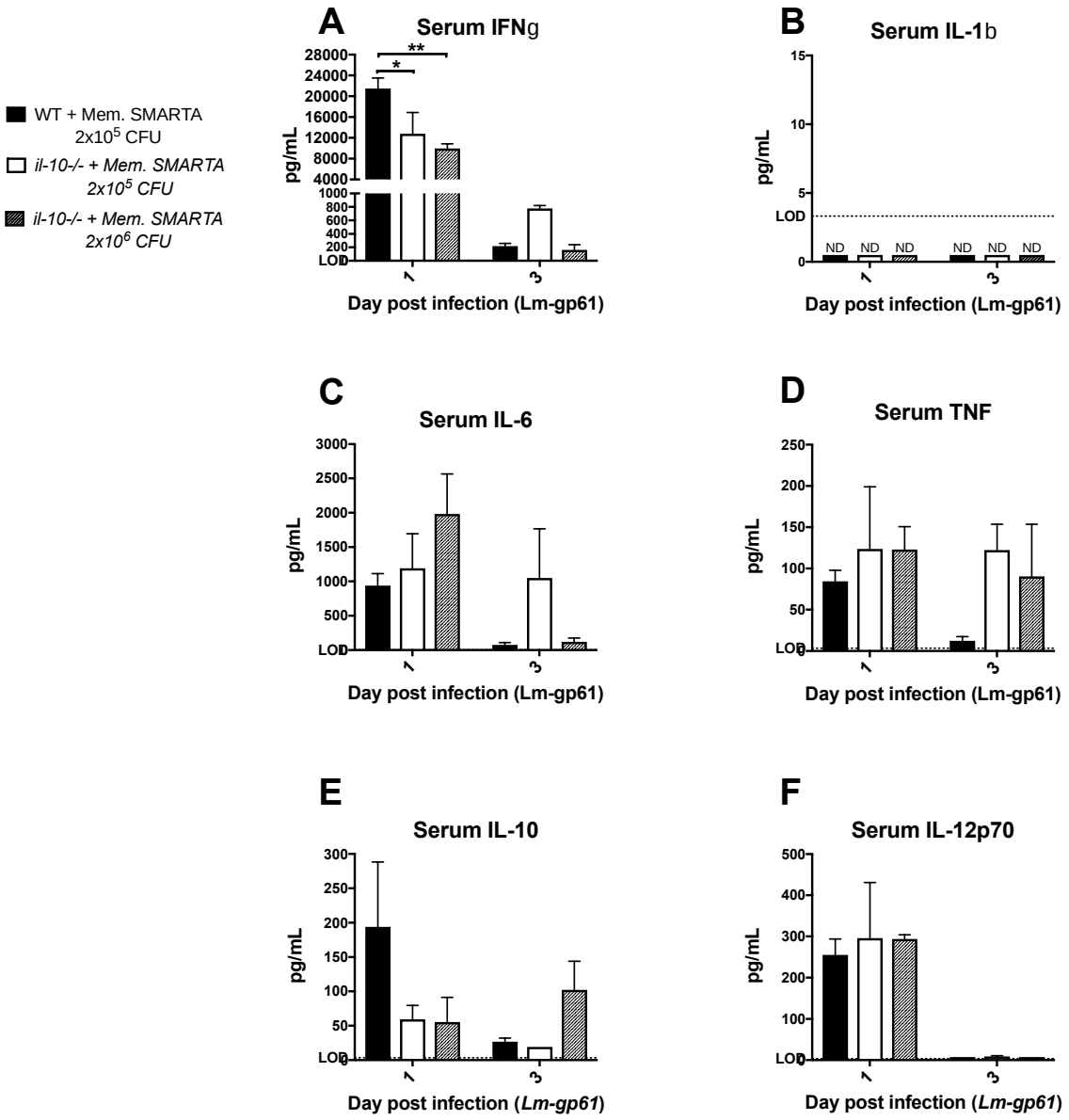

Figure 2.8. Systemic inflammation in WT and il10-/- animals after Lm-gp61 challenge. Serum was obtained on days 1 and 3 postchallenge and analyzed via LegendPlex multiplex kits (A-F). n=3 mice/group

compared to WT animals (Fig. 2.8A). Other similarities include no significant differences in IL-1β, IL-6, and day 1 levels of TNF (Fig. 2.8B-D). Day 3 levels of TNF are elevated in the absence of IL-10, which is most likely due to the lack of antiinflammatory signaling due to a lack of Treg function (Fig. 2.8D). As expected, IL-10 levels are lower in *il10*$^{-/-}$ animals, but there are detectable levels of this cytokine even in the knock-out animals (Fig. 2.8E). This is either due to the presence of SMARTA cells and the plasticity of differentiated T helper cells to produce cytokines not typically attributed to their differentiated helper subset (33, 34), or potentially due to nonspecific binding in the detection assay. Surprisingly, IL-12 levels were not affected by the absence of IL-10 signaling (Fig. 2.8F). This indicates that IFN-I signaling in our model is likely not influencing IL-12 levels via IL-10 induction, as we would expect an equivalent increase in IL-12 in both genotypes if that were the case. Ultimately, this serum data indicate that IL-10 and IFN-I utilize different mechanisms to regulate T cell function, though there may be some overlap.

Discussion

The role of Signal 3 cytokines in CD8$^+$ T cell function is well-described, particularly concerning IL-12 and IFN-I. As with much of the T cell canon, the emphasis of study typically falls on CTLs, rather than CD4$^+$ T helper cells, leaving the role of Signal3 cytokines during secondary effector CD4$^+$ responses poorly understood. Here we used a TCR transgenic mouse model to study the role of TCR-independent cytokine signaling in programming secondary effector CD4$^+$ T cell function. We observe two different but potentially related mechanisms of controlling these responses, via either IFN-I signaling or IL-10 signaling. IFN-I inhibits IL-12 production, which in turn decreases overall IFNγ

production and T cell effector function. Inhibition of this pathway via receptor inhibition or knock-out results in increased early systemic IL-12 and IFNγ, as well as increased T cell effector function. IL-10, on the other hand, does not appear to impact IL-12 levels, but reduced IL-10 levels increase T cell function. Overall, we observe a significant role for Signal 3 cytokines in the secondary effector differentiation of memory $CD4^+$ T helper cells that is independent of their TCR affinity for antigen.

Further study is required to understand how these and other Signal 3 cytokines will influence T cell responses in different infectious settings. Here we use a *Listeria* infection model, but it is well appreciated that different types of infections can induce different cytokine signaling. As mentioned, IFN-I signaling is detrimental for protection from bacterial infections such as *Listeria* but is required for protection in a viral setting. However, we are currently unsure of the role of IFN-I on T cell function in a viral setting, particularly in regards to secondary effector differentiation. We observe that primary effector $CD4^+$ T cells fail to properly expand and form memory in response to challenge with LCMV when they lack IFNAR1, indicating a requirement for direct IFN-I signaling to $CD4^+$ T cells during primary viral infection that is consistent with published data concerning $CD8^+$ T cells. To study the role in a secondary viral infection, we must use different experimental tools, specifically a different heterologous secondary challenge model. Primary infection with Lm-gp61 fails to produce a robust primary memory population, so simply inverting the order in which we use LCMV and Lm-gp61 will not be sufficient for these studies. Our lab has a strain of vaccinia virus that also expresses gp61-80 (VV-gp61), meaning we can use this virus in place of Lm-gp61 in our heterologous rechallenge model to study the role of Signal 3 cytokines in memory $CD4^+$ T cell-mediated

secondary responses to a virus. Regardless of which tools are used, it is clear that further study is required to determine whether the role of IFN-I signaling in regards to T cell function is dependent upon the infection.

Additional questions remain based on the data we have presented here. First, it is likely that *Ifnar*$^{-/-}$ and *il10*$^{-/-}$ do not have the same bacterial burden throughout infection compared to WT animals, even with increased infectious doses, due to differences in their kinetics of clearance. Examining the bacterial burden and T cell functional avidity maturation throughout the entire response to acute infection will determine if T cell functional differences are truly an antigen-independent effect of IFN-I and IL-10 signaling. Additionally, augmenting the clearance of WT animals via antibiotics such as ampicillin would demonstrate whether or not increased clearance rates are contributing to our observed phenotype. Second, the cytokine concentrations observed at the systemic level may not accurately represent the inflammatory environment at the site of infection, in this case the spleen and to a lesser extent the liver. More targeted analysis at the site of infection through qRT-PCR would give a more accurate representation of the cytokine milieu that is surrounding the T cells in question. Many other questions can be raised based on the data presented here to further our understanding of Signal 3 cytokines in this context, but these proposed follow-up studies will help to cement or reshape the conclusions we have drawn thus far. Increased comprehension of the roles of IFN-I, IL-12, and IL-10, along with other cytokines, in secondary T cell differentiation and subsequent memory formation will provide much-needed information in bolstering and augmenting vaccination and adjuvant therapies. Our studies will specifically provide information that can be utilized in the development of secondary vaccination or booster strategies.

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CHAPTER 3

IFN-GAMMA-DEPENDENT AND INDEPENDENT MECHANISMS OF $CD4^+$ MEMORY T CELL-MEDIATED PROTECTION FROM *LISTERIA* INFECTION

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IFN-Gamma-Dependent and Independent Mechanisms of CD4$^+$ Memory T Cell-Mediated Protection from *Listeria* Infection

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Abstract: While CD8$^+$ memory T cells can promote long-lived protection from secondary exposure to intracellular pathogens, less is known regarding the direct protective mechanisms of CD4$^+$ T cells. We utilized a prime/boost model in which mice are initially exposed to an acutely infecting strain of lymphocytic choriomeningitis virus (LCMV), followed by a heterologous rechallenge with *Listeria monocytogenes* recombinantly expressing the MHC Class II-restricted LCMV epitope, $GP_{61–80}$ (Lm-gp61). We found that heterologous Lm-gp61 rechallenge resulted in robust activation of CD4$^+$ memory T cells and that they were required for rapid bacterial clearance. We further assessed the relative roles of TNF and IFNγ in the direct anti-bacterial function of CD4$^+$ memory T cells. We found that disruption of TNF resulted in a complete loss of protection mediated by CD4$^+$ memory T cells, whereas disruption of IFNγ signaling to macrophages results in only a partial loss of protection. The protective effect mediated by CD4$^+$ T cells corresponded to the rapid accumulation of pro-inflammatory macrophages in the spleen and an altered inflammatory environment in vivo. Overall, we conclude that protection mediated by CD4$^+$ memory T cells from heterologous *Listeria* challenge is most directly dependent on TNF, whereas IFNγ only plays a minor role.

Keywords: immunological memory; secondary challenge; CD4$^+$ T cells; *Listeria monocytogenes*

1. Introduction

A hallmark of adaptive immunity is the formation of memory following immunization or infection. Memory T cells, once formed, survive stably in both mice and humans and are a key component of protective immunity, responding more rapidly and robustly to secondary challenge [1–5]. A large number of studies have assessed the effector functions by which CD8$^+$ memory T cells mediate protection from secondary exposure to viral or cytosolic bacterial pathogens, but studies of the role of CD4$^+$ memory T cells have more closely focused on their helper role in enhancing CTL and antibody responses. In order to directly analyze the properties and functions of secondary CD4$^+$ T cell responses, we utilized a prime/boost model in which mice are initially exposed to an acutely infecting strain of lymphocytic choriomeningitis virus (LCMV), followed by a heterologous rechallenge 6–8 weeks later with *Listeria monocytogenes* recombinantly expressing the immunodominant I-A^b-restricted LCMV epitope, $GP_{61–80}$ (Lm-gp61). Heterologous Lm-gp61 rechallenge resulted in robust secondary expansion of $GP_{61–80}$-specific CD4$^+$ memory T cells, enhanced secondary effector function as compared to homologous rechallenge with LCMV, and stable long-lived persistence of secondary CD4$^+$ memory T cells [2,6]. Furthermore, we found that CD4$^+$ memory T cells were capable of providing direct protection from a heterologous Lm-gp61 rechallenge [7]. Because protection in this model system was independent of CD8$^+$ T cells and antibodies, in the current study we have employed it to test mechanisms of direct protection mediated by CD4$^+$ memory T cells against the intracellular bacterium *Listeria monocytogenes* (Lm).

Several properties highlight the enhanced effector functions of $CD4^+$ memory T cells. First, $CD4^+$ memory T cells are highly sensitive to low antigen concentrations, allowing them to respond rapidly upon secondary infection [5,7,8]. Second, $CD4^+$ memory T cell are able to immediately produce multiple cytokines, including IFNγ, IL-2 and TNF, leading to a profound alteration of the early inflammatory environment following infection [7], and the presence of $CD4^+$ memory T cells with the ability to produce multiple cytokines is correlated to enhanced protection from secondary challenge [9,10]. Third, $CD4^+$ memory T cells can home to tissue sites of infection. For example, $CD4^+$ memory T cells can be detected in the liver many months after Lm infection and tissue-homing and/or tissue-resident $CD4^+$ memory T cells have been characterized for a variety of infections in the lung, skin and reproductive tract [11–16]. Lastly, $CD4^+$ memory T cells can rapidly re-express effector molecules such as Granzyme B and exert cytolytic function [17–19], although these findings are mostly limited to anti-viral responses.

In a prior study we found that the secondary effector function of $CD4^+$ memory T cells was highly dependent on the inflammatory environment, as disruption of IL-12 and Type I IFN had opposing effects on effector differentiation of $CD4^+$ memory T cells [7]. Further exploration of how the inflammatory environment influences secondary responses by $CD4^+$ T cells will allow for a better understanding of how current vaccine strategies, particularly booster vaccinations which result in secondary responses and secondary memory formation, impact $CD4^+$ memory T cell protective function.

While $CD4^+$ T cells are required for optimal generation and maintenance of $CD8^+$ memory T cells [20–23], protection from primary and secondary Lm infection mediated by $CD8^+$ T cells plays a more dominant role than $CD4^+$ T cells. $CD8^+$ T cells can mediate primary and protective immunity in a manner that is independent of Perforin and IFNγ but dependent on TNF [24–26], although other studies have indicated a potential role for both Perforin and IFNγ in CD8-mediated immunity [27,28]. The key role of TNF is further reinforced by the finding that patients receiving TNF inhibitor treatment for chronic inflammatory conditions are more susceptible to infection with intracellular bacteria, including Lm [29,30]. In contrast, $CD4^+$ T cell-mediated protection was reported to require IFNγ [31]. However, these studies depended on adoptive transfer models that may not have replicated the in vivo inflammatory environment induced by endogenously arising $CD4^+$ T cell responses. Both IFNγ and TNF are key components of the innate response, with genetic disruption of these pathways resulting in early lethality following Lm infection [26,32,33]. IFNγ is a well-studied inducer of macrophage activation, and Type I IFN, a known antagonist of protective immunity to Lm [34,35], acts in part by inducing down-regulation of the IFNγR on macrophages [36]. Clearly, both TNF and IFNγ play key roles in the adaptive and innate arms of the protective immune response to Lm. However, it remains unclear whether endogenously arising $CD4^+$ memory T cells mediate protective immunity via IFNγ, as during the primary response, or whether they adopt distinct mechanisms of protection.

Due to our observation that $CD4^+$ memory T cells are sufficient to mediate rapid clearance of secondary Lm infection, we sought to define the mechanisms behind their protective effect. In a variety of model systems, the mechanism underlying the protective function of both innate and adaptive immune cells, including $CD4^+$ T cells, are distinct during the primary versus secondary immune response [37–39]. We utilized a heterologous rechallenge model wherein mice are initially infected with LCMV and allowed at least 30 days for pathogen clearance and memory formation. Subsequently, mice were rechallenged with Lm-gp61, allowing for the specific induction of recall responses by I-A^b/$GP_{61–80}$-specific $CD4^+$ memory T cells without substantial contribution from the $CD8^+$ memory T cell compartment. Because previous studies with Lm have highlighted the critical roles of IFNγ and TNF in protective $CD4^+$ or $CD8^+$ T cell function during primary responses, we hypothesized that $CD4^+$ memory T cells would mediate protection following heterologous rechallenge primarily via the production of IFNγ. Conversely, TNF is required for secondary protection mediated by $CD8^+$ T cells [24,25]. Additionally, SCID mice showed impaired clearance at day 3 post-infection when treated with TNF neutralizing antibodies, and by day 5 most anti-TNF treated animals had succumbed to

infection [40], showing a requirement for TNF in both the innate and adaptive arms of the immune response. Therefore, we also considered the possibility that $CD4^+$ memory T cells could mediate enhanced Lm clearance via early production of TNF.

We found that $CD4^+$ memory T cell-dependent protection from heterologous challenge with Lm-gp61 was only somewhat dependent on the presence of IFNγ. Even macrophage-specific deletion of IFNγR resulted in only a modest loss of protection following secondary challenge. Conversely, neutralization of TNF resulted in a severe reduction in protection following heterologous rechallenge. Accumulation and activation of IFNγR-expressing M1 phenotype macrophages during the secondary response required the presence of $CD4^+$ memory T cells and TNF. Overall these results point to a substantially greater role for TNF than IFNγ in $CD4^+$ memory T cell-mediated protection from heterologous Lm-gp61 rechallenge and suggest that the mechanisms of protection mediated by $CD4^+$ T cells during primary and secondary challenges differ.

2. Results

2.1. $CD4^+$ Memory T Cells Induce Rapid Clearance of a Heterologous Lm-gp61 Challenge

We sought to define the protective capacity of $CD4^+$ memory T cells in LCMV-immune mice receiving a heterologous rechallenge with Lm-gp61. In our previous studies, we performed adoptive transfer experiments to demonstrate a direct role for $CD4^+$ memory T cells in mediating protection. However, protection in our heterologous prime/boost model (LCMV → Lm-gp61) could be complicated by the presence of a CTL response to a previously described minor MHC Class I-restricted epitope within $GP_{61–80}$ [41]. To address this issue, B6 mice were infected with LCMV, then challenged with Lm-gp61 >30 days later following treatment with anti-CD4-depleting or PBS control. By day 3 post-challenge with Lm-gp61, LCMV-immune mice cleared infection more rapidly in both the spleen and liver (Figure 1A,B). Addition of an isotype control during either primary infection or heterologous rechallenge had no effect on clearance (data not shown). The protective effect was dependent upon the presence of $CD4^+$ T cells, as mice treated with CD4-depleting antibody prior to infection had bacterial loads similar to a primary infection (Figure 1A,B). This effect is specifically due to the presence of memory $CD4^+$ T cells in immune mice and not myeloid cells expressing CD4, as depletion of $CD4^+$ T cells in a naïve host had no effect on pathogen load and bacterial clearance (Figure 1C). Depletion of $CD8^+$ T cells prior to rechallenge resulted in no significant difference in protection (data not shown), confirming the dominant role for $CD4^+$ memory cells in protection from heterologous rechallenge with Lm-gp61. There was no change in the total number of $CD4^+$ or $CD8^+$ T cells when comparing primary versus secondary challenge (Figure 1D) but rather an increase in the number of antigen-specific secondary effector cells. In untreated LCMV-immune mice large numbers of secondary effector $CD4^+$ T cells were present in the spleen and able to produce high levels of multiple cytokines upon restimulation, including IFNγ, TNFα and IL-2 (Figure 1E). These results confirm that rapid clearance following heterologous rechallenge is dependent on the presence of $CD4^+$ memory T cells.

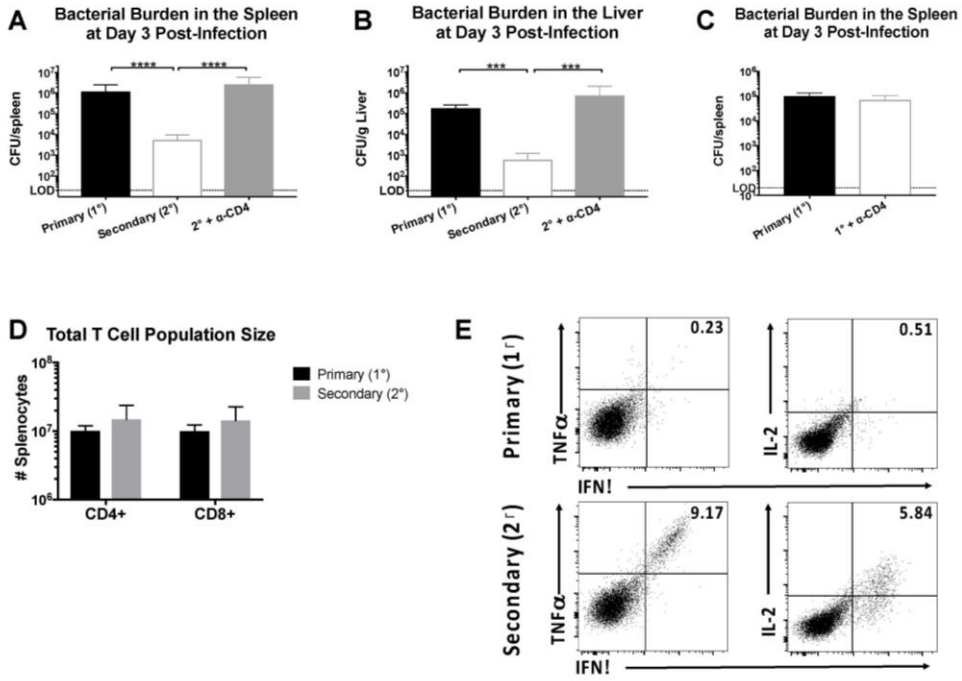

Figure 1. CD4$^+$ memory T cells induce rapid clearance following Lm-gp61 rechallenge. Naïve or lymphocytic choriomeningitis virus (LCMV)-immune (>day 30 post-infection) B6 mice were infected with Lm-gp61. Additionally, some LCMV-immune mice were treated with anti-CD4 antibody to deplete CD4$^+$ T cells prior to challenge. Bacterial burden in the (**A**) spleen (CFU/spleen) and (**B**) liver (CFU/g) was measured 3 days post-challenge; (**C**) Bacterial burden in the spleen of naïve mice with and without CD4 depletion, infected with Lm-gp61 was measured 3 days post-challenge; (**D**) Total numbers of CD4$^+$ and CD8$^+$ T cells in the spleen at day 3 post-challenge of naïve and LCMV-immune mice; (**E**) At day 3 post-challenge, splenocytes were stimulated *ex vivo* with the LCMV peptide $GP_{61–80}$ in the presence of Brefeldin A, then fixed and permeabilized and stained with antibodies to detect the presence of intracellular cytokines. Representative flow plots are gated on total CD4$^+$ T cells. n = 4–5 mice/group, data are representative of two separate experiments.

2.2. Heterologous Secondary Challenge with Lm-gp61 Induces an Altered Inflammatory Environment Compared to Primary Lm-gp61 Infection

To assess the impact of the rapid response of CD4$^+$ memory T cells on the early inflammatory environment following rechallenge, we measured the levels of inflammatory cytokines in the serum after primary Lm-gp61 challenge or secondary heterologous Lm-gp61 challenge. Heterologous rechallenge of CD4$^+$ memory T cells responses induced significantly higher levels of IFNγ. TNF in the serum was not significantly different by 24 h post-infection, as compared to primary challenge with Lm-gp61. This may reflect differences in local versus systemic concentrations of TNF. In contrast, heterologous challenge resulted in reduced induction of systemic IL-12 and IL-6, with no significant differences in the levels of IL-1 and IL-10 (Figure 2A–F). It has long been appreciated that IFNγ is a critical cytokine in mediating protection from primary Lm infection [26] and higher serum IFNγ levels correlate to more rapid clearance in our model. In contrast, levels of IL-12p70 are significantly lower in a secondary infection, indicating that CD4$^+$ memory T cell-dependent induction of elevated IFNγ is likely IL-12-independent (Figure 2B). Despite the previously described central role of TNF in protection from Lm, differences in systemic TNF levels between primary and secondary infections were not significant (Figure 2C). Although IL-6 is another key cytokine required for protection from primary Lm infection [42,43], we observed lower levels of systemic IL-6 following heterologous

Lm-gp61 challenge, as compared to primary infection (Figure 2C). Overall, these differences highlight the unique inflammatory environment induced by the secondary response of $CD4^+$ memory T cells, suggesting the possibility that inflammatory cytokines could have unique roles in these settings.

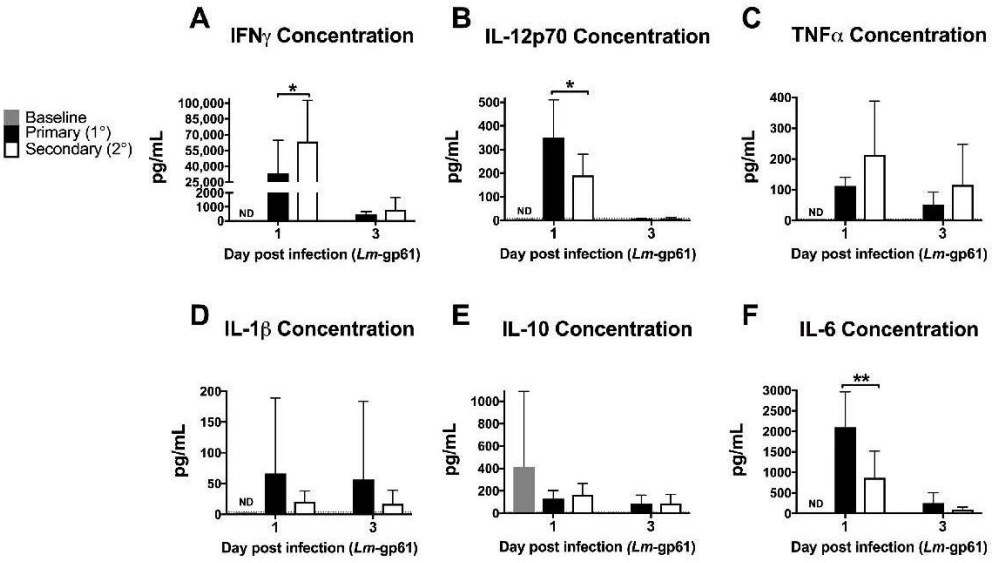

Figure 2. Heterologous challenge with Lm-gp61 induces increased levels of serum IFNγ. Naïve and LCMV-immune mice were challenged with Lm-gp61. Cytokine concentrations in the serum at day 1 and 3 post-infection were assessed using a cytokine bead array for (**A**) IFNγ; (**B**) IL-12p70; (**C**) TNF; (**D**) IL-1β; (**E**) IL-10; and (**F**) IL-6. $n = 8$ mice/group, data are pooled from two separate experiments. Dotted lines indicate limit of detection for the assay.

2.3. Rapid Clearance Following Heterologous Lm-gp61 Challenge Is Highly Dependent on TNF But Only Partly Dependent on IFNγ Signaling to Myeloid Cells

As noted previously, both IFNγ and TNF are produced at high levels by $CD4^+$ memory T cells after heterologous challenge. To test the role of these cytokines in this setting, we challenged LCMV-immune mice with Lm-gp61 as before, with some groups receiving TNF- or IFNγ-neutralizing antibody treatments, or PBS control. While IFNγ neutralization resulted in a somewhat higher bacterial load by day 3 post-infection, the effect was modest and not statistically significant. In contrast, TNF neutralization resulted in a complete loss of protection (Figure 3A,B). Neutralization of TNF or IFNγ during a primary Lm-gp61 infection resulted in dramatically increased susceptibility to infection in LCMV-naïve mice, indicating that neutralization of either cytokine was sufficient to reduce protection from a primary response (Figure 3C,D, data not shown). Addition of isotype controls did not impact clearance (data not shown). Bacterial loads in untreated animals were consistent with our previously published results [7]. Given the complex immunomodulatory roles of IFNγ in vivo, we further tested whether IFNγ signaling directly to macrophages played a key role in protection. We crossed mice containing LoxP sites flanking the IFNγR1 locus with mice expressing Cre under the control of the LysM promoter in order to generate a scenario in which only IFNγ signaling to myeloid cells is disrupted. We then infected these mice with LCMV, which clear primary LCMV infection normally [44]. Upon heterologous challenge of these mice with Lm-gp61 30 days later, we observed a partial loss of protection in mice lacking IFNγR1 expression on myeloid lineage cells. Overall, we concluded that TNF plays a dominant role in $CD4^+$ memory T cell-mediated protection from secondary challenge, whereas IFNγ signaling to macrophages (and other myeloid cells) plays a significant but less dominant role. The TNF-dependent role in the protective effect mediated by $CD4^+$ memory T cells is a function

previously attributed to $CD8^+$ T cells. These results also highlight key differences in the requirement for IFNγR1 expression by macrophages during primary versus secondary Lm infection [45].

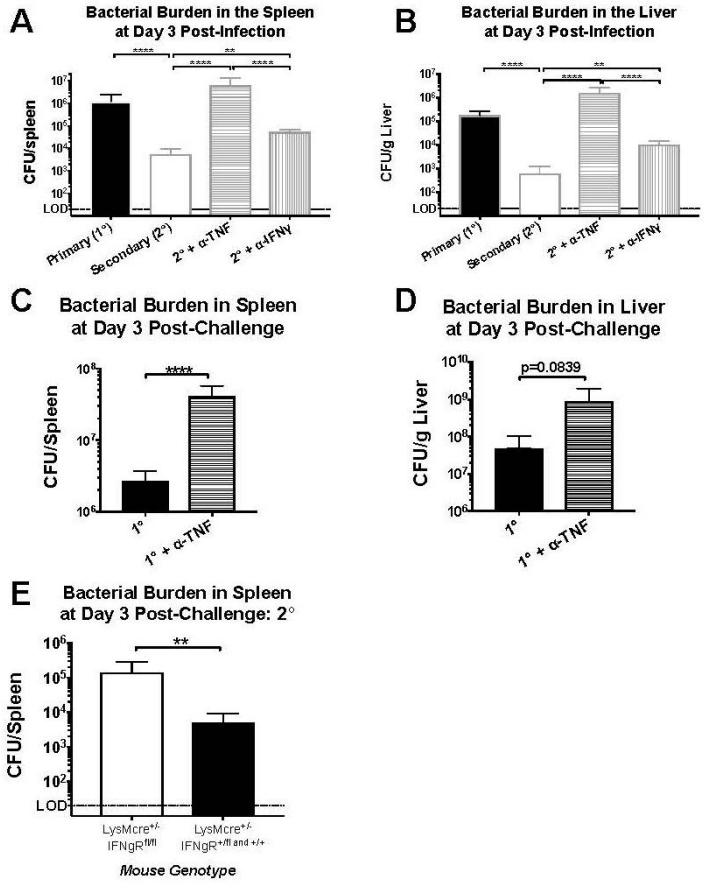

Figure 3. Protection mediated by $CD4^+$ memory T cells is heavily dependent on TNF but only partly dependent on IFNγ. Naïve and LCMV-immune mice were challenged with Lm-gp61 and some groups of mice were additionally treated with neutralizing antibodies to IFNγ or TNF. Bar graphs indicate bacterial load in the (**A**) spleen and (**B**) liver day 3 following primary or secondary challenge (n = 4–7 mice/group); (**C**,**D**) Bar graphs indicate bacterial load in the spleen and liver, as indicated, on day 3 following primary Lm-gp61 infection with or without TNF neutralization (n = 4 mice/group); (**E**) Bar graph shows bacterial burden in the spleen of LCMV-immune $LysM^{Cre}/Ifngr1^{fl/fl}$ mice day 3 after rechallenge with Lm-gp61 (n = 4 mice/group). Data are representative of at least two separate experiments.

2.4. TNF But Not IFNγ Is Required for Macrophage Activation Early in Secondary Infection

To determine what impact TNF signaling has on macrophage activation in vivo, we examined the accumulation of IFNγR-expressing macrophages in the spleen as well as the upregulation of IFNγR1 on their cell surface. We observed both a decrease in the frequency of IFNγR1-expressing macrophages in the spleen as well as a decrease in the intensity of IFNγR1 cell surface staining on day 3 following TNF neutralization during heterologous Lm-gp61 challenge (Figure 4A,B). In contrast IFNγ neutralization resulted in no change in the percent of $IFN\gamma R1^+$ macrophages, though the surface expression increased on a per cell basis. This may be a result of cytokine neutralization, as binding of IFNγ to its receptor results in receptor internalization and lower surface expression [46]. Differences

in the accumulation of IFNγR-expressing macrophages occurs only early in infection, as by 8 days post-infection there are no significant differences between treatment groups (Figure 4A,B).

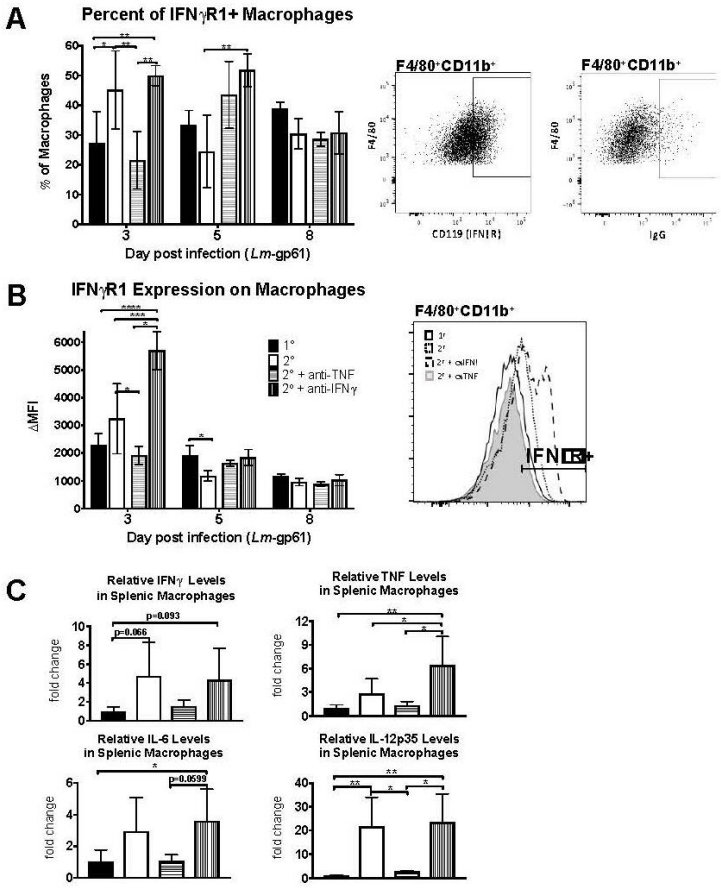

Figure 4. Neutralization of TNF prevents accumulation of activated macrophages in the spleen. Naïve and LCMV-immune mice were challenged with Lm-gp61 and some groups of mice were additionally treated with neutralizing antibodies to IFNγ or TNF. (**A**) Splenocytes were gated for F4/80$^+$CD11b$^+$ macrophages. The bar graph indicates the frequency of macrophages expressing IFNγR1 (CD119) for each treatment group. Accompanying flow plots indicate representative gating for CD119$^+$ macrophages, as compared to isotype control. Frequencies were obtained by subtracting the percentage that were stained by the isotype control; (**B**) The bar graph indicates the change in MFI of CD119 staining on F4/80$^+$CD11b$^+$ macrophages for each treatment group. The change in MFI was obtained by subtracting the MFI following isotype control staining from the MFI following CD119 staining. The accompanying flow plot indicates representative staining for CD119 on macrophages for each treatment group; (**C**) RNA from FACS-sorted F4/80$^+$CD11b$^+$ macrophages was analyzed by semi-quantitative RT-PCR for changes in cytokine transcript levels. Bar plots indicate the relative fold change in expression between treatment groups for the indicated transcripts. $n = 3$–5 mice per group.

To examine functional changes in macrophages, we sorted F4/80$^+$CD11b$^+$ cells from the spleens of LCMV-immune mice 3 days after heterologous challenge with Lm-gp61 and isolated RNA for semi-quantitative RT-PCR analysis. While we did not observe significant differences in expression of IFNγ, TNF or IL-6, as compared to macrophages isolated after primary Lm-gp61 infection, heterologous challenge resulted in a significant induction in IL-12p35 expression by splenic macrophages. This induction was completely abrogated following TNF neutralization but

unaffected by IFNγ neutralization (Figure 4C). These findings further support our conclusion that TNF neutralization results in impaired accumulation and activation of macrophages following heterologous Lm-gp61 challenge.

2.5. *IFNγ-Dependent Regulation of IL-6 Does Not Impact Protection from Heterologous Lm-gp61 Challenge*

In order to better understand the impact of TNF and IFNγ on systemic inflammatory responses, we measured the concentrations of serum cytokines on days 1 and 3 following heterologous rechallenge in the presence of TNF or IFNγ neutralizing antibodies (Figure 3). Neutralization of TNF resulted in no change to systemic IFNγ levels (Figure 5A) at day 1 post-challenge. Conversely, IFNγ neutralization resulted in a significant decrease in circulating TNF levels (Figure 5B). This again reflects differences in the systemic and local inflammatory environments, as the serum concentration of TNF does not reflect the increase in TNF production by splenic macrophages (Figure 4C). TNF and IFNγ had opposite effects on IL-6 production, with IFNγ neutralization resulting in a significant increase in IL-6, with concentrations similar to that of a primary Lm-gp61 infection. We additionally neutralized IL-6 during heterologous rechallenge, with no effect on bacterial clearance (data not shown). Previous work had indicated an important protective role for IL-6 during in primary infection [42,43] and these results suggest that whereas IFNγ may be an important regulator of IL-6 during primary infection, its role is diminished due to the effector function of secondary $CD4^+$ effector T cells induced by heterologous rechallenge.

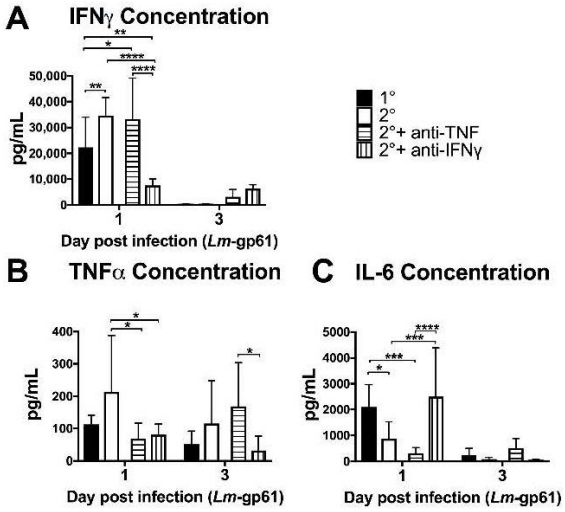

Figure 5. Neutralization of IFNγ and TNF alter the inflammatory environment induced by secondary challenge. Naïve and LCMV-immune mice were challenged with Lm-gp61 and some groups of mice were additionally treated with neutralizing antibodies to IFNγ or TNF. Bar plots show the concentration of (**A**) IFNγ; (**B**) TNF and (**C**) IL-6 in the serum of the indicated treatment groups at day 1 or 3 after Lm-gp61 challenge. $n = 7$–8 mice per group, data are representative of two separate experiments.

3. Discussion

We demonstrate that $CD4^+$ memory T cells alone are sufficient to protect from a Lm challenge in a manner independent of both CTLs and antibodies. Additionally, we have provided evidence that both TNF and IFNγ contribute to this protective effect, with TNF playing a dominant role and IFNγ playing a minor role. While much of the IFNγ is contributed by the innate immune response, IFNγ-dependent mechanisms are a key component of the contribution of Th1 cells to control of primary Lm infection [31]. We observe a quite different role for IFNγ in protection mediated by $CD4^+$ memory T cells following heterologous rechallenge. $CD4^+$ memory T cells mediate protection

in a largely IFNγ-independent fashion and the presence of $CD4^+$ memory T cells is sufficient to render IFNγ dispensable for Lm clearance. Our data suggest that the contribution of $CD4^+$ memory T cells to protection from secondary challenge may be more similar to that of $CD8^+$ T cells, with TNF playing a dominant role. These findings particularly highlight that the mechanisms of protection mediated by T cells during primary and secondary responses can differ, illustrating the need to better understand the functional mechanisms underlying memory T cell-mediated bacterial clearance in the development of effective vaccination strategies. Based on our results, we propose a model in which protection mediated by $CD4^+$ T cells during primary Lm infection is primarily IFNγ-dependent, whereas protection mediated by $CD4^+$ memory T cells following heterologous rechallenge is primarily TNF-dependent. It will be critical in future studies to determine whether similar mechanisms of protection are employed by $CD4^+$ memory T cells during the antiviral response.

As evidenced here, TNF is of particular importance in $CD4^+$ memory T cell-mediated protection, as the absence of TNF results in a loss of protection during heterologous secondary challenge. Studies involving primary Lm responses describe a role for TNF in primary protection, as TNF neutralization or infection of gene knock-out mice result in increased bacterial burden and increased mortality [24,25]. Our data agree with this, as neutralization of TNF during primary Lm-gp61 infection resulted in increased bacterial burden 3 days post-challenge. While we observe a loss of protection at day 3 post-infection during secondary challenge in the absence of TNF, the levels of bacteria are similar to those of a normal primary infection, indicating an altered mechanism of action during a secondary infection compared to a primary infection. Additionally, TNF-dependent mechanisms of protection appear localized to the site of infection, as we observe no significant systemic changes in TNF during heterologous rechallenge but do observe induction in splenic macrophages and memory T cells. TNF neutralization results in impairment of macrophage activation, as evidenced by decreased expression of IL-12 and IFNγR1, a marker for classically activated pro-inflammatory macrophages. Like IFNγ, TNF plays key roles in both the adaptive and innate facets of the anti-Lm immune response. While TNF may play a role in regulating the IFNγ-dependent response, the relative role of IFNγ in mediating protection is minor. Future studies are required to determine whether it is the contribution of $CD4^+$ memory T cells to the TNF response, the action of TNF on $CD4^+$ memory T cells, or some other mechanism that promotes faster bacterial clearance. Our findings suggest the likelihood of additional TNF-independent, $CD4^+$ memory T cell-dependent mechanisms of protection from heterologous Lm challenge.

While TNF, IL-12 and IFNγ are classically associated with protective immunity to *Listeria*, other cytokines have also been shown to play a role. Of note, IL-6 has been demonstrated to promote protection from primary Lm infection, with IL-6 neutralization resulting in increased bacterial burden [42,43]. In contrast, it has also been shown that acquired immunity does not require IL-6 [47], a finding that we confirm in the heterologous rechallenge model. Other cytokines may also play an important role in $CD4^+$ T cell-mediated protection, possibly independently of TNF. While IL-12, IL-6 and IFNγ are all inducible by TNF, IL-18 is a cytokine upstream of TNF that has been described as playing a role in protection from *Listeria* infection [48]. Additional investigation of these cytokine pathways will further elucidate the role of TNF-dependent and independent cytokine signaling in $CD4^+$ T cell-mediated protection. Overall, our observations support the idea that secondary $CD4^+$ T cell responses are sufficient to alter the anti-Lm immune response in key ways. First, they induce altered cytokine production systemically in the very earliest stages of the anti-Lm response. Second, they lead to a more rapid accumulation of activated macrophages in the spleen. Third, they are positioned to contribute to the inflammatory response at a time point that is normally dominated by innate immune cells. Understanding these and other differences between secondary and primary $CD4^+$ T cell responses and the unique mechanisms by which $CD4^+$ T cells mediate protection, will allow for fine-tuning of vaccines and immunotherapeutics in order to better manipulate $CD4^+$ T cell activity in vivo.

4. Materials and Methods

Mice and Infections. 6–8-week-old C57BL/6J (B6) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). C57BL/6N-IFNγr1$^{tm1.1Rds}$/J (IFNγR^{fl}; stock number 025394) mice were purchased from Jackson Laboratories and bred to mice expressing Cre under the control of the LysM promoter [49]. LCMV-Armstrong and Lm-gp61 were stored and propagated as previously described [2]. For primary infections with LCMV-Arm, mice were injected intraperitoneally (i.p.) with 2×10^5 plaque forming units (PFU). For infections with Lm-gp61, bacteria were first grown to log phase in BHI media as determined by the O.D. at 600 nm (0.3–0.7). Mice were then injected intravenously (i.v.) with 2×10^5 colony forming units (CFU). Primary infections with LCMV were done between 6–9 weeks of age, while primary and secondary infections with Lm-gp61 were done 6 weeks later, when mice were 10–15 weeks old. All experiments using animals were performed under a protocol approved by the University of Utah IACUC (Protocol #15-09004, approved 23 September 2015).

Neutralizing antibody treatments. 200 µg anti-CD4 depleting antibodies (BioXCell, Clone GK1.5) were given i.p. on day-2 and -1 prior to infection with Lm-gp61. 0.5 mg anti-TNF neutralizing antibodies (BioXCell, XT3.11) or anti-IL-6 (BioXCell, MP5-20F3) were given i.p. 1 day prior to infection and then every other day after that until sacrifice. 1 mg neutralizing antibodies to IFNγ (BioXCell, XMG1.2) were given i.p. 1 day prior to infection and then every 4 days after that until sacrifice. Treatment efficacy was confirmed by flow cytometry or by the ability of neutralizing cytokines to impair clearance of primary Lm infection. PBS control was not different from IgG1 or IgG2b isotype controls given at equivalent amounts as neutralizing or depleting antibodies and we have utilized this method of control previously [7], so PBS injection served as a control for all antibody treatments.

Serum Cytokine Analysis. Mice were bled on days 1 and 3 post-infection. Blood was allowed to clot at room temperature then spun at max speed in a microcentrifuge for 20 min. Serum was collected and stored at −20 °C. Serum cytokine concentrations were measured using a custom 6-plex LEGENDplex bead-based cytokine assay (Biolegend, San Diego, CA, USA; IFNγ 740153, IL-1B 740157, IL-6 740159, IL-10 740158, IL-12 (p70) 740156, TNF-a 740154, Standard 740371, Detection antibodies 740165, buffer set B 740373).

Tissue and cell preparations. Whole spleens and liver portions were collected in the tissue culture hood in 2 mL sterile PBS. Livers were weighed and all organs were dissociated using frosted microscope slides. For assessment of bacterial load, serial 1:10 dilutions were performed in sterile PBS and aliquots were plated on brain heart infusion agar (BHI) agar plates. Plates were incubated at 37 °C overnight. Colony counts were reported as CFU/spleen or CFU/g of liver. For all other cell preparations, dissociated tissues were places in single cell suspension in DMEM containing 10% fetal bovine serum (FBS), L-glutamine and Pen/Strep. For detection of intracellular cytokines, splenocytes were incubated with GP_{61-80} peptide (GLKGPDIYKGVYQFKSVEFD) for 4 h in the presence of Brefeldin A (Golgi Plug), followed by fixation and permeabilization, according to the manufacturer's instructions (BD Biosciences).

Flow Cytometry and Cell Sorting. Cells were suspended in PBS + 1% FBS, then stained with fluorescent dye-conjugated antibodies (anti-CD4, anti-CD8, anti-IFNγ, anti-TNFα, anti-IL-2, anti-CD11b, anti-F4/80, anti-IFNγR) (Biolegend, San Diego, CA, USA) for 20–40 min. Samples were collected on a LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo (TreeStar, Ashland, OR, USA). For cell sorting, splenocytes were stained with antibodies specific to CD4, CD8, CD11b and F4/80 for sorting by a BD FACSAria II (BD Bioscience) at the University of Utah Flow Cytometry Core Facility. Macrophages were sorted by gating on $CD4^{neg}CD8^{neg}CD11b^{+}F4/80^{+}$ cells and sorting directly into Qiazol (Qiagen, Germantown, MD, USA).

RNA Isolation and RT-PCR. RNA macrophages were isolated using the miRNeasy kit (Qiagen, Germantown, MD, USA). cDNA was generated using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed with the SYBR Green Master Mix (Thermo Fisher Scientific) and primers specific for our genes of interest (DNA Synthesis Core, University of Utah) using an LC480 PCR LightCycler

(Roche Diagnostics, Indianapolis, IN, USA). The following primer sequences were used: 1L12p53: F- TGCCTTGGTAGCATCTATGAGG, R- CGCAGAGTCTCGCCATTATGAT; TNF: F-ATGAGCACAGAAAGCATGA, R- AGTAGACAGAAGAGCGTGGT; IFNγ: F- TTCTTCAGCAACAG CAAGGC, R- TCAGCAGCGACTCCTTTTCC; IL6: F-CCTCTGGTCTTCTGGAGTACC; R- ACTC CTTCTGTGACTCCAGC [50].

Statistical Analysis. Statistical significance was determined using the student's *t*-test for two groups and ANOVA for more than two groups using GraphPad Prism 7 software (Graphpad, La Jolla, CA, USA). Graphs depict mean ± SD, with a p value of less than 0.05 being considered significant. p values are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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CHAPTER 4

DISCUSSION

In this dissertation, we explore cytokine-mediated control of $CD4^+$ T cell responses. We observe a TCR-independent role for cytokine signaling in determining the strength of the recall response for $CD4^+$ T cells. Furthermore, the requirement for individual cytokines is determined by the invading pathogen, as evidenced by differential requirements for specific cytokines in the immune response to either viral or bacterial pathogens. Our work has defined the requirement for a variety of pro and antiinflammatory cytokines during secondary challenge with *Listeria monocytogenes*, a facultative intracellular bacterium, when protection is mediated by $CD4^+$ T helper cells. We illustrate the roles of multiple cytokines induce by infection with *Listeria* and how these molecules control $CD4^+$ T cell secondary effector responses (Fig. 4.1).

In Chapter 2, we explore the roles of the two most well-studied Signal 3 cytokines: IL-12 and type I interferons (IFN-I's). We show that, as with $CD8^+$ T cells, these cytokines can produce effects on the function of T helper cells. Loss of IL-12 signaling leads to reduced expansion and decreased functional avidity of secondary effector $CD4^+$ T cells, even in a monoclonal TCR population such as SMARTA $CD4^+$ T cells (1). Overall, we conclude that IL-12 induction of IFNγ correlates to $CD4^+$ T cell functional avidity and expansion during an acute bacterial infection. It is important to note that the differences in effector function caused by interruption of IL-12 signaling only occur when LCMV-immune memory cells are transferred to a naïve mouse. While there is a proportional decrease in circulating IFNγ when naïve or LCMV-immune mice are treated with IL-12 neutralizing antibodies, the level of IFNγ is still much higher in the immune mouse than the baseline in the naïve mouse.

Work previously published by our lab showed that normal IFN-I signaling induced

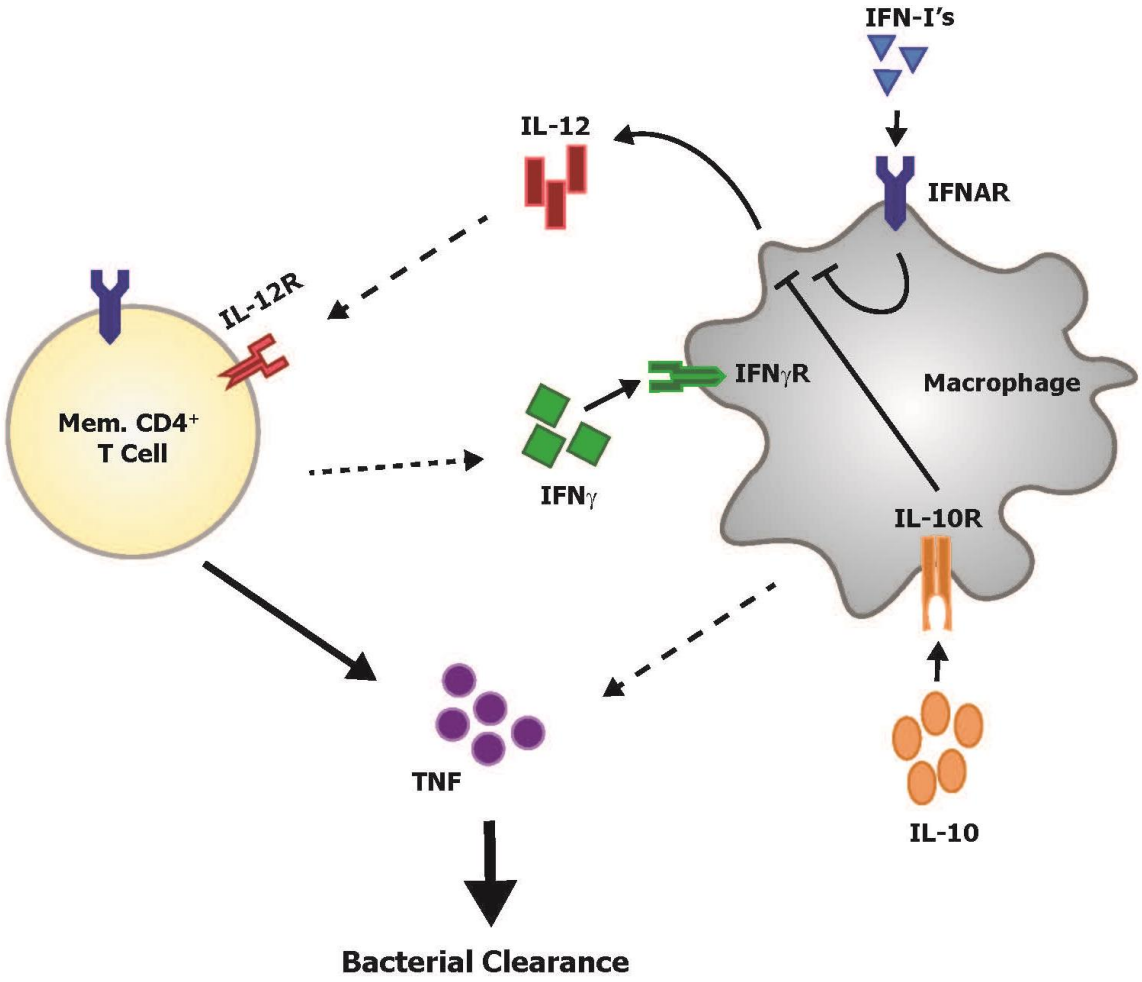

Figure 4.1. Visual summary of signal 3 cytokine signaling during secondary $CD4^+$ T cell responses described in this dissertation. IL-12 production can be inhibited by IFN-I and IL-10, which results in changes to T cells. Additionally, IFN-I can directly influence T cell responses specifically concerning expansion kinetics. Additionally, TNF produced by T cells and, possibly to a lesser extent, macrophages, is critical for bacterial clearance. IFNγ, while critical for protection in primary responses to *Listeria*, proves to be expendable for early protection during a $CD4^+$ T cell-mediated secondary response. Large dashed lines indicate mechanisms inferred based on the current literature.

by *Listeria* infection curtailed secondary $CD4^+$ T cell effector responses, as blockade of the IFN-I receptor (IFNAR) produced secondary effector $CD4^+$ T cells with significantly higher functional avidity. This corresponded to a large increase in circulating IL-12 and IFNγ in the absence of IFN-I signaling, supporting the role of both of these cytokines in productive effector responses. Use of knock-out animals allowed us to study the role of IFN-I on secondary T cell responses in a more targeted way. We determined that this effect was T cell-indirect, as when all cells except for the T cells lacked IFNAR we still observed increased functional avidity. This effect appears to be independent of antigen as well as TCR, as a similar increase in functional avidity is observed at normal and 10-fold higher than normal infectious doses of *Listeria*.

However, IFN-I may play a direct role in expansion kinetics of secondary effector $CD4^+$ T cells, as differences in expansion and contraction are observed only when IFNAR signaling on T cells is disrupted. While loss of IFNAR signaling on T cells leads to more robust expansion of secondary effector cells, a greater degree of contraction is observed during secondary memory formation. Blocking antibodies to IFNAR were only given once directly prior to infection, but the duration of antibody binding to receptor is not defined and was not investigated. Future work on this subject should verify the duration of the blockade to better describe the role of this pathway. If the blockade persists for the duration of the acute response, it would indicate a beneficial requirement for IFN-I to minimize the contraction phase. Others have shown that IFN-I can in fact extend the presentation of the IL-2Rα on the surface of $CD8^+$ T cells to prevent contraction, which may likely be important for $CD4^+$ T cells as well. As expansion is greater in the absence of early IFN-I signaling, there could alternatively be an increased differentiation to the short-lived

effector cell (SLEC) subset, leading to greater cell death after pathogen clearance (2). Analysis for SLEC versus memory-precursor effector cell (MPEC) markers such as KLRG1 and CD127, as well as IL-2Rα (CD25), will determine whether IFN-I is important for controlling the early differentiation of effector cells.

Work by others indicated a potential pathway of immune evasion induced by *Listeria*, wherein IFN-I's signal to macrophages to induce IL-10 production, which subsequently decreases macrophage-produced IL-12 (3). Use of *il10*$^{-/-}$ mice in place of *ifnar*$^{-/-}$ mice produces a significant but smaller increase in functional avidity. More importantly, there is no change in circulating IL-12 levels in the absence of IL-10 compared to WT hosts, a stark contrast to the significant increase observed in *ifnar*$^{-/-}$ mice. These data, as well as differences in the number of CD4^{+} T cells with multicytokinic potential, indicate that while both IL-10 and IFN-I control secondary CD4^{+} T cell effector responses, they do so to different degrees and through different mechanisms during *Listeria* infection.

In Chapter 3, we show that CD4^{+} memory T cells are able to confer protection from secondary heterologous challenge, but that this is entirely dependent upon TNF signaling. Importantly, this illustrates one of the many distinctions between primary and CD4^{+} T cell-mediated secondary protection to *Listeria*. While IFNγ is generally considered to be the most important cytokine for primary protection, we observe that it is TNF and not IFNγ that is of particular importance during the secondary response. Additionally, TNF is also important for a primary response, but TNF-dependent protection is not identical between primary and secondary infections. In a primary infection, there is a loss of protection leading to a significant increase in CFU compared to control treated animals. During a secondary infection with CD4^{+} memory T cell-mediated protection, the CFU are reduced

to around naïve levels, not higher. Additionally, previous work indicates that neutralization of TNF during a primary infection leads to death of all mice by day 4 postinfection (4). We observe very little mortality after neutralization of TNF in the presence of $CD4^+$ memory T cells during a secondary heterologous challenge (data not shown), indicating that though $CD4^+$ T cell-mediated protection requires TNF, overall survival may not be as dependent upon TNF in immune mice. Alternatively, incomplete neutralization may allow for survival of these animals, and thus repeating these experiments with increased application of TNF-neutralizing antibodies will determine if survival during heterologous secondary challenge is also dependent upon TNF.

Both Chapters 2 and 3 indicate that IFNγ is important during the immune response to *Listeria*, though there appears to be a complicated role for this cytokine in secondary effector $CD4^+$ T cell responses. In Chapter 2, we observe what appears to be a positive correlation between effector function and circulating IFNγ levels. This appears to be confirmed in Chapter 3, as increased protection also correlates to elevated levels of circulating IFNγ. However, neutralization of IFNγ does not result in a severe defect in protection as would be expected based on previous publications (5–7). Additionally, neutralization of TNF does not result in a change in circulating IFNγ. While this may indicate that IFNγ is effectively dispensable for $CD4^+$ T cell-mediated protection from secondary challenge, we do observe that neutralization of TNF reduces the expression of the IFNγ receptor on macrophages. This implies IFNγ has at least a minor role in protection from secondary challenge, at least in the macrophage compartment. The focus of this chapter was on the very early response to *Listeria*, ending with analysis at day 3. We have studied this neutralization over a longer time course just once and do not observe any

significant differences in clearance throughout the acute infection. Other work has indicated that perhaps loss of protection may not be observable by day 3 postinfection (8), so future exploration of this with a specific focus on the latter phase of the acute response will determine if our observation holds true for the duration of the immune response or if there is in fact a defect in later clearance in the absence of IFNγ.

Together, the data presented here show that Signal 3 cytokines control effector T cell responses in a TCR and possibly antigen-independent manner, and that it is possible to manipulate the cytokine milieu in such as a way as to produce more effective secondary effector T cell responses. Beyond simple effects on individual cells, we observe differences in protection when cytokine signaling is interrupted. Importantly, the roles of these cytokines are dependent on the pathogen, as many have reported differences between the roles of IL-12 and IFN-I's in viral and bacterial infections. This dissertation focuses exclusively on infections with *Listeria*, but our lab does possess a strain of vaccinia virus expressing the MHCII-restricted epitope of LCMV GP_{61-80} (VV-gp61) much like the strain of *Listeria* used in the work presented here (Lm-gp61). Future studies implementing a VV-gp61 secondary challenge could be used to determine what role the cytokines presented here play in $CD4^+$ T cell-mediated protection from secondary viral infection. We would expect an opposite role for IFN-I's during a viral infection as has been extensively published, but IL-12, TNF, and IFNγ in particular could have similar roles on $CD4^+$ T cell function in both viral and bacterial settings, though whether this translates to controlling the protective effect of these memory cells is another matter.

In addition to ascertaining the role of Signal 3 cytokines during $CD4^+$ T cell-mediated protection in a viral setting, many questions remain regarding the mechanisms

by which TNF and other cytokines control memory $CD4^+$ T cell-mediated protection. For example, we have published data describing a protective role for $CD4^+$ T cells but have yet to fully determine what factors mediate that protective effect. We have shown that protection is dependent upon TNF, rather than IFNγ, but observe no significant differences in TNF expression between naïve and LCMV-immune mice infected with Lm-gp61. Study of cytokine signaling at the site of infection, in this case the spleen, will likely provide more insightful data to illustrate a mechanism for $CD4^+$ T cell-mediated protection. Techniques such as RNA-sequencing or more targeted quantitative PCR will allow us to analyze gene expression patterns with and without the presence of LCMV-immune $CD4^+$ memory T cells specifically at the site of infection. This will allow us to observe whether there is in fact an increase in TNF in the spleen that is not reflected by systemic cytokine concentrations. Additionally, it will allow us to better determine what other cytokines are important in regards to memory $CD4^+$ T cell-mediated protection, such as IL-18 (9). Importantly, a 7-day time course should be examined as cytokine signaling is not static throughout infection. Analysis of individual cell populations such as T cells and macrophages can then be performed using flow cytometry and qPCR to examine phenotypic differences in these cells that are affected by the different inflammatory environments. Markers could include the IL-2Rα, which has been correlated to decreased contraction, or markers for SLECs and MPECs to determine the fate of effector T cells.

Our attempts to determine whether IFN-I and IL-12 are required to signal directly to T cells during secondary effector responses have so far failed due to the tools available to us. Fortunately, there are tools available to study this, though they are not currently cost-effective. The Knockout Mouse Program has an IL12Rb2 floxed mouse that, in conjunction

with an inducible cre driver under the CD4 promoter, would result in T cells that can have the IL12 receptor specifically deleted after primary memory formation. However, the floxed mouse is only available via cryo recovery as of yet and therefore is a prohibitive expense for that experiment. While studying IL-12 signaling to T cells continues to be problematic based on the available tools, there is now an IFNAR floxed mouse available through Jackson Laboratories that could be of use to further test our hypothesis that IFN-I is required to minimize contraction during secondary memory formation. Additionally, this would allow for deletion of the receptor not just after primary memory formation, but at any time during the secondary response to determine at what stage IFN-I programming of the contraction phase occurs.

Subsequent work will be required to determine if IL-12 and IFN-I play a role in $CD4^+$ T cell-mediated protection. As mentioned, differences in effector function caused by disruption of IL-12 or IFN-I signaling are only observed in naïve hosts, which is likely due to the high concentrations of IFNγ in LCMV-immune hosts. Even though this is the case, it would be interesting to examine the effects of IL-12 neutralization or IFNAR blockade during heterologous challenge of LCMV-immune hosts without any adoptive cell transfer. This will also allow for direct comparison to the TNF and IFNγ neutralization experiments, and study without secondary transfer will more accurately determine if there is any biological significance to the potential for these cytokines to program secondary $CD4^+$ T cell responses. Importantly, early attempts at determining what role of IL-12 and IFN-I play in protection were inconclusive. It is therefore important to elucidate this fact through neutralization of IL-12 or IFNAR blockade in our heterologous rechallenge model. We have additionally hypothesized, based on previous work by our lab and others, that more

robust secondary responses programmed by optimal Signal 3 cytokine signaling lead to better memory formation. We have examined memory only after secondary adoptive transfer, which may not be completely biologically accurate. Determining the role that all of these cytokines play in secondary memory formation will indicate whether there are long-term effects on $CD4^+$ T cells that are programmed by early Signal 3 cytokine signaling.

As mentioned, our published work concerning TNF and IFNγ in $CD4^+$ T cell-mediated protection focus primarily on the early protection and signaling events. We have yet to explore the effects that depletion of these cytokines have on secondary memory formation, such as the contraction phase and memory maintenance. While we would expect that stronger effector responses would correlate with more rapid clearance, and subsequently more robust memory formation, this may not be the case. Specifically, while TNF depletion results in slower clearance and we would therefore expect greater contraction to memory or poor maintenance of secondary memory, IFNγ may not follow those same principles. As Th1 can be activated by IFNγ signaling as well as IL-12, there may be some effects on the secondary memory population that do not present as a defect in protection. Additionally, we have yet to determine the effect blocking these signaling pathways have on the functional avidity of the secondary effector population. Subsequent study in this area should start here, as it will help to complete the story we have started to tell concerning the role of TNF and IFNγ in controlling CD^+ T cell-mediated protection.

Finally, future studies should examine the potential of direct cell killing by secondary effector $CD4^+$ T cells. Since protection is mediated by these cells, it is possible that secondary effector Th1 cells also have cytolytic potential that leads to their directly

killing infected cells, resulting in the more rapid clearance of pathogen that we observe. Cytolytic $CD4^+$ T cells have been recently described, with similar granzyme B and perforin cell-killing mechanisms as $CD8^+$ CTLs (10–14). Additionally, CD4+ CTLs utilize the Fas-FasL pathway to mediate cell killing. Future use of intracellular and surface staining for granzyme B, perforin, and FasL will help to define the potential cytolytic phenotype of these memory $CD4^+$ T cells. Furthermore, *in vivo* cell killing assays can be employed to directly examine the cell-killing function of these cells. Though mechanisms regulating the programming of these cytolytic functions are not identical to those determining differentiation to the Th1 subset, it is generally held that $CD4^+$ CTLs are typically Th1 cells. Memory $CD4^+$ T cells with cytolytic capacity could be acting as both coordinators of the immune response as well as direct mediators of cell killing. Therefore, determining what impact Signal 3 cytokines have on $CD4^+$ T cell-mediated cytotoxicity via *in vivo* assay will indicate whether $CD4^+$ memory T cell-mediated protection is via immune coordination alone or whether they have a more direct protective role.

While the data presented here focuses on T cell function in a nontranslational manner, there are important implications for understanding the inflammatory cues that can influence T cell responses. Importantly, the development of vaccines and immunotherapies require an advanced understanding of mechanisms regulating productive immune responses and long-lived immune memory. We have previously touched on the broadly immunoregulatory function of $CD4^+$ T cells, and thus the memory population generated should include $CD4^+$ T cell memory. By understanding how the inflammatory milieu interacts with T cells during the response to acute infection or vaccination, it is subsequently possible to adjust said milieu via adjuvant therapies such as the addition of

recombinant cytokines or neutralization of other cytokines (15–17). In fact, mouse studies have been performed utilizing a mutant form of TNF as an adjuvant in both vaccinations for HIV and cancer therapies (18–20). It is this potential for use in cancer immunotherapies that is particularly interesting, as the importance of $CD4^+$ T cells in the antitumor response has become more appreciated as of late (21, 22). The work presented in this dissertation explores just a few of the multitude of cytokines with potential regulatory roles in $CD4^+$ T cell effector function, but this work provides an important initial understanding as to the ideal inflammatory environment for the generation of optimal effector $CD4^+$ T cells that may later be applied to the development of immunotherapeutics. Similarly, our lab has collaborated with a number of clinicians to examine how immune profiles are altered during the course of treatment for metastatic melanoma. We are particularly interested in determining if there are any specific markers, such as elevated levels of specific cytokines in the serum, which correlate either to response to therapy or continued progression of disease. While much of this work is ongoing and has yet to be published, the following Appendix gives one example of this correlative work. Ongoing and future work relating to these projects will further explore the potential for these potential diagnostics.

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APPENDIX

A PHASE I STUDY OF INTRATUMORAL IPILIMUMAB AND INTERLEUKIN-2 IN PATIENTS WITH ADVANCED MELANOMA

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A phase I study of intratumoral ipilimumab and interleukin-2 in patients with advanced melanoma

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ABSTRACT

Purpose: Intratumoral interleukin-2 (IL-2) is effective but does not generate systemic immunity. Intravenous ipilimumab produces durable clinical response in a minority of patients, with potentially severe toxicities. Circulating anti-tumor T cells activated by ipilimumab may differ greatly from tumor-infiltrating lymphocytes activated by intratumoral ipilimumab in phenotypes and functionality. The objective of this study was to primarily assess the safety of intratumoral ipilimumab/IL-2 combination and to obtain data on clinical efficacy.

Results: There was no dose limiting toxicity. While local response of injected lesions was observed in 67% patients (95% CI, 40%-93%), an abscopal response was seen in 89% (95% CI, 68%-100%). The overall response rate and clinical benefit rate by immune-related response criteria (irRC) was 40% (95% CI, 10%-70%) and 50% (95% CI, 19%-81%), respectively. Enhanced systemic immune response was observed in most patients and correlated with clinical responses.

Experimental Design: Twelve patients with unresectable stages III/IV melanoma were enrolled. A standard 3+3 design was employed to assess highest tolerable intratumoral dose of ipilimumab and IL-2 based on toxicity during the first three weeks. Escalated doses of ipilimumab was injected into only one lesion weekly for eight weeks in cohorts of three patients. A fixed dose of IL-2 was injected three times a week into the same lesion for two weeks, followed by two times a week for six weeks.

Conclusions: Intratumoral injection with the combination of ipilimumab/IL-2 is well tolerated and generates responses in both injected and non-injected lesions in the majority of patients.

INTRODUCTION

With an estimated 73,870 new cases and 9,940 deaths, melanoma was the leading cause of skin cancer death in the US in 2015 [1]. For patients with localized melanoma the five year survival was 91% and for patients with regional and metastatic melanoma it declines to 63% and 16%, respectively [1].

Activation of transmembrane inhibitory receptor cytotoxic T lymphocyte associated antigen-4 (CTLA-4) *via* binding to B7.1 or B7.2 downregulates T-cell activation [2-6]. This results in inhibition of interleukin-2 (IL-2) secretion and T-cell proliferation. Additionally, CTLA-4 enhances the function of regulatory T cells (Treg). Blockade of CTLA-4 *via* anti- CTLA-4 antibody allows unopposed T-cell activation thereby breaking tolerance to tumor antigens [7, 8]. Ipilimumab (Ipi), a fully human IgG1 anti-CTLA-4 monoclonal antibody, was approved by the Food and Drug Administration (FDA) for metastatic melanoma in 2011 [9, 10]. Ipi lowers the threshold for T cell activation by blocking CTLA-4 expressed on activated T cells. Ipi has a response rate of approximately 11 % and is the first drug shown to significantly improve overall survival for metastatic melanoma [9, 11, 12]. However, since Ipi has limited tissue distribution and remains in the vasculature [13], circulating anti-tumor T cells activated by this drug may differ greatly from tumor-infiltrating lymphocytes (TIL) activated by intratumoral (IT) Ipi in terms of quantity and quality. With systemic Ipi associated with a low response rate and life threatening toxicities, [14] alternative combination and routes of administration of this drug are warranted.

Interleukin-2 (IL-2) is a glycoprotein discovered initially as a T cell growth factor [15, 16]. Activated CD4+ T cells, CD8+ T cells and dendritic cells (DC) are the main source of IL-2. IL-2 has been found to stimulate and enhance the function of cytotoxic T lymphocytes (CTL), natural killer cells and B cells [17-21]. While its in-vivo role is more complex, IL-2 plays key roles in driving T cell expansion, Treg function, and enhancing the differentiation, survival and effector function of long-lived memory CTL [22-25]. Administration of high dose systemic IL-2 was FDA approved for treatment of metastatic melanoma in 1998 [26]. The response rate of high dose IL-2 has been approximately 16%, with half achieving long term durable responses but it can cause severe toxicities [2]. To avoid toxicities, several studies have assessed the efficacy of IT IL-2 in melanoma patients [27-29]. While treatments were well tolerated, with only grades 1 and 2 toxicities and complete response of treated lesions in 62.5%- 69% of patients [27, 28] , there were no systemic responses observed in uninjected lesions. The absence of abscopal effect, defined as a response in at least 1 non-injected lesion, may reflect a likely failure to boost systemic immunity by IT IL-2 despite an impressive local effect.

Intratumoral (IT) administration of Ipi has the potential to enhance local activation of tumor infiltrating lymphocyte (TIL) and effectively induce systemic activation of tumor-specific T cells. We hypothesized that the combination of IL-2 and Ipi administered IT would effectively hyper-activate TIL to induce a systemic immunity with minimal toxicities.

RESULTS

Patients

Twelve patients were treated at the Huntsman Cancer Institute, Salt Lake City, Utah between November 2012 and July 2014 in this phase I study. Nine of 12 patients had received prior treatment. The patient characteristics are listed in Table 1. The duration of exposure of the IT drug combination was 53 days. All patients receiving treatment were evaluated for dose limiting toxicities (DLT) during the first three weeks of treatment. The first three patients received IL-2 (3 mIU) and 0.5 mg Ipi over 8 weeks (dose level +1) as per protocol design. With 0 of 3 patients reaching DLT, three more patients were evaluated for dose level +2 of IL-2 (3 mIU) and 1 mg of Ipi over 8 weeks. With no DLT reported in any of the three patients in dose level +2, three more patients were evaluated at dose level +3 of IL-2 (3 mIU) and 2 mg of Ipi over 8 weeks. With no DLT reported and level +3 being the highest planned dose level, three additional patients were enrolled at the same dose level without evidence of DLT.

Toxicities

Of the 12 patients who received 216 individual treatments, none experienced DLT and all completed the treatment phase of the study. The patients were followed up on months 1, 4 and 7 after the last treatment. One patient in dose level +1 died during follow up, almost seven months since the end of his treatment, due to progression of disease and was not attributed to study medication. A total of 57 individual treatment-related AEs were reported. No patient in the study or the follow up phase developed a grade 4-5 AE. Treatments were well tolerated and toxicities are summarized in Table 2. The only related grade 3 toxicity observed was injection/tumor site ulceration/necrosis, which was not a DLT per protocol. One patient had grade 3 hyponatremia, which was present prior to treatment and thus unrelated. Other toxicities were grade 1 or grade 2 in nature. None of the toxicities could be clearly attributed to an autoimmune phenomenon.

Table 1: Patient Characteristics (n = 12)

Characteristics	
Sex	
Male	6
Female	6
Age (in years)	
Median	63.5
Range	43-88
Disease Stage	
IIIb	1
IIIc	5
IV M1a	3
IV M1b	1
IV M1c	2
Performance Status	
ECOG 0	10
ECOG1	2
Previous treatment	
High dose interleukin-2	3
Systemic ipilimumab	4
Other therapy*	5

*Carboplatin, IT BCG, TIL therapy with fludarabine/ cyclophosphamide pre-conditioning, GM-CSF, melphalan limb perfusion

Tumor response

The secondary objective of this trial was to determine the clinical efficacy of the IT combination of IL-2 and Ipi. Ten of 12 patients were evaluable for objective response by immune-related response criteria (irRC). There were three partial response (irPR, 30%), one stable disease (irSD 10%), and six progression of disease (irPD, 60%). Hence, the overall objective response rate was 40% (95% CI, 10%-70%). This is summarized in Table 3. Two patients, who were unevaluable per irRC due to size criteria (lesions <10 mm), had regression of multiple skin lesions.

Local response of the injected lesion, by measurement and/or pathology (resection or biopsy), was seen in eight patients (seven local complete response (CR) and one local PR, 67% (8/12) (95% CI, 40%-94%)). Four local PD was observed; however, these four lesions were not biopsied for confirmation.

Interestingly, an abscopal effect, was seen in eight of nine patients (88.9%) (95% CI, 68%-100%) at locoregional and distant sites. Three patients were not evaluable for abscopal effect since they had only one lesion at baseline.

Four of ten evaluable patients, patients 5, 8, 9 and 11, (40%) (95% CI, 10%-70%) achieved objective responses based on imaging and/or pathology. Patient 5, with progression on prior intratumoral BCG injections, was enrolled at dose level+2. She had a partial response at one month follow up, but chose to enroll in another trial even without disease progression. Therefore, duration of response was undeterminable in her case. She died from disease progression more than 19 months from her enrollment in our study.

Patients 8, 9 and 11 were enrolled in dose level +3. Patient 8 was found to have disease progression of the right supraclavicular mass and a new lesion based on physical examination and imaging at one month following the last injection. However, pathology of the resected lesions showed mixed inflammation and necrosis but no residual viable melanoma. Therefore, patient 8 had a pathologic CR despite being classified as irPD and continues to have no evidence of disease (NED). Patient 9, who had progressed on HD IL-2 prior to enrollment,

Figure 1: CT scan of abdomen and pelvis of non-injected lesion for patient 11. (shown by the arrow) at **A.** screening **B.** 1 months post last injection and **C.** 4 months post last injection. Initial progression followed by regression was observed in (B) and (C), respectively. This figure reflects both pseudo-progression and abscopal response.

Table 2: Treatment-related Toxicity (total events = 57)

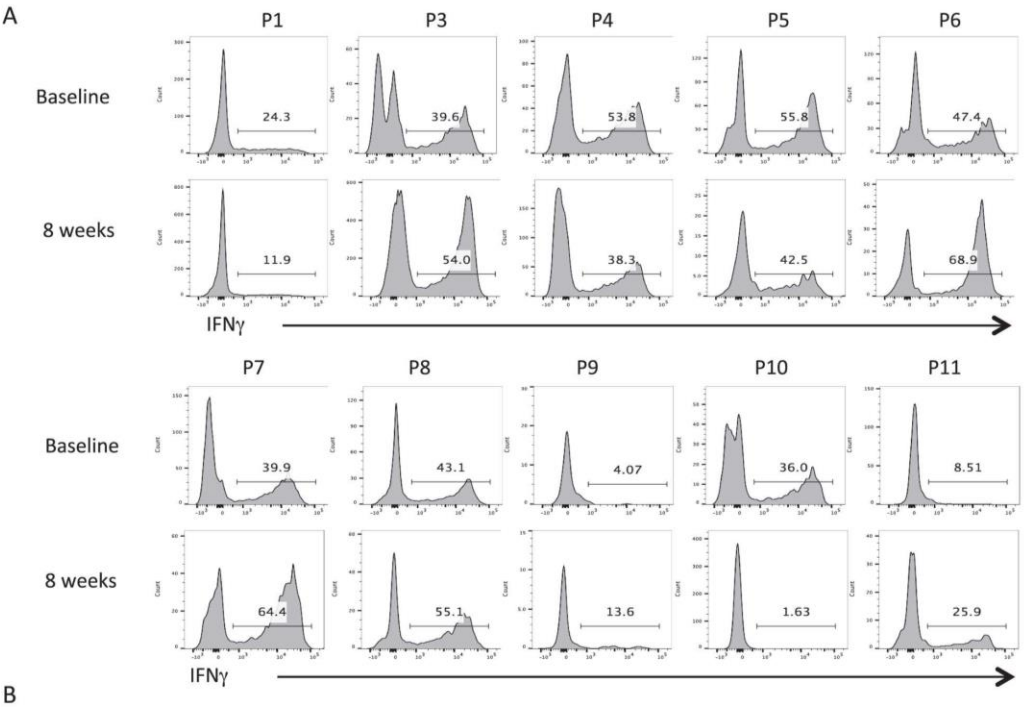
Treatment related Toxicity	No. of instances of adverse event by grade (CTCAE V 4.0)			
Toxic effect	1	2	3	4
Anemia	1	0	0	0
Arthralgias	1	0	0	0
Cellulitis right leg	1	0	0	0
Chills	4	0	0	0
Creatinine increased	1	0	0	0
Crusting lesion (an injected lesion)	1	0	0	0
Dehydration	1	0	0	0
Dry mouth	1	0	0	0
Dry skin	1	0	0	0
Erythemia around scab	1	0	0	0
Eyelid Edema	1	0	0	0
Facial Swelling	1	0	0	0
Fatigue	6	0	0	0
Flu like symptoms	5	0	0	0
Hyponatremia	0	0	1	0
Injection site reaction	7	0	0	0
Itchy lesion	1	0	0	0
Nausea	1	0	0	0
Pain at injection site	5	1	0	0
Pruritis	1	0	0	0
Rash	2	0	0	0
Right eye edema- eyelid	1	0	0	0
Right femoral leg pain	1	0	0	0
Soft tissue infection	0	2	0	0
Swelling right ear canal	0	1	0	0
Tingling in right leg	1	0	0	0
Ulceration at injection site	0	0	5	0
White blood cell count decreased	1	0	0	0

Table 3: Tumor Response [*n* = 12, 10 subjects evaluable, 2 subjects non-evaluable per immune related response criteria (irRC)]

Best response	Injected lesion	irRC	irRC with pathology correlation
CR	7/12	0/10	1/10
PR	1/12	3/10	3/10
SD	-	1/10	1/10
PD	4/12	6/10	5/10

achieved irPR. She had a residual melanoma lesion resected and has been NED since. Patient 11 had irPR as best response. The non-injected right femoral lymph node had increased by 147% of baseline at month 1 follow-up, without further treatment decreased by 75% of baseline at month 4 follow-up (Figure 1). This lymph node

A



B

Patients	Fold Increase				Abscopal Response	Any Response
	IFNg	Granzyme B	Perforin	Tbet		
1	-	-	-	-	+	+
2	ND	ND	ND	ND	NA	-
3	1.4	1.9	1.6	1.3	+	+
4	-	-	-	-	+	+
5	-	-	-	1.1	+	+
6	1.5	-	-	1.1	NA	-
7	1.6	1.9	1.8	1.7	+	+
8	1.3	-	-	-	NA	+
9	3.0	2.0	1.5	3.0	+	+
10	-	1.5	1.3	-	-	-
11	3.3	-	1.3	2.3	+	+
12	ND	ND	ND	ND	+	+

Figure 2: Circulating IFNg-producing CD8 T cells (P/I stimulation 4 h). PBMCs at baseline and end of treatment were analyzed by flow cytometry as described. Intracellular cytokine staining for factors relating to T lymphocyte activity were compared between the start and cessation of treatment for CD8+ CTLs **A.** Fold increase in the percentage of CD8+ cells that also stain positive for these markers over time are summarized in **B.** Fold increase in total circulating CD8+ T cells expressing IFNg: 2.02 (95% CI, 1.31-2.73); Granzyme B: 1.83 (95% CI, 1.61-2.05); Perforin: 1.50 (95% CI, 1.32-1.68); Tbet: 1.75 (95% CI, 1.14-2.36). ND: not done; NA: not applicable as the patient had 1 lesion at baseline. Any response includes objective, abscopal and/or pathologic response.

completely regressed with further follow up. We used a PR designation for her response since the CR occurred after the study follow up period. She continues to be free of disease. Thus 3/4 patients (75%) who had objective response by imaging and/or pathology are still alive and currently free of disease, more than 31+ months from their enrollment in our study. Of interest, these three patients were in the last cohort of six patients (50%) who received drug at the highest planned dose level.

Immunologic response

In order to assess the systemic immune response, we examined the intracellular expression of IFNγ, granzyme B, perforin, Tbet and FoxP3 in circulating T cells in ten patients with adequate blood samples. An increase in frequency of CD8+ T cells expressing IFNγ and Tbet was observed in 6 patients (60%), with fold-increase of 2.02 (95% CI, 1.31-2.73) and 1.75 (95% CI, 1.14-2.36). An increase in frequency of CD8+ T cells expressing granzyme-B and/or perforin was observed in four (40%) and five (50%) patients, respectively, with fold-increase of 1.83 (95% CI, 1.61-2.05) and 1.50 (95% CI, 1.32-1.68), respectively.

Seven patients (70%) had an increase in CD8+ T cells expressing at least one of these effector molecules. A similar pattern was also observed when correlated with patients who experienced any response. That is, six of nine (67%) patients with any response demonstrated increased levels of peripheral CD8+ T cells expressing at least one of these activation markers (Figure 2). There were no significant changes in CD4+ T cell parameters Foxp3 or IFNγ.

DISCUSSION

This study was to determine the toxicities and clinical response associated with the IT injection of IL-2 and Ipi. Ipi while enhances the immune response against tumors [30] frequently unleashes immune related toxicities. Two of four patients in our study who had prior-systemic Ipi treatment had discontinued because of immune related toxicities. IT injection with the combination of Ipi/IL-2 was well tolerated, including those who already had prior exposure to systemic Ipi and high dose IL-2. Most toxicities were grade 1 or 2 in nature.

While clinical cases of abscopal effects in response to radiotherapy have been reported since 1973, more recently radiotherapy has been reported to be synergistic with immune stimulatory drugs like anti-CTLA-4 and anti-PD1 [31]. The inflammatory microenvironment and the cytotoxic immune system have been suggested as the main drivers of spontaneous regression [32-34]. In the present study an abscopal effect was seen in 8 of 9 patients (89%). Abscopal effect impacts distant tumor growth and originates, at least in part, from enhanced immune functions such as DC and CTL activation. DCs present tumor peptides *via* major histocompatibility complex (MHC) and deliver co-stimulatory signals to naïve CD4 and CD8 T cells that mediate tumor-specific cell killing [35].

We hypothesize that with IT Ipi/IL-2, T cells are activated at two sites. The first site of activation is within the tumor itself. After adoptive cell transfer, tumor-specific naive CD8+ T cells have been shown to migrate to the tumor masses, become activated and proliferate there independently of the draining lymph nodes [36]. Mutual activation of DCs and T cells has been shown to occur within tertiary lymphoid structures in tumors, resulting in tumor-specific immunity, which was inhibited by regulatory T cells (Treg) present in the tumor-associated tertiary lymphoid structures. Depleting Treg enhanced anti-tumor response [37]. We believe the second site of activation is in the draining lymph nodes. DCs pick up and process tumor antigens within the tumors and travel to the draining lymph nodes for presentation to naïve and resting T cells. Further, it is possible that some of the Ipi/IL-2 injected IT drains to the locoregional lymph nodes as similarly seen in sentinel lymph node mapping procedure [38], resulting in further activation of T cells in the lymph nodes. An interesting observation was the lack of immune related toxicity, which is probably due to the miniscule amount of Ipi used in our study and possibly due to the inability of Ipi to penetrate the vasculature from the injection site. This suggests that the systemic effect of the treatment was due to trafficking of the activated lymphocytes rather than a leak of some of the Ipi into the systemic circulation.

Another phenomenon observed with cancer immunotherapy is pseudoprogression due to inflammatory changes in the tumor, which is reported in 7.2% of melanoma patients treated with pembrolizumab [39]. In our study, this was observed in two patients (8 and 11). Therefore, physicians and investigators should be aware of the difficulty in accurate assignment of response status during treatment with immunotherapy, whether it is systemically or locally administered.

The relative contributions of IL-2 and Ipi in our treatment model are unclear. A previous preclinical study in mice showed that IT anti-CTLA-4 antibody depleted regulatory T cells and enhanced antitumor immunity and improved survival only when combined with anti-CD25 antibody [40]. Therefore, it is possible that IT injection of Ipi as a single agent may be insufficient to generate systemic immune responses and may require the addition of other agents such as IL-2, anti-PD-1, anti-PD-L1, or anti-OX40.

In our trial in which only one lesion was injected with Ipi/IL-2, we observed complete or partial regression of the injected lesion in 66.7% of our patients. This is comparable to the results seen with IT IL-2 alone [27,

28]. However unlike those studies our observation of strong abscopal effect is quite promising. These responses were correlated with an increase in the frequency of total peripheral IFN-γ or Tbet producing CD+8 T cells in 7 of 10 patients. This result suggests that local intratumoral therapy with IL-2 and Ipi engendered a systemic immune response in these patients.

MATERIALS AND METHODS

Patients were eligible if they had unresectable stage III, resectable but declined resection or stage IV melanoma with accessible cutaneous, subcutaneous, and/or nodal lesions, according to the AJCC Staging Manual, 7th Edition, 2011. Other inclusion criteria were age of at least 18 years; Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2; adequate bone marrow, kidney and liver function. Patients agreed to use an appropriate method of birth control while on study. Exclusion criteria were concurrent therapy with any other non-protocol anti-cancer therapy; prior local therapy within 2 weeks or prior systemic therapy within 4 weeks of starting protocol treatment, history of any other malignancy requiring active treatment, pre-existing autoimmunity, clinically significant cardiovascular disease, active systemic infection or known history of HIV infection or chronic hepatitis B or C.

The protocol was approved by the institutional review board at the University of Utah and was conducted in accordance with the ethical principles originating from the Declaration of Helsinki and with Good Clinical Practice as defined by the International Conference on Harmonization. All patients gave written informed consent. The trial was registered with *http://www.ClinicalTrials.gov* identifier NCT01672450.

Study design

This was a single center, open label phase I dose escalation study. This study assessed the highest tolerable intratumoral dose of Ipi with IL-2. The objective was to primarily assess the safety of the drug combination and to obtain preliminary data on the clinical efficacy of the combination.

The standard 3+3 design was used for the dose escalation phase. Patients were accrued to each dose level in cohorts of up to 3-6 patients. Escalation continued until a dose limiting toxicity (DLT) was observed or the highest dose-level was reached. The goal was to ensure the safety and tolerability and not to determine the maximum tolerated dose (MTD). The study was to test three dose levels of 0.5 mg/0.1 mL (dose level +1), 1 mg/0.2 mL (dose level +2), 2 mg/0.4 mL (dose level +3) Ipi, with a fixed dose of IL-2 (3 mIU).

Patients were treated with Ipi on day 1 of every week for 8 weeks and IL-2 on days 1, 3, 5 on weeks 1-2 and days 1 and 4 of weeks 3-8. Drugs were administered IT, using separate 30-gauge needles for superficial injections. Intra-patient dose escalation was not permitted since toxicity was monitored throughout the duration of treatment and follow-up.

Immune characterization

Peripheral blood mononuclear cells (PBMCs) were isolated from patient whole blood samples using Ficoll-Paque (GE Healthcare) gradient centrifugation. Isolated PBMCs were resuspended in freezing media (45% DMEM 45% FBS 10% DMSO) and frozen. For cellular restimulation and intracellular cytokine stains, frozen PBMCs were rapidly thawed and washed with RP-10 (RPMI media + 10% FBS + pen/strep) then resuspended in RP-10 and transferred to a 96-well plate. Brefeldin A was added to a concentration of 10 uM (BD GolgiPlug™), as was PMA (20 ng/mL) and ionomycin (1ug/mL) to stimulate cytokine production [41-43]. The analysis was performed as described by manufacturer.

Assessments

Each adverse event (AE) was evaluated to determine the severity grade based on National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE version 4.0, grade 1-5). Relationship of AE was assessed for IL-2 and Ipi and whether it constituted an immune related AE or serious adverse event (SAE). Protocol guidelines for the management of immune-related AE included the administration of corticosteroids (orally or intravenously), a delay in a scheduled dose, or discontinuation of therapy.

Dose-limiting toxicity (DLT) was defined as any of the following AE/SAE as assessed in the reporting period by the investigator: Any grade > 3 (non-autoimmune) toxicity suspected to be related to the study drugs, any grade 3 autoimmune event that did not resolve with intervention (steroids), to a grade 1 or less within 21 days. A grade 3 ulcerated or necrotic lesion located at the injection site was not to be considered a DLT. Patients who were not assessable for toxicity in the first three weeks were to be replaced, however patients not assessable for efficacy were not substituted.

Statistical analysis

The primary endpoint was to assess the safety data of all patients receiving at least 1 dose of study treatment. The secondary end points were to assess the clinical response of the treated and untreated lesions. Secondary endpoints were summarized for all treated

patients together. There was no formal hypothesis tests for secondary endpoints. The disease control rate, overall response rate and response rate was summarized by the observed proportion and exact 95% binomial confidence interval. PMBC was analyzed for T cell subsets using flow cytometry. The change in the fraction of T cells was reported from baseline to the eighth week of treatment.

CONCLUSIONS

IT Ipi/IL-2 given to patients with non resectable stage III and IV melanoma is well tolerated. Most toxicities were Grade 1 or 2 in nature (fatigue, headache, pain, chills, rash, etc). The clinical benefit rate was 50%, with 10% CR, 30% PR and 10% SD. A PD turned out to be a pseudoprogression with a pathologic CR. An abscopal effect was seen in 8/9 patients (89%). Pseudoprogression was observed in two patients. We plan to conduct a phase II trial using IT Ipi/IL-2 in conjunction with systemic immunotherapy such as an anti-PD-1 or PD-L1 monoclonal antibody.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget.

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