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Investigating the Role of Lipopolysaccharide and Polyinosinic:Polycytidylic Acid in Boosting CD4 T cell Responses

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Investigating the Role of Lipopolysaccharide and Polyinosinic:Polycytidylic Acid in Boosting CD4 T cell Responses

Paurvi Ravindra Shinde, Ph.D

University of Connecticut, 2017

Most commonly used adjuvants in vaccines are effective at elevating serum antibody titers but do not elicit significant CD4 or CD8 T cell response. CD4 T cells are important in protection against challenging infectious diseases such as HIV, malaria and tuberculosis and also important for anti-tumor immunity. Therefore, our goal is to evaluate how adjuvants lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly I:C) induced mechanisms enhance the CD4 T cell immunity. LPS, a known Toll like receptor 4 (TLR4) ligand, when injected with or shortly after a T cell antigen, enhances T cell clonal expansion, long-term survival and Th1 differentiation. Importantly, LPS can synergize with a potent costimulatory agonist, OX40/CD134 (anti-CD134 mAb) to further enhance CD4 T cell expansion, survival, and memory. The mechanism behind this synergy is unknown. Our preliminary data suggests that Ag, LPS and anti-CD134 immunization results in enhanced production of type I IFN (IFN- β) which corresponds with the increased T cell expansion this vaccine induces. Therefore, we hypothesized that LPS and anti-CD134 mediated synergistic CD4 T cell response could depend on IFN α R signaling.

Depending on the model used, we show that absence of IFN α R signaling results in minor to major reduction in specific CD4 T cell expansion in peripheral lymph node (pLN) and liver tissue.

More importantly, LPS induced type I IFN, promotes synthesis of chemokines, CXCL9 and CXCL10, suggesting their role in the LPS and CD134 costimulation response. Indeed, blocking CXCL9 highlighted the importance of this chemokine in promoting specific CD4 T cell accumulation in pLN and liver. CXCL9 impacted the T cell accumulation in spite of no difference in serum levels of type I IFN, suggesting that type I IFN induced downstream chemokines could play a more dominant role in promoting T cell accumulation.

We next evaluated the role of synthetic dsRNA analog, poly I:C in enhancing CD4 T cell immunity. Recent studies, have shown that poly I:C synergizes with CD40 agonist Ab, to promote enhanced effector CD4 T cell response, where poly I:C induced IFN α R signaling was shown to enhance CD134L expression on dendritic cells (DC's).

Therefore, we hypothesized that poly I:C could be directly combined with CD134 costimulation to promote enhanced CD4 T cell immunity. Indeed our results show that, administration of peptide Ag with poly I:C and anti-CD134 enhanced the Ag-specific CD4 expansion and Th1 differentiation and it was dependent on poly I:C induced IFN α R signaling. Thus, the vaccine combination of poly I:C and anti-CD134 should be evaluated for its efficacy in therapeutic vaccines against infectious diseases or cancer where CD4 Th1-type immunity is crucial. Additionally, we also show that CD134 costimulation induced CD4 Th1 response was also dependent on IFN α R signaling. Understanding, the mechanism of how CD134 costimulation triggers IFN α R signaling to promote CD4 T cell response will be important for the efficient targeting of CD134 in preclinical cancer trials.

**Investigating the Role of Lipopolysaccharide and Polyinosinic:Polycytidylic Acid
in Boosting CD4 T cell Responses**

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Master of Science, Biotechnology
Texas Tech University, 2010

A Dissertation

Submitted in Partial Fulfillment of the

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Doctor of Philosophy

at the

University of Connecticut

2017

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Investigating the Role of Lipopolysaccharide and Polyinosinic:Polycytidylic Acid in Boosting CD4 T cell Responses

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CHAPTER I: INTRODUCTION

UNDERSTANDING HOW LPS AND POLY I:C INDUCED RESPONSES

IMPACT THE CD4 T CELL IMMUNITY

(I) Use of Adjuvants in Vaccines

Vaccines have been one of the most successful medical inventions that have led to eradication and protection against several infectious diseases in the last 100 years. Current vaccines make use of purified protein components of infectious organisms to produce more targeted immune response. However, the lack of the immunostimulatory components of the whole pathogen results in a weaker immune response. Hence, adjuvants are added to vaccines to intensify the host immune response against the infectious agent. Although adjuvants have been used in vaccines for the past century, few have been approved for use in humans.

Aluminum salt in the form of aluminum hydroxide, aluminum phosphate or alum has been the predominant components used in licensed human vaccines such as hepatitis A and B, human papillomavirus (HPV) and diphtheria-tetanus-pertussis (DTP) [1-4]. Alum based adjuvants, predominantly induce Th2 response. However, vaccines against some of the major causes of mortality worldwide including malaria, human immunodeficiency virus (HIV), tuberculosis, require a productive B-cell and T-cell response [5-7]. CD4 T cells are important in protection against these diseases as they can help B cells synthesize specific antibodies as well as stimulate cytotoxic T cells to kill the infected cells. Bacterial LPS, is one such adjuvant that enhances both humoral and T cell immunity [8, 9]. In terms of CD4 T cell response, LPS can enhance T cell clonal expansion, effector differentiation, and long-term survival [10, 11]. Such

an adjuvant could be beneficial in vaccines to induce long-term immunity to fight primary as well as secondary infections. The toxicity of LPS, however, prevents its human use. Pioneering work from Ribi, led to the development of a less toxic derivative of LPS, monophosphoryl lipid A (MPL) [12]. Although both LPS and MPL signal via TLR4, the latter signals mainly via TRIF [13] whereas the former signals via both MyD88 and TRIF, which results in enhanced proinflammatory cytokines and possible toxicity [13]. MPL formulated with alum (AS04), is now a component of licensed vaccines for HPV (Cervarix[®]) and Hepatitis B (Fendrix[®]) [14]. AS04 stimulates a polarized Th1 cell response in contrast to the mixed Th1-Th2 cell response induced by alum alone [14]. Subsequent studies, investigating less toxic derivatives of LPS such as MPL and RC-529 demonstrated that even though MPL can enhance primary CD4 T cell expansion and Th1 differentiation, long-term CD4 T cell response were better promoted with LPS than MPL [15]. For this reason, we used LPS as an adjuvant in our vaccine model. Furthermore, the addition of costimulation, also increases the effectiveness of T cell priming. For example, OX40 (CD134) costimulation supplied in the form of agonist antibody (anti-CD134) enhances CD4 T cell clonal expansion, survival, and memory [16-18]. Importantly, LPS can synergize with anti-CD134 to further enhance the CD4 T cell expansion and survival [19]. A fundamental understanding of how LPS and CD134 agonist induced responses promote T cell survival can be beneficial in development of an optimal T cell inducing vaccine. With the aim of understanding LPS induced mechanisms, previous studies in our lab showed that, LPS induced TRIF (TIR-domain-containing adapter-inducing interferon- β) signaling is essential for promoting effector CD4 T cell accumulation to non-lymphoid tissues of liver and lung [20]. However, TRIF induced cytokines TNF- α , IL-10, IFN- γ did not play a role in the T cell accumulation. Following this, a recent study suggested that lipid A-TRIF induced type 1 IFN

signaling (IFN α R signaling) is important for Ag-specific CD4 T cell expansion. We therefore, hypothesized that LPS induced type I IFN could play a dominant role in promoting the synergy between, LPS and CD134 agonist. Thus, in the 1st chapter of thesis we will focus on understanding the role of LPS induced IFN α R signaling in promoting Ag-specific CD4 T cell response in the vaccine model combining Ag with LPS and CD134 agonist.

Another adjuvant, poly I:C, a synthetic analog of viral dsRNA, has been studied in vaccines developed against infectious diseases such as HIV, malaria, HPV as well as in certain cancers [21-26]. Poly I:C was shown to be a superior adjuvant to MPL in the induction of CD4 Th1-type response to dendritic cell–targeted HIV gag protein vaccine [21]. Importantly, poly I:C induced CD4 T cell immunity depended on IFN α R signaling [22]. Furthermore, the combination of poly I:C and CD40 agonist that promotes synergistic CD4 T cell expansion and Th1 differentiation requires functional IFN α R signaling in DC's [27]. Authors in this study showed that IFN α R signaling induced CD134L expression on CD8⁺ DC's is essential for the synergistic effect of poly I:C and anti-CD40. Therefore, we hypothesized that combining poly I:C directly with CD134 costimulation could promote enhanced CD4 T cell response. Thus, in the 4th chapter of thesis, we will present the study of combined administration of Ag, poly I:C and anti-CD134 mAb and its effect on specific-CD4 T cell expansion and Th1 differentiation. Knowledge gained from this study could be useful in optimal development of vaccines against diseases such as HIV, HPV, malaria and certain forms of cancer where poly I:C is already been shown to be a promising adjuvant.

A. LPS as an adjuvant

TLR4 recognizes pathogen associated bacterial LPS, the primary cell wall component of the

outer membrane of gram-negative bacteria [28]. Bacterial LPS consists of a hydrophobic lipid A, a hydrophilic core polysaccharide chain and O-antigen repeating units [29]. The lipid A portion of the molecule is responsible for exerting the bacteria's endotoxic effects. Initial studies showed that Lipid A stimulates macrophages via TLR4 resulting in the secretion of proinflammatory cytokines leading to the pathogenesis of septic shock [30, 31]. However, the ability of LPS to induce several cytokines, induce APC maturation and directly promote CD4 T cell survival, established it as a valuable vaccine adjuvant [3, 11]. Since then a large body of research has been devoted towards investigating the adjuvant effects of LPS on lymphocytes.

1. LPS Detection

LPS recognition by innate receptors involves the association of LPS binding protein (LBP) with LPS molecules followed by its transfer to CD14 receptor [28]. CD14 lacks a transmembrane domain and does not signal in the presence of LPS. Rather, CD14 directs LPS to TLR4, which initiates the signaling process. Another important molecule is myeloid differentiation factor 2 (MD-2), which is associated with TLR4 on the cell surface [32]. MD-2 directly binds LPS, thereby conferring TLR4 responsiveness. B cells also express the receptors RP105 and MD-1, structurally related to TLR4 and MD-2, which are important for their LPS response [33].

2. LPS induced TLR4 Signaling

The intracellular portion of TLR4 contains a Toll/interleukin-1 receptor (TIR) domain that is common to all members of the TLR family [34]. This TIR domain recruits cytoplasmic adaptors, named myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor protein/MyD88-adaptor-like (TIRAP/Mal), TIR domain-containing adaptor inducing

IFN- β /TIR-domain-containing molecule 1 (TRIF/TICAM 1) and TRIF-related adaptor molecule/TIR-domain-containing molecule 2 (TRAM/TICAM 2). TIRAP recruits MyD88 [35, 36], while TRAM recruits TRIF to TLR4 [37], resulting in two distinct signaling pathways (see Figure. 1-1). Upon sensing LPS, TIRAP is thought to interact with TLR4 at PI(4,5)P₂-rich plasma membrane subdomains and recruit cytosolic MyD88, which eventually leads to the assembly of myddosome, a protein complex consisting of TIRAP, MyD88, and IRAK family kinases. This complex promotes proinflammatory cytokine production. The MyD88 pathway begins with the phosphorylation of IL-1 receptor associated kinases (IRAK), IRAK4 and IRAK1, leading to activation of TNF receptor-associated factor 6 (TRAF6). TRAF6 activates transforming growth factor- β -activated protein kinase 1 (TAK1). TAK1 then activates IKK complex and MAP kinase pathway, leading to degradation of I κ B and activation of C-Jun N-terminal kinase (JNK) and p38. LPS also activates extracellular signal-regulated kinase (ERK) through MEK1 and MEK2. MyD88-dependent signaling ultimately results in the activation of NF- κ B and AP-1 transcription factors [28, 38].

TLR4 could be internalized into endosomes where it engages TRAM, to recruit TRIF [39]. Importantly, the plasma membrane signaling of TLR4 could be uncoupled from its endosomal signaling. The TRIF pathway begins with the recruitment of receptor interacting protein 1 (RIP1) and TRAF6, both of which cooperate to activate NF- κ B. In addition, TRIF activates TANK-binding kinase 1 (TBK1) and IKKi, resulting in the phosphorylation of interferon regulatory factor 3 (IRF3) [38, 40]. Initial IFN- β production is IRF3-mediated and IFN- β amplifies type I IFN production further through induction of IRF7. IRF3 and IRF7, along with NF- κ B and ATF2/c-Jun, induce the transcription of IFN- β . Thus, MyD88 and TRIF signaling pathways fully account for the adjuvant activity of LPS [40, 41].

3. Role of the adjuvant LPS in CD4 T cell activation and effector differentiation.

Considering the central roles of CD4 T cells in assisting both B cell and CD8 T cell responses, the effects of LPS on CD4 T cells is of considerable interest. By tracking Ag-stimulated T cells *in vivo* our understanding of LPS adjuvanticity has greatly increased. The superantigen model is best suited for studying endogenous CD4 T cell responses, where Staphylococcal Enterotoxin A (SEA) binds and activates V β 3 receptor expressing T cells by linking them with MHC class II molecules on APCs [42]. Injecting SEA from *staphylococcus aureus* into mice results in significant expansion of V β 3 T cells followed by their contraction over the next few days [43]. Importantly, injecting LPS 24 hours after SEA, rescues V β 3 T cells from dying and results in about five-fold increase in their numbers [10]. Subsequent reports demonstrated that LPS can also increase the accumulation of peptide-stimulated CD4 T cells [44, 45]. The ability of LPS to enhance T cell survival was attributed to MyD88 dependent induction of inflammatory cytokines such TNF and IL-1 [46]. However, injecting TNF or IL-1 along with Ag does not result in T cell survival, to the same level as LPS, suggesting involvement of other factors for full adjuvant effect of LPS [44]. In line with this observation, CD11c⁺ cells were shown to be important for production of IFN- γ by T cells and their Th1 differentiation [11]. In an attempt to examine other MyD88-independent factors that contribute to LPS induced effector differentiation of CD4 T cells, TRIF signaling was shown to be important for accumulation of Ag-specific CD4 T cells to non-lymphoid tissues of liver and lung and their Th1 differentiation [20]. A similar study, using Lipid A instead of LPS, suggested that TRIF mediates its adjuvant effect on T cells through induction of type I IFN [47]. It is known that type I IFN can enhance T cell response by either directly acting on CD4 T cells and enhancing their survival [48, 49] or indirectly by inducing expression of costimulatory molecules on APC's [50-52]. Therefore, we

hypothesized that LPS induced IFN α R signaling can promote Ag-specific CD4 T cell expansion and or accumulation in our vaccine model, which could explain how LPS and anti-CD134 synergize to enhance the CD4 T cell expansion and survival.

B. Role of the adjuvant poly I:C in T cell response

Many laboratories use synthetic dsRNA to study the immune response against dsRNA viruses or once that generate dsRNA intermediates during viral replication. One such example is poly I:C, a nucleic acid duplex consisting 1 strand of polyriboinosinic acid and a complementary strand of polyribocytidylic acid. Poly I:C can activate both Toll like receptor 3 (TLR3) and melanoma differentiation associated gene 5 (MDA5) signaling pathways [53-57]. Activation of both TLR3 and MDA5 optimizes the magnitude and durability of CD4 Th1-type immunity as well as CD8 T cell immunity compared to either pathway alone. This highlights a central feature of potency of poly I:C as compared to other TLR adjuvants. Below I will explain, how poly I:C and its derivatives are used as adjuvants to promote T cell response in several infectious diseases and cancer.

1. Poly I:C and its derivatives as vaccine adjuvants

Poly I:C as an adjuvant

Poly I:C's role as an adjuvant has been tested in several experimental vaccine models. Two recent studies demonstrated that poly I:C induces durable and protective CD4 T cell immunity when used as an adjuvant in vaccines that targets DEC-205 DCs [21, 22]. In these settings, poly I:C boosted DC activation and CD4 T cell response by promoting the systemic release of type I IFN from both non-hematopoietic (stromal) and hematopoietic cells (macrophages and

DCs). Inclusion of poly I:C in an HIV vaccine consisting of purified recombinant gp120 antigen has shown to promote the generation of MHC class I-restricted CD8 T cells *in vivo* [58]. Whereas a study testing poly I:C as a mucosal adjuvant, showed that intranasal administration of poly I:C, induces enhanced levels of CXCL10 in the airways, correlating with the increased migration of CXCR3⁺ CD4 and CD8 T cells [59]. This suggests the potential use of poly I:C as an adjuvant in vaccines against mucosal infections. In the quest for vaccines against cancer, poly I:C has been shown to enhance tumor specific T cell responses [60]. However pre-clinical studies in primates showed that poly I:C is easily degraded by serum nucleases, with subsequent reduction in type I IFN secretion and anti-tumor activity [61]. Increasing the dosage of poly I:C has not been successful, as it is not well-tolerated. Thus, several derivatives of poly I:C have been synthesized and tested for their safety and adjuvanticity, such as poly I:CLC and poly I:C₁₂U.

Poly I:C₁₂U as a vaccine adjuvant

Poly I:C₁₂U (Ampligen®) is a modified version of poly I:C, with mismatched uracil and guanosine residues [62]. The mismatch in the molecule's backbone results in its reduced toxicity and decreased half-life *in vivo*. Poly I:C₁₂U signals exclusively through TLR3 but not through MDA-5. Intranasal administration of poly I:C₁₂U with hemagglutinin (HA)-based H5N1 influenza vaccine, has shown to enhance the levels of protective, specific mucosal IgA and systemic IgG responses than the adjuvant-free vaccine [63]. In phase II and III clinical trials for HIV vaccines, and in phase I and II cancer vaccine studies, poly I:C₁₂U was shown to induce maturation of myeloid DCs, secretion of IL-12, inhibition of IL-10, and enhanced Ag-specific CTL and Th1-type CD4 T cell responses [64, 65]. In both cases, poly I:C₁₂U has been deemed

safe for use. In 2009, a study was conducted to compare the efficacy, between poly I:C₁₂U and poly I:CLC as adjuvants in a protein based vaccine utilizing keyhole limpet hemocyanin (KLH) as an Ag [66]. Both, poly I:C₁₂U and of poly I:CLC potentiated CD4 T-cell proliferation; however, three-times as much poly I:C₁₂U by mass was required to obtain the same Ag-specific CD4 T-cell proliferation in the host (rhesus macaques) as with poly I:CLC, suggesting that poly I:CLC can be utilized in less quantity to obtain protective CD4 T cell immunity.

Poly-ICLC as a vaccine adjuvant

Poly I:CLC (Hiltonol®), is a synthetic derivative of poly I:C stabilized with poly-L-lysine in carboxymethyl cellulose to make it less sensitive to serum-degradation [67]. The ability of poly I:CLC to elicit a strong Th1 response makes it a very attractive adjuvant [21, 68]. In RTS,S vaccine for malaria developed against *plasmodium falciparum* infection, it was shown that mice immunized with poly I:CLC + RTS,S induced significantly higher Th1 CD4 T-cell responses than mice vaccinated with RTS,S + GLA/SE (glucopyranosyl lipid adjuvant-stable emulsion), and poly I:CLC + RTS,S combination provided approximately 50% protection [23]. Moreover, the poly I:CLC + RTS,S combination was also immunogenic in non-human primates resulting in production of Ag-specific, polyfunctional CD4 T cells and antibody production [69]. In an HIV vaccine study, inclusion of poly I:CLC in a gag recombinant protein vaccine where gag p24 was fused to DEC-205 resulted in polyfunctional CD4 and CD8 T-cell responses in rhesus macaques [70]. Additionally, in phase I ovarian cancer trial, patients treated with NY Eso peptides + poly:ICLC formulated with Montanide-ISA-51 showed enhanced peptide-specific antibody, CD8 and CD4 T cell responses [71]. Currently several ongoing Phase I and II clinical trials of studying intratumoral administration of poly:ICLC in patients with liver cancer,

pancreatic cancer, melanoma, sarcoma, and head and neck cancer have demonstrated the safety of this approach, but efficacy data are not yet available.

2. Poly:I:C detection

Toll-like Receptor 3 (TLR3)

TLR3 was the first identified as a dsRNA receptor while studying poly I:C responsiveness in the NFkB reporter cell line transfected with different TLRs [53]. In humans, TLR3 is expressed both on surface and intracellularly in non-immune cells such as fibroblast and epithelial cells or intracellularly in monocyte derived immature DCs and CD11c⁺ DCs [54, 72-74]. Whereas, in mice, TLR3 is expressed intracellularly in CD8⁺ DC's and macrophages [53, 75]. Whether its expressed on the surface or intracellularly, TLR3 signaling, occurs intracellularly as it requires endosomal maturation [73]. The intracellular location of TLR3 poses a challenge to ligand recognition. A recent study demonstrated that CD14 enhances dsRNA-mediated TLR3 activation by directly binding to poly I:C and mediating its cellular uptake [74]. The internalized poly I:C colocalizes with CD14 and TLR3. As extracellular domain of CD14 consists of leucine rich repeat (LRR) [76], poly I:C might be transferred from CD14 to TLR3.

TRIF-signaling pathway

Among all the members of the TLR family, TLR3 is the only one that uses TRIF but not the MyD88 signaling pathway [77, 78]. Upon ligand-binding, TRIF is recruited to the cytosolic TIR domain of TLR3, similar to TLR4-TRIF (see Figure. 1-2). TRIF recruits RIP1 and TBK1 downstream. RIP1 is phosphorylated and polyubiquitinated by the E3 ubiquitin ligase, Peli1. Polyubiquitinated RIP1 forms a complex with TRAF6 and TAK1. TRAF6 is an ubiquitin ligase

whose substrates include TRAF6 itself and NF- κ B essential modifier (NEMO). Ubiquitinated TRAF6 and NEMO recruit and activate TAK1. Additionally, TAK1 is part of a protein kinase complex that further activates two pathways [79]. On one end, TAK1 phosphorylates and activates MAPK, to phosphorylate AP-1. AP-1 dimerizes and enters the nucleus, contributing to the transcription of cytokine genes. On the other end, TAK1 activates the NEMO-IKK α -IKK β complex. This protein complex phosphorylates I κ B, targeting it for proteosomal degradation and releasing NF κ B (p50/RelA dimer) that enters the nucleus and induces cytokine gene transcription.

TRIF mediated IFN- β induction requires activation of TBK1 kinase. This activation step is achieved through recruitment of TRAF3. TRIF associates with TRAF3, NAK-associated protein 1 (NAP1), TANK-binding kinase 1 (TBK1) and IKK-i (I κ B kinase-i). Activated TBK1 and IKK-i phosphorylates IRF3 that dimerizes and enters the nucleus to initiate IFN- β gene transcription [80, 81]. Transcriptional activation of pro-inflammatory cytokine genes requires the binding of NF κ B and AP-1 dimer to the promoter region, whereas transcription of type I IFN genes is more stringent, requiring the combined activities of IRF3, NF κ B and AP1 [77]. Additionally, phosphoinositide 3-kinase (PI3K) is required for the full activation of IRF3 and NF κ B [82].

RNA helicases MDA5, RIG-I and LGP2

Unlike TLR3, whose expression is more restrictive, RNA helicases RIG-I, MDA5 and LGP2 are expressed ubiquitously [84-86,171]. Long chain poly I:C (> 2kb) can bind both TLR3 and MDA5, however, RIG-I recognizes relatively short dsRNA (up to 1kb) with 5' triphosphate [87, 88]. Thus, if poly I:C was to be shortened by RNaseIII to less than 1kbp, then it can bind RIG-I [89]. The protein structures of these receptors contain two important domains that are essential

for their function: the caspase recruitment domain (CARD) and the DExD/H box RNA helicase domain, with the exception of LGP2, which does not have a CARD domain. The DExD/H domain situated at the N terminal is the ligand-interacting domain and essential for unwinding the RNA substrate that is powered by ATP hydrolysis. The CARD motif is responsible for mediating downstream signaling. LGP2 does not have the CARD motif [84] and hence fails to signal [90]. Similar to RIG-I, LGP2 interacts with both ssRNA and dsRNA [91], but does not induce type I IFN response or NFkB transcription factor [90]; hence, it was thought to act as a negative regulator of RIG-I signaling. Several models were proposed for the mechanism of dsRNA recognition inhibition by LGP2 [92]. However, recent studies demonstrated that LGP2 works upstream of MDA5 and RIG-I to facilitate activation of these receptors [93]. The ATPase function of LGP2 is essential for this function, but the exact molecular events remain unknown.

RIGI/MDA5 signaling pathway

MDA5 and RIG-I helicases recognize different RNA substrates but their downstream signaling pathways are similar since they both act through IFN- β promoter stimulator 1 (IPS-1) (also known as MAVS), a mitochondrial outer membrane-associated protein [94, 95]. In addition to ligand sensing, RIG-I activation requires ubiquitination by TRIM25. The interaction of RIG-I and MDA5 with MAVS is mediated through their CARD domain. From this point, MAVS associates with TRAF3 (a ubiquitin ligase) and activates TBK1 in a similar fashion to the TRIF pathway (Figure. 1-2). Three proteins that are functionally similar to each other, TANK, NAP1 and SINTBAD, are important in facilitating the type I IFN response mediated by both TRIF and MAVS [96]. Additionally, MAVS interacts with FADD and RIP-1 via non-CARD region to facilitate NF-kB activation. It was found that FADD interacts with both MAVS and caspase 8

and 10 and processed forms of caspase 8 and 10 are important for NFkB activation as well as cell apoptosis [97].

In Summary

The studies reviewed above, strongly suggest that poly I:C or its derivatives can induce multifunctional CD4 and CD8 T cell responses and induce maturation of DCs. Importantly, if poly I:C is combined with other signaling pathways, it may result in further enhancement of the T cell response. Indeed, specific CD4 T cells were shown to be synergistically activated by administration of Ag together with poly I:C and a CD40 agonist. This synergistic effect was dependent on expression of IFN α R and CD134L on DC's. Thus, in 4th chapter of thesis we will study if poly I:C can be combined directly with CD134 costimulation to generate enhanced CD4 T cell expansion and Th1 subtype differentiation. If poly I:C and anti-CD134 synergize to promote enhanced CD4 Th1 type cell response, this combination could be used to develop an effective Th1 response inducing vaccine.

As this thesis focuses on studying how LPS and poly I:C induced type I IFN impacts CD4 T cell response, below we will address the general characteristics of type I IFN and what's known about its role in enhancing CD4 T cell immunity.

(II). Type I IFN Interferon

A. Role of Type I Interferon

Type I IFNs were first identified more than 50 years ago as an “inhibitory factor” of viral replication [98]. They belong to a family of closely related cytokines and consist of the products of numerous IFN- α genes, a single IFN- β gene (in mice and humans) and several other genes

that are expressed variably in different species (IFN ω , $-\tau$, $-\kappa$, $-\epsilon$, $-\delta$ and limitin) [99]. Type I IFN can be produced by all nucleated cells and although all type I IFN members signal through the same receptor complex [100, 101], their signaling can result in diverse effects depending on the engaged type I IFN, the responding cell and timing of the signaling (before or after T cell receptor (TCR) activation)) [102, 103]. The anti-viral effects of type I IFN are ascribed to the induction of interferon stimulated genes (ISGs) that result in production of proteins such as, 2'-5' oligoadenylate synthases (OAS), dsRNA-dependent protein kinase (PKR) and Mx. [104-106]. 2'-5' OAS catalyzes the formation, of 2'-5' oligoadenylates to activate the ribonuclease RNase L that degrades the viral and cellular RNA. Whereas PKR, is a serine/threonine kinase that binds dsRNA and phosphorylates eukaryotic translation initiation factor alpha subunit (eIF2a) to block viral RNA translation. The Mx protein family are large GTPases that complex with dynamin to disrupt the trafficking or activity of viral polymerase and thereby interfere with viral replication. The importance of type I IFNs in viral infections has been demonstrated in mouse models, where infection of mice lacking the IFN α R1 fail to control viral replication of vesicular stomatitis virus (VSV), semliki forest virus (SFV), vaccinia virus (VV) and lymphocytic choriomeningitis virus (LCMV) [107].

Apart from induction of the anti-viral protein, type I IFN receptor (IFN α R) signaling also activates other immune cells, including phagocytes [108-110], NK cells [111, 112] and T cells [48, 113]. Type I IFNs can act directly on T cells and NK cells, leading to their activation and acquisition of effector functions for elimination of the pathogen. Additionally, type I IFNs can act on APCs to promote their full activation and maturation [114, 115].

B. Type I IFN Signaling

All type I IFN family members signal through the complex, consisting of IFN α R1 and IFN α R2 subunits [101] (See, Figure. 1-3). Binding of type I IFN leads to the dimerization of these two subunits bringing Janus kinase 1 (Jak1) associated with IFN α R2 into close proximity with the tyrosine kinase 2 (Tyk2) [116, 117]. This leads phosphorylation and activation of Tyk2, which then cross phosphorylates Jak1. Jak1 then phosphorylates IFN α R1, providing a docking site for signal transducer and activator of transcription (STAT) proteins. Binding of STAT leads to their phosphorylation, dissociation from the receptor complex and translocation to the nucleus leading to regulation of IFN induced genes [101]. The major transcription factor formed in response to type I IFN is interferon-stimulated gene factor 3 (ISGF3), a heterodimer of STAT1 and STAT2 combined with IFN-regulatory factor 9 (IRF9) [118]. ISGF3 binds to interferon regulated response elements (ISREs), contained in the promoters of ISGs. Although STAT1 and STAT2 are the most important mediators, IFN α R can also activate STAT1 and STAT3 homodimers and heterodimers in most cell types and activate STAT4, STAT5 and STAT6 in certain cell types [119]. Interestingly, different STATs have different biological effects. IFN α / β provides pro-survival and mitogenic signals, possibly through STAT3 and STAT5, rather than antiproliferative signals through STAT1 [120], [121]. On the other hand, type I IFN induced STAT4 is required for IFN- γ production from CD8 T cells during lymphocytic choriomeningitis virus (LCMV) infection, whereas, STAT1 negatively regulates IFN- γ production [122, 123]. The relative abundance of these STATs, which may vary depending on cell type and activation state is likely to have a major impact on the overall response to type I IFN.

C. Role of Type I IFN signaling in adaptive immune response

1. Influence of type I IFN signaling on DC's

Several studies in both human and mouse systems suggest that type I IFN can influence the adaptive immune cell response by acting on DCs. Type I IFN act on immature DCs to enhance their cell-surface expression of MHC and co-stimulatory molecules (CD80 and CD86) endowing them with a better ability to stimulate T cells [51, 52, 124]. Type I IFN also promotes the ability of DCs to cross present Ags to CD8 T cells during viral infections, such as vaccinia virus and LCMV infections [115, 125]. Additionally, type I IFN may promote migration of DCs to lymph nodes, by up regulating their chemokine receptor expression, to help prime and activate T cells [126, 127]. DCs are potent producers of IL-12, which is important for driving Th1 differentiation and IFN- γ production by T cells. In some settings, IFN α R signaling has been shown to induce IL-12 from DCs following PRR stimulation [128]. However, high but physiological levels of type I IFN can also inhibit IL-12 production during MCMV and LCMV infections [129, 130]. This mechanism may have developed to favor optimal cytotoxic responses by T cells and NK cells in response to virus, while limiting the pathological effects of excessive IL-12 production [131]. Overall, type I IFN can influence T cell response by inducing DC maturation, their expression of costimulatory or chemokine receptors, and secretion of cytokines such as IL-12.

2. Influence of type I IFN signaling on T cells

The role of type I IFN in modulating T cell responses first became evident from studies investigating the effects of injecting type I IFN, together with Ag's in experimental models. Timing of type I IFN exposure is important in deciding the fate of T cell response, where pre-

exposure to type I IFN was found to inhibit, whereas type I IFN given at same time and/or after antigen was shown to augment CD4 and CD8 T cell response [132].

Role of type I IFN in T cell survival

Definitive evidence that T cells were direct cellular targets of type I IFN came from studies with adoptive transfer of transgenic T cells lacking IFN α / β R receptor against different MHC class I- or II-restricted peptides of LCMV virus into IFN α / β R^{+/+} mice. This resulted in dramatic reduction of specific CD4 and CD8 T cell expansion and generation of memory as compared to transfer of IFN α / β R⁺ control T cells [133-135]. The reduced accumulation of T cells was due to poor survival of activated T cells rather than their inability to proliferate. Moreover, reduced accumulation of IFN α R1^{-/-} CD8⁺ T cells was associated with lower expression of the anti-apoptotic proteins Bcl-xL and Bcl-2 [136]. Direct evidence that type I IFN can prevent the death of activated CD4⁺ and CD8⁺ T cells *in vitro* has been reported for mouse [48] and human T cells [49]. In these studies, activated T cells exhibited a lower rate of death when cultured in the presence of type I IFN than when placed in medium alone.

In contrast to the evidence that type I IFN can enhance the survival of activated T cells, it has also been reported to increase T cell death under certain conditions. For instance, this has been shown in an *in vitro* model of activation induced cell death (AICD), in which pre-activated human T cells were exposed to anti-CD3, triggering a certain degree of apoptosis. Here, addition of IFN- α with anti-CD3 induces a greater proportion of apoptotic T cells than anti-CD3 alone [137]. In this case, the pro-death effects of type I IFN could be a feedback mechanism to prevent T cell-mediated pathology such as that seen during chronic infection, where high levels of viral Ags and type I IFN persist beyond the acute stage of infection. Consistent with

this, injection of IFN- α into mice relatively late during an immune response (day 8) has shown to augment expression of programmed cell death-1 (PD-1), a negative regulator of TCR signaling [138].

Role of type I IFN in T cell proliferation

Like T cell survival, the effect of type I IFN on cell proliferation also depends on timing and context of signaling. It has been shown that pre-exposure to type I IFN significantly inhibits the proliferation of both purified mouse [139] and human T cells [140]. Further evidence of this came from a study, where human T cells that were pretreated with IFN- α for 18–20 h before anti-CD3/CD28 mediated stimulation showed reduced proliferation, however addition of IFN- α to activated T cells did not inhibit the proliferation [141]. This outcome was associated with enhanced expression of IL-2R α as well as genes associated with cell proliferation (c-myc) and cell survival (pim-1) after adding IFN- α to activated T cells [142], whereas T cells treated with IFN- α before stimulation had reduced IL-2 and IL-2R expression [140]. Additional confirmation that the nature of signaling through IFN α R can be altered following T-cell activation came from study, showing that in response to LCMV infection in mice the proliferating CD8 T cells downregulate STAT1 expression, which is associated with anti-proliferative activity of type I IFN [143].

These findings suggest that the timing of exposure to type I IFN relative to antigen is critical in determining if T cell proliferative response is enhanced or suppressed.

Role of type I IFN in effector T cell differentiation

Role of type I IFN in T cell differentiation came from initial studies showing that IFN- α/β induces differentiation of APC-activated naive mouse T cells into CTL, increasing their cytolytic activity, IFN- γ expression, and perforin and granzyme B expression [144]. Similarly, type I IFN was shown to promote the cytolytic activity and IFN- γ secretion by CD3/CD28-stimulated human CD8⁺ T cells [145]. In comparison *in vivo*, work showed that type I IFN unresponsive mouse CD8⁺ T cells responding to LCMV infection were found to secrete less IFN- γ and degranulate less effectively than WT cells [136].

In terms of CD4 T cells, although type I IFN on its own appears to be insufficient to serve as a polarizing factor for Th1 differentiation, it can act in concert with other cytokines to promote Th1 differentiation. For instance, IFN- α has been shown to increase the production of IFN- γ by anti-CD3/CD28 stimulated human CD4⁺ T cells in the presence of IL-12 that was associated with IFN- α -mediated enhancement of IL-12R β 2 expression [146]. Similarly, treatment of anti-CD3-activated human T cells with IFN- α was shown to increase the expression of IL-18R, which correlated with increased production of IFN- γ in response to treatment with IL-12 + IL-18 together [147]. IFN- α has also been shown to increase IL-21 expression when added with anti-CD3, or when added to anti-CD3/CD28 pre-activated human T cells [148]. IL-21 can act in concert with IL-18 to enhance IFN- γ , suggesting another possible mechanism by which type I IFN could promote Th1-type responses [149].

While contributing positively to Th1 polarization, type I IFN has been shown to be inhibitory on other T cell differentiation fates. Type I IFN has been reported to inhibit the differentiation of human naive CD4 T cells towards a Th2 phenotype and to reduce production of Th2 cytokines from pre-committed Th2 cells [150]. IL-5 appears to be the Th2 cytokine most strongly affected

by type I IFN [151, 152]. IFN- α has been shown to regulate the response of human CD4 and CD8 T cells to IL-4 in a complex manner, enhancing IL-4R mediated signaling at early time points (6 h), but inhibiting this response at later times (12–24 h) [153]. IFN- α has also been reported to inhibit the differentiation of mouse and human naive CD4 T cells *in vitro* (activated with anti-CD3/CD28 under Th17 polarizing conditions) into Th17 cells [154]. Taken together, these results indicate that positive effects on type I IFN on Th1 differentiation and negative effects on Th2 and Th17 polarization likely influences its capacity to drive type 1 responses *in vivo*.

Role of type I IFN in memory T cell responses

Finally, in addition to modifying effector T-cell differentiation, there is evidence that direct stimulation of T cells by type I IFN contributes to the generation of memory T cells. This was suggested indirectly in a study where the role of IFN α R mediated signaling in CD8 T cells was examined after LCMV infection [134]. Here, the numbers of both effector and memory CD8 T cells were greatly reduced for type I IFN unresponsive T cells compared to the control cells. However, given that memory T cells can arise from effector T cells, the reduction in memory cells may be simply a consequence of diminished initial T-cell expansion. Indeed, the generation of functional memory cells was confirmed from IFN α R1^{-/-} CD8⁺ T cells in a similar LCMV infection model [155]. Here the absence of IFN α R1 in CD8 T cells resulted in reduced generation of short-lived effector T cell (SLEC), however, generation of memory precursor effector cells (MPEC) that gave rise to functional memory CD8 T cells was unaffected. By contrast, experiments studying the response of IFN α R1^{-/-} CD8 T cells in a bacterial, *Listeria monocytogenes* infection model demonstrated a markedly reduced generation of memory cells

despite strong primary expansion [156]. A recent study showed that when human CD8 T cells were activated by IFN- α and IL-12 together, IL-12 programs development of effector memory cells (TEM) by induction of IL-12R on actively dividing cells. In contrast, IFN- α slowed the cell division in some cells, resulting in their acquisition of central memory (TCM) phenotype [157]. In terms of CD4 T cells, there have been very few reports showing that type I IFN can promote memory CD4 T cell responses. Recently, it was shown in an *in vitro* study that IFN- α synergizes with IL-12, when added to anti-CD3/CD28 stimulated naive human CD4⁺ T cells, to produce IL-2-secreting CD4 T cells that display the characteristics of TCM cells and generate IFN- γ upon secondary activation [158]. These results are different than seen for human CD8 T cells, where IL-12 programs the development of TEM cells, whereas IFN- α results in development of TCM cells. However, overall type I IFN alone or in combination of cytokines such as IL-12 can promote the development of both CD8 and CD4 memory T cells, where most reports suggest that type I IFN promotes the development of central memory cells.

In summary

The overall role of type I IFN in promoting T cell responses could rely on its direct effect of T cells by influencing their survival, proliferation and differentiation or it could be coupled with its indirect effects on other cell types such as DC's. Alternatively, it is possible that type I IFN induced downstream signaling pathways influence the overall quality and magnitude of the T cell response, where Type I IFN signaling may enhance the expression of chemokine receptors on T cells to allow their accumulation to certain peripheral tissues. Several studies have reported that CD4 and CD8 T cells recovered from inflamed peripheral tissues in human autoimmune diseases were highly enriched in CXCR3 expression relative to T cells found in

the blood [159-161]. In addition, CXCR3 ligands were also highly expressed in these same diseased tissues. In fact, previous studies in our lab, showed that LPS induced TRIF signaling in host cells, promotes CXCR3 expression on specific-T cells. Up-regulated CXCR3 expression might explain how TRIF mediates T cell accumulation to peripheral tissue such as liver. LPS used in our vaccine model can result in development of IFN- γ producing Th1 cells and Th1 cells are almost exclusively CXCR3 positive [162, 163]. Hence, one of our aims is to understand, if LPS-TRIF induced IFN α R signaling can enhance the expression of CXCR3 on specific-CD4 T cells to promote their migration or accumulation to peripheral tissues of liver and lung. Below I will review, the role of CXCR3 and its ligands in promoting T cell responses in various diseases.

III. Role of CXCR3 and its ligands in promoting CD4 T cell response

A. CXCR3 and its ligands

CXCR3 is composed of 368 amino acids (aa) and is a seven-transmembrane G-protein coupled receptor [164]. It is mainly found in two alternatively spliced forms, CXCR3-A [165] and CXCR3-B [166]. Recently a third splice variant, CXCR3-alt was discovered [167]. Ligands for CXCR3 include the chemokines, CXCL9, CXCL10, and CXCL11, formerly known as monokine induced by interferon- γ (Mig), interferon- γ inducible 10-kDa protein (IP-10), and interferon-inducible T cell α chemoattractant (I-TAC) respectively [159, 165, 168]. Although both forms of CXCR3-A and CXCR3-B can bind CXCL9, CXCL10 and CXCL11, the CXCR3-A has a higher affinity for them and can outcompete CXCR3-B [166]. These two forms of the receptor operate by different signaling pathways, and thus lead to different outcomes when activated. It is likely that CXCR3-A binds to the CXCL9 and CXCL10 in our vaccine model,

however we will not be addressing which receptor specifically binds to the chemokines in our study. CXCR3-alt encodes a truncated version of the receptor that is 260 aa in length and is predicted to have four or five transmembrane domains [167]. Chemotaxis studies show that this change in structure abolishes CXCR3-alt's ability to interact with CXCL9 and CXCL10, but retain's its activity to interact with CXCL11. It is important to note that, C57BL/6 mice does not possess a functional CXCL11 gene, since a point mutation and single-base deletion in its leader sequence, results in a reading frame shift introducing a stop codon early within the gene [169, 170]. Hence we will not be addressing the role of CXCL11 in promoting T cell response.

B. Chemokine induction

It is important to note that the CXCR3 ligands are differentially induced. CXCL9 is strongly induced by IFN- γ , but most studies suggest that type I IFN does not directly induce CXCL9 but rather synergizes with cytokines such as IL-18 to produce IFN- γ which may then induce CXCL9 [171, 172]. Again TNF cannot alone induce, but rather synergizes with IFN- γ to induce CXCL9 [173]. Whereas, CXCL10 is strongly induced by IFN- γ as well as by the type I IFN and weakly by TNF, although TNF can synergize with type I IFN to induce CXCL10 [171, 174]. In our model LPS and poly:IC induced IFN- γ (produced by Th1 cells) may result in induction of CXCL9 and CXCL10 or type I IFN may synergize with cytokines such as IL-12, IL-18 or IL-21 to induce IFN- γ which may then induce CXCL9. Whereas, CXCL10 can be directly induced by type I IFN. Several reports suggest that, CXCL9 and CXCL10 are released by stromal cells, macrophages, DCs, neurons or endothelial cells, depending on the kind of pathogen involved and site of infection [175-179]. In our study as well, these cells could contribute to production

of CXCL9 and CXCL10, however, we have not specifically analyzed the cells responsible for induction of these chemokines.

C. CXCR3 signaling

CXCR3 is a seven-transmembrane-spanning glycoprotein receptor that mediates its action through a G-protein coupled signaling pathway [164]. Heterotrimeric G proteins consist of $G\alpha$, $G\beta$ and $G\gamma$ subunits, of which the latter two proteins are tightly bound [180]. The $G\alpha$ subunit harbors a GTP hydrolase catalytic site that is occupied by GDP when the G protein is in the inactive state. On binding of a G protein to an activated receptor, GDP dissociates from the G protein and is replaced by GTP. Subsequently, the G protein dissociates from the receptor and the $G\alpha$ -GTP and $G\beta\gamma$ complexes may each go on to initiate signal transduction cascades [180, 181]. $G\beta\gamma$ activates the membrane-associated enzyme phospholipase C β 2 (PLC2) [182] which in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂) to form the intracellular second messengers phosphatidylinositol 1,4,5-triphosphate (IP₃) and diacyl-glycerol (DAG). IP₃ mobilizes calcium from intracellular stores, whereas DAG acts in conjunction with calcium to activate various isoforms of protein kinase C (PKC). The activation of PKC and of various calcium-sensitive protein kinases catalyze protein phosphorylation, which activates a series of signaling events that eventually lead to cell movement and other responses [183-185].

D. Role of CXCR3 in inflammatory diseases

Expression of CXCR3 on activated T cells grants them entry into sites otherwise restricted. This has been particularly shown for infections in the brain. CXCR3 is required on CD8 T cells to be recruited into brain during *Plasmodium berghei* ANKA infection for the development of

cerebral malaria symptoms and CXCR3^{-/-} mice are protected from cerebral malaria [175, 186]. This protection is mediated by CXCL9 and CXCL10, as mice deficient in either of these ligands showed partial disease protection [175]. In other models of brain inflammation, CXCL10 appeared to have a dominant role in the recruitment of T cells into the brain. In West Nile virus (WNV) infection, CXCL10 expressed by neurons, induced the migration of CD8 T cells into the brain [187]. Moreover CD8 T cell infiltration into vaginal mucosa in Herpes Simplex Virus-2 (HSV-2) infection for protection against the disease required CXCR3 expression [188, 189]. Additionally, CXCR3 expressing cells were first characterized in autoimmune rheumatoid arthritis synovium of human patients [160] and subsequently in murine models [190, 191]. CXCR3 mediated T cell infiltration is also known to play a role in development of autoimmune insulinitis diabetes [192] and systemic lupus erythematosus (SLE) [193].

E. Role of CXCL9 and CXCL10 in diseases

Dominance of CXCL9 versus CXCL10 in diseases

In some inflammatory models, the requirement of one CXCR3 ligand dominates over other and its deficiency cannot be compensated by the presence of the other ligands. For example, some infections in the brain show importance of CXCL10 ligand over others. In dengue virus infection, CXCR3 and CXCL10 are more important for trafficking of CD8 effector T cells into the brain to provide protection [194]. Similarly, as mentioned previously, in WNV infection, CXCL10 expressed by neurons directs the migration of CD8 T cells into the brain [187]. Similarly in a model that uses acute LCMV infection of transgenic mice that expresses LCMV glycoprotein in the cells of the islets of Langerhans, both CXCL9 and CXCL10 were up regulated, but CXCL10 was expressed throughout the infiltrated islet whereas CXCL9

expression was limited [192, 195]. Mice treated with CXCL10 neutralizing antibodies showed delayed onset of insulinitis, whereas CXCL9 neutralizing antibodies had no effect on disease progression.

In some autoimmune diseases however CXCL9 could play a dominant role. For example, CXCR3 and CXCL9-deficient, but not CXCL10-deficient, Murphy Roth Large/lpr mice were protected from autoimmune lupus-like inflammation of the kidney [193]. Also, during acute infection with *toxoplasma gondii*, CXCL10 was shown promote CD4 and CD8 T cell infiltration to several peripheral organs and control the spread of disease [196], but, during the chronic infection, CXCL9 was more important for recruiting T cells to brain and providing protection [177].

Collaboration of CXCL9 and CXCL10 in diseases

In some disease models, cooperation between CXCL9 and CXCL10 is required for full T cell infiltration and development of effector responses. Studies by Groom et al, recently showed using the model of OVA-pulsed DCs injected with LPS and poly I:C as well as with LCMV Armstrong infection, that CXCL9 and CXCL10 binding to CXCR3 was important for promoting T cell-DC interaction in dLN and their subsequent differentiation into Th1 cells [197]. As previously mentioned, CXCR3 expression on CD8 T cells was required for their infiltration into the brain during *P. berghei* ANKA infection for the development of cerebral malaria symptoms. In this study, CXCL9 and CXCL10 were equally important, mouse deficient in one of these ligands showed partial disease protection [175]. The cooperation between CXCR3 ligands appears to be due to expression of these ligands by different cell types, where endothelial cells predominantly produced CXCL9, whereas neurons were the main source of CXCL10.

Similarly, in a mouse model of herpes simplex virus-2 infection, expression of both CXCL9 and CXCL10 were necessary for optimal recruitment of NK cells and CTLs into the spinal cord and control of the infection [189]. Moreover, both CXCL9 and CXCL10 were shown to provide the chemotactic signals for recruitment of CXCR3⁺ central memory CD8 T cells and their localization in the periphery of dLN to mount effective anti-viral responses using during LCMV infection [198].

Tissue specific expression of CXCL9 and CXCL10, has been shown to orchestrate the movement of CXCR3⁺ T cells from the lymphoid compartment to the peripheral tissue in a murine model of granulomatous liver disease induced by *Propionibacterium acnes* [199]. In this model, LN DCs produced CXCL10, whereas hepatic granuloma cells including the kupffer cells in liver parenchyma produced CXCL9. It was suggested that CXCL10 instructs CD4⁺ Th1 cells to stay in the LN interacting with DCs, whereas CXCL9 expression in the periphery drives the recruitment of these cells out of the LN and into the liver.

In conclusion

Overall, the role of chemokines in promoting T cell recruitment could be dependent on several factors, such as their preferential expression in certain tissues, the type of infection, the cell types that produce them and availability of cytokines such as type I IFN and IFN- γ that could be secreted by T cells, NK cells, macrophages, stromal cells or several others following encounter with the pathogen.

Having looked at how chemokine receptor or chemokines induced by the adjuvants, LPS, poly I:C or type I IFN impact T cell recruitment or accumulation to different tissues, will next review how combining, the adjuvants LPS and poly I:C with costimulatory pathways such as OX40

can be beneficial in promoting T cell expansion and survival.

IV. Combining TLR signals with OX40 signaling

Combined administration of Ag, LPS and costimulatory signals, has been shown previously to enhance CD4 T survival than Ag and LPS alone. Studies using SEA model showed that, CD40 costimulation, which primarily acts on APCs could further enhance the T cell survival induced by SEA and LPS [200]. Similarly using SEA as well as the DO11.10 adoptive transfer model, it was shown that combination of LPS with OX40 agonist mAb that primarily acts on T cells, also resulted in a massive synergistic effect on T cell expansion and survival of long-term memory T cells [19]. However, combining LPS with both anti-OX40 and anti-CD40 did not further increase T cell survival, suggesting that they may work through similar or redundant mechanisms [200].

Moreover, the combination of poly I:C and CD40 agonist has also been shown to enhance generation of effector CD4 T cells in several DEC-205 targeted vaccine models [201-203]. Furthermore, combining a dominant epitope of RSV with poly I:C and anti-CD40 enhanced the generation of RSV-specific effector memory CD8 T cells in lung airways, protecting the mice against infection [204]. Similarly, administration of poly I:CLC and CD40 agonist together with HIV envelop glycoprotein peptides in a NHP model of rhesus macaques resulted in enhanced generation of effector CD4 and CD8 T cells against different HIV envelop peptides in the BAL fluid and lung [205]. Subsequent studies addressing the mechanism of synergy between poly I:C and CD40, suggested that poly I:C and CD40 induced Ag-specific CD4 and CD8 T cell responses were dependent on OX40L and IFN α R signaling in DC's [206]. Importantly, enforced OX40 costimulation was able to rescue the reduced CD4 T cell expansion in IFN α R^{-/-}

mice to WT levels in poly I:C and CD40 immunization model [27], suggesting that CD40 costimulation may work by enhancing OX40L expression and subsequent OX40 (CD134) signaling in T cells. Hence in the 4th chapter of the thesis we will study, if combining Ag together with poly I:C and anti-CD134 promotes enhanced Ag-specific CD4 T cell expansion and Th1 differentiation, similar to poly I:C and CD40 combination. If this combination generates enhanced CD4 T cell response than poly I:C and anti-CD134 alone, it could be further tested in vaccines against HIV, malaria, HPV and certain cancers where poly I:C or its derivatives have shown to provide protection in mouse or non-human primate models by promoting enhanced T cell responses.

Before I address, how LPS and poly I:C induced signaling pathways impact CD4 T cell response, I will review what's known about OX40 costimulation and why using it together with the adjuvants LPS and poly I:C could be beneficial in promoting optimal CD4 T cell immunity.

V. OX40 costimulation

A. OX40 and OX40L

The OX40 costimulatory molecule (also called as CD134) is a member of the TNF receptor superfamily [17, 207, 208]. OX40 signaling has been suggested to sustain the signaling pathways induced by TCR, CD28, and IL-2R [17, 209, 210]. Following Ag stimulation OX40 is transiently induced on activated naive CD4 and CD8 T cells [207, 211]. Although TCR signals are sufficient for inducing OX40 expression, CD28-B7.1/2 interactions augment and sustain its expression [212, 213]. T cell and APC-derived cytokines like IL-1, IL-2, and TNF may further modulate the extent and length of expression. OX40 is not constitutively expressed, but it's induced after TCR stimulation, and its expression peaks around 2-5 days after activation [17,

211, 214]. OX40 was originally characterized as a T cell activation marker, with preferential expression on CD4 T cells [207, 214, 215]. However under strong antigenic stimulation, OX40 can also be expressed on CD8 T cells [216, 217] and gut CD8⁺ intraepithelial cells [218]. OX40 ligand (OX40L) was originally identified on human T cell leukemia virus type 1 (HTLV-1) transformed T cells [219, 220]. OX40L is expressed on activated APCs, Ag and/or CD40 activated B cells and CD40L activated DCs and macrophages [208, 221-223]. In some cases, OX40L is expressed on NK cells [224], mast cells [225] and vascular endothelial cells [226, 227].

B. Role of OX40 in T cell expansion, survival, and memory

A direct role of OX40 signaling in promoting T response was demonstrated in OX40 deficient CD4 T cells, where the initial proliferation and cytokine production was normal, however they were unable to maintain the response after 3-5 days and showed a defect in long-term survival and maintenance of effector function [17, 212, 228, 229]. Furthermore, OX40 deficiency results in fewer memory T cells, suggesting that it either promotes survival of effector cells that enter the memory pool or induce effector cells to differentiate into memory T cells [230]. Studies, evaluating the role of OX40 signaling in T cell survival stem from the initial observation that OX40 costimulation allowed T cells to proliferate and survive *in vitro* [209]. Subsequent studies employed superantigen model to study the role of OX40 signaling, since the expansion and contraction phase of Ag-specific T cells is well characterized in this model [43]. Administration of agonist anti-OX40 after sAg injection resulted in approximately 10-fold increase in CD4 T cells surviving the contraction phase [19]. The increased survival was accentuated by the addition of a danger-signal, LPS, where combination of a LPS and anti-OX40 led to a 60-fold

increase in the generation of Ag-specific memory T cells. In another study using adoptive transfer of TCR transgenic CD4 T cells followed by immunization of peptide in adjuvant, addition of anti-OX40 promoted accumulation of effector cells and importantly, resulted in accumulation of functionally competent memory cells, 35 days after immunization [230]. These data confirms the role of OX40 signaling in clonal expansion and accumulation of effector cells which foster the development of functional memory T cells.

Anti-apoptotic proteins induced by OX40 signaling can also inhibit AICD in T cells. In vitro stimulation of WT CD4 T cells with anti-OX40 mAb was shown to enhance the expression of Bcl-2 and Bcl-xL, and retroviral transduction of Bcl-2 and Bcl-xL in OX40 deficient CD4 T cells restored the survival defect in T cells [212]. Additional studies show that, OX40 signals maintain the active form of Protein Kinase B (PKB), which is known to promote Bcl-2 expression [228] and enhance the expression of survivin [229], a protein known to regulate G1-S transition and hence T cell division. In summary, these data suggests that OX40 signaling promotes T cell survival beyond initial T cell priming by enhancing anti-apoptotic protein expression.

C. OX40 in T cell differentiation

Initial studies suggested a role of OX40 signaling in driving Th2 differentiation [17, 213, 231-236], but later reports indicated that OX40 signals could also enhance Th1 differentiation [16, 237-239]. The role of OX40 signaling in Th2 differentiation was shown *in vitro* with naive human CD4 T cells that were stimulated with anti-OX40 resulting in production of IL-4, and differentiation into Th2 cells which further produced high levels of IL-4 [232]. Additionally CD4 T cells, stimulated with OX40L expressed on activated murine B cells, also produced IL-4 and

differentiate into Th2 cells [235]. OX40 costimulation could drive both Th1 and Th2 differentiation in response to same antigen, where using TCR transgenic CD4 T cells specific for pigeon cytochrome C peptide, OX40 costimulation was shown to induce IL-2, IFN- γ and IL-5 production from CD4 T cells [17]. Recently, in a transgenic mouse model where Ag is persistently expressed, OX40 costimulation was shown to promote Th1 differentiation of CD4 T cells when stimulated *in vitro* with IL-12 and IL-18 [210]. Moreover, while OX40 costimulation is important in driving Th2 responses and lung pathology in asthma models [236, 240], it also results in CD4 T cell-mediated pathology in rheumatoid arthritis (RA) [237] and experimental autoimmune encephalomyelitis (EAE) [226, 241], diseases that are associated with Th1 or Th17 cell activation. Overall these reports, suggest that OX40 signaling does not directly influence T cell polarization to Th1 or Th2 phenotype, but rather enhances effector cell programs established early in T cell priming, perhaps as dictated by the TCR affinity for antigen or the surrounding cytokine milieu.

D. OX40 signaling in disease

Role in infectious diseases

Potential role of OX40 signaling in enhancing T responses suggests that OX40 could be potentially targeted in vaccination strategies or therapeutic applications to promote protection against pathogens. In terms of fungal infection, treatment with stimulatory OX40L.Ig fusion protein has been shown to promote IFN- γ production by CD4 T cells and reduced eosinophilia and *C. neoformans* burden in the lung [242]. Moreover, immunotherapy with OX40L-Fc chimeric fusion protein in combination with anti-CTLA4 was shown to promote CD4 T cell proliferation, granuloma maturation, and killing of *Leishmania donovani* [243]. Anti-OX40 also

promoted SIV gp130-specific T cell and antibody responses in rhesus monkeys [244], and, similarly, inclusion of the OX40L gene in a plasmid DNA vaccine encoding hepatitis B surface antigen (HBsAg) enhanced primary CD4 and CD8 T cell responses against this antigen, as well as long-term memory [245]. Lastly, OX40-OX40L interactions were important for generation of anti-viral T cell responses to provide protection during mCMV [246] and vaccinia virus infection [247].

Role in tumor immunity

The ability of OX40 agonists to regulate immune responses, as well as the expression of OX40 on CD4 and CD8 T cells from the tumors and tumor-draining lymph nodes in mice and humans led investigators to examine OX40 manipulation as a treatment for cancer patients [248-250]. Initial pioneering work from Andrew Weinberg's group showed that OX40 agonist mAb augments protection against growth of melanoma, sarcoma, breast and colon carcinoma, in the mouse resulting in significant improvement in the percentage of tumor-free survivors (20–55%) [251]. Following preclinical studies have demonstrated that treatment of tumor-bearing hosts with OX40 agonists, including anti-OX40 mAb or OX40L-Fc fusion proteins, resulted in tumor regression in several tumors [248, 249, 252-254]. In addition to promoting effector T cell expansion, OX40 agonists have the ability to directly regulate Treg cells. There are conflicting reports on whether these agonists promote or diminish Treg cell responses. It is suggested that anti-OX40 can push Treg cells in both directions, depending upon the context of stimulation and the cytokine milieu [253, 255, 256] and overall immunological effects of anti-OX40 therapy can vary based on the tumor model and multiple mechanisms can be involved for the anti-tumor activity of OX40 agonists.

The use of anti-OX40 monotherapy in a Phase 1 clinical trial in patients with solid tumors, showed promising results, where twelve out of 30 patients receiving OX40 agonist had regression of at least 1 metastatic lesion with only 1 cycle of treatment [257]. Patient toxicities were much milder for anti-OX40 mAbs compared to more severe toxicities, i.e., autoimmune-like disease, colitis, etc., caused by treatment with CTLA-4 blockade (ipilimumab), and most frequently resulted in temporary lymphopenia. Patients receiving the OX40 agonist had an expansion of CD4 (non-Treg cells) and CD8 T cells with concomitant expression of activation markers CD38 and HLA-DR. Unlike treatment with ipilimumab, OX40 agonist treatment did not induce expansion of Treg cells in the blood or tumor. Moreover two out of three patients had IFN- γ -producing CD8 T cells following stimulation with autologous tumor cell lines *in vitro*, suggesting a tumor-specific T cell response, though the antigens they recognize remain unknown. Recently, two new OX40-based clinical trials have been initiated at the Providence Cancer Center, Phase I/II trial that combines high-dose fractionated radiation with anti-OX40 treatment in breast cancer patients (NCT01862900) and Phase Ib trial combining chemotherapy (cyclophosphamide) and radiation with anti-OX40 Ab treatment in prostate cancer patients (NCT01303705). The anti-OX40 Ab used in these trials is of mouse origin. The recent development of humanized OX40L:Ig fusion protein that binds to OX40 with greater affinity could result in better outcomes and less side effects in the future studies using OX40 treatment [258]. Indeed, the use of OX40 agonists in the clinic represents an exciting chapter in cancer immunotherapy. However, it is unlikely that anti-OX40 alone will be sufficient to cure all the patients or all tumor types. There is great promise that combination immunotherapy incorporating OX40 with other checkpoint inhibitors (PD-1 and CTLA-4), or recombinant IL-2, intratumoral TLR ligands, radiotherapy, and chemotherapeutics may be able to do what single

agents alone cannot.

In conclusion

Our aim is to understand if the signaling pathways induced by adjuvants, LPS and poly I:C when combined with CD134 costimulation can promote enhanced CD4 T cell expansion, cytokine differentiation, and memory. For this we will first analyze if LPS induced IFN α R1 signaling and downstream chemokine pathways promote CD4 T cell expansion or accumulation in different lymphoid and non-lymphoid tissues. We have seen before that LPS induced TRIF signaling promotes T cell accumulation in non-lymphoid tissues of liver and lung, however the mechanism behind this is unknown. Hence studying LPS induced signaling pathways of type I IFN and downstream chemokines, could help us explain (1) How LPS and CD134 agonist synergize together to augment CD4 T cell expansion and survival (2) How TRIF signaling promotes T cell migration into peripheral tissues of liver and lung. Following this will determine, if poly I:C and CD134 agonist can be combined together, similar to poly I:C and anti-CD40 to promote enhanced CD4 T cell expansion and Th1 differentiation. We will also analyze if poly I:C and anti-CD134 combination relies on IFN α R1 signaling to promote enhanced T cell expansion and Th1 differentiation. For fighting challenging infectious diseases such as HIV, malaria and tuberculosis, Th1-type T cell immunity is crucial. Hence understanding, the signals induced by adjuvants, LPS and poly I:C that promote CD4 T cell expansion, survival, subset differentiation will allow us to harness these pathways in the future for design of optimal Th1 response inducing vaccines.

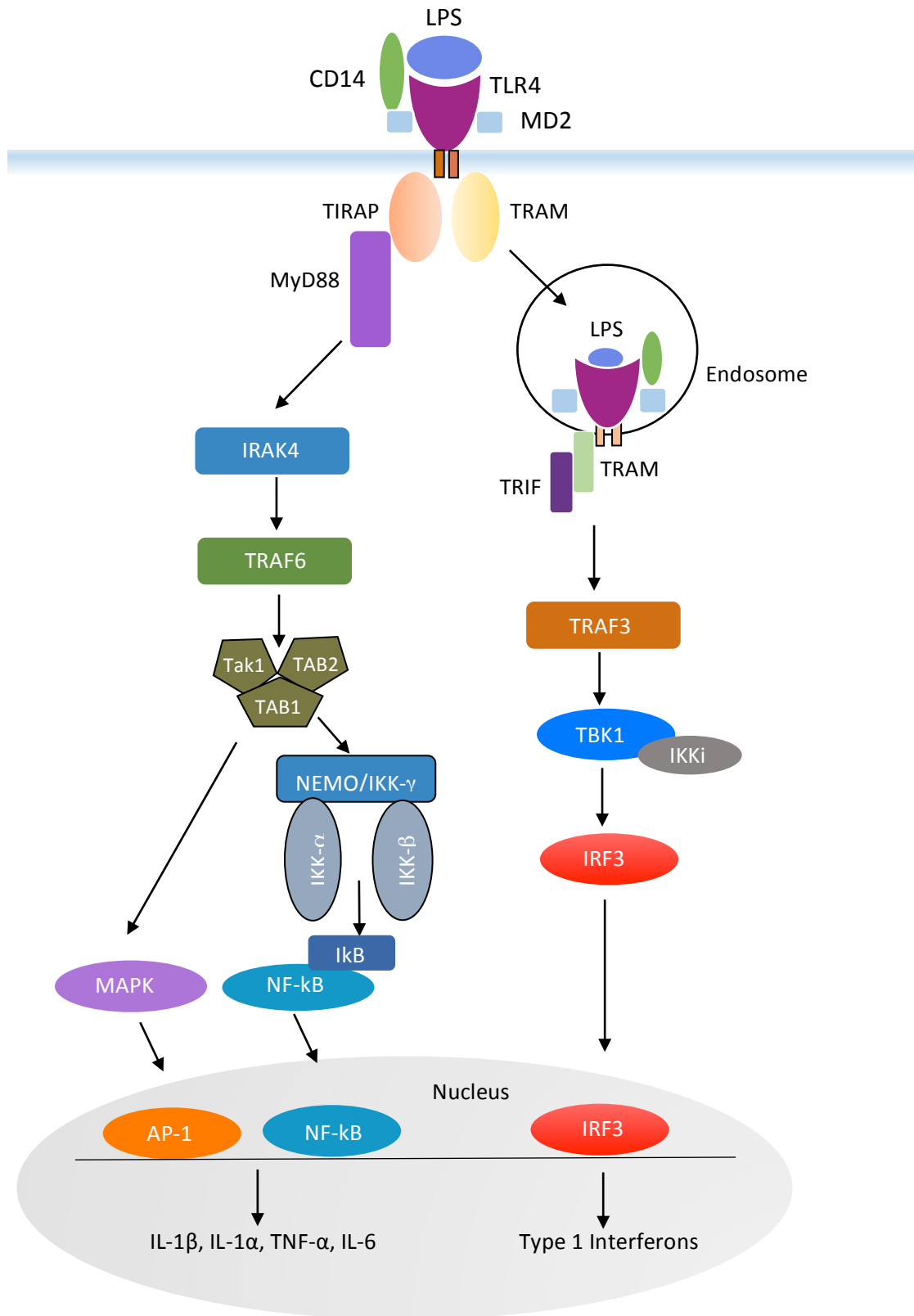


Figure 1-1 Simplified version of LPS induced TLR4 Signaling: TLR4 activation by LPS involves the participation of several molecules (LBP, CD14 and MD-2). Once activated, TLR4 induces two downstream signaling pathways, MyD88-dependent and MyD88-independent. The MyD88-dependent pathway is triggered by MyD88 recruitment of TRAF6 and IRAKs, which in turn results in activation of TAK1. TAK1 phosphorylates IKK β , leading to degradation of I κ B α and activation of NF κ B. NF- κ B translocates to the nucleus to promote the transcription of several cytokine genes. TAK1 also activates MAPK pathway leading to activation of JNK and p38 (not depicted). This leads to activation of AP-1 that dimerizes and translocates to the nucleus to activate cytokine genes. AP-1 and NF κ B together induce transcription of several proinflammatory cytokines such as IL-1 α/β , IL-18, IL-6, and TNF α . The MyD88-independent pathway relies on TRIF recruitment of TRAF3. TRAF3 activates IRF3 through TBK1 and IKKi, inducing transcription of type I interferons (IFNs) and IFN-inducible genes.

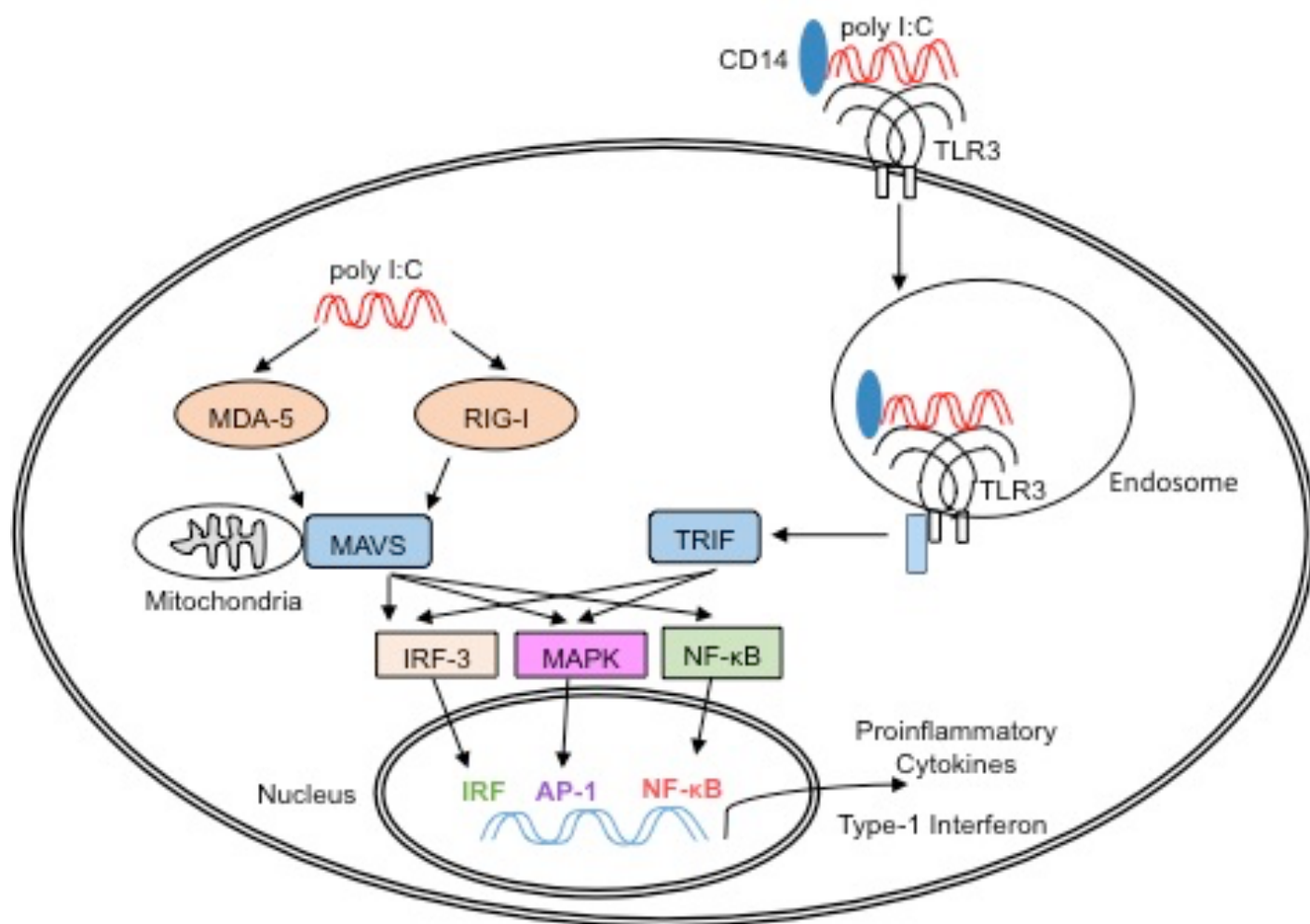


Fig 1-2. Poly I:C induced signaling pathways. Figure depicts a simplified version of signaling pathways triggered by poly I:C. Poly I:C could be sensed by multiple receptors at different cellular location. TLR3 in the endosome detects extracellular poly I:C with the help of a cell surface co-receptor, CD14 and uses TRIF as its signaling adaptor. TRIF recruits TRAF6 and TRAF3. Ubiquitinated TRAF6 binds NEMO to activate TAK1. TAK1 phosphorylates and activates MAPK, which then goes on to phosphorylate AP-1. AP-1 dimerizes and enters the nucleus, to activate transcription of cytokine genes. On the other end, TAK1 activates the NEMO-IKK α -IKK β complex and subsequently phosphorylates and degrades I κ B, freeing NF κ B to enter the nucleus and induce gene transcription. Additionally TRIF through TRAF3 recruits and activates TBK1 kinase (TBK1 and IKKi) that phosphorylates IRF3. IRF3 dimerizes and enters nucleus to induce gene transcription. RIG-I and MDA5 are RNA helicases that recognize poly I:C based on the structural differences. RIG-I detects poly I:C with 5'triphosphate cap and requires ubiquitination to be activated. MDA5 senses long chain poly I:C. LGP2 another RNA helicase (not depicted here) lacks signaling domain and is therefore believed to play a structural role in facilitating ligand recognition by RIG-I and MDA5. Both MDA5 and RIG-I, uses IPS-1 or MAVS located on mitochondrial membrane as signaling adaptor. MAVS interacts with TRAF3 and activates TBK1 to initiate IRF activation but depends on FADD and caspase 8 and 10 proteolysis to activate NF κ B. As a result of this signaling pathways NF κ B and AP-1 induces pro-inflammatory cytokines whereas induction of type I IFN is more stringently controlled (specially of IFN β) by coordinated actions of IRF, NF κ B and AP-1.

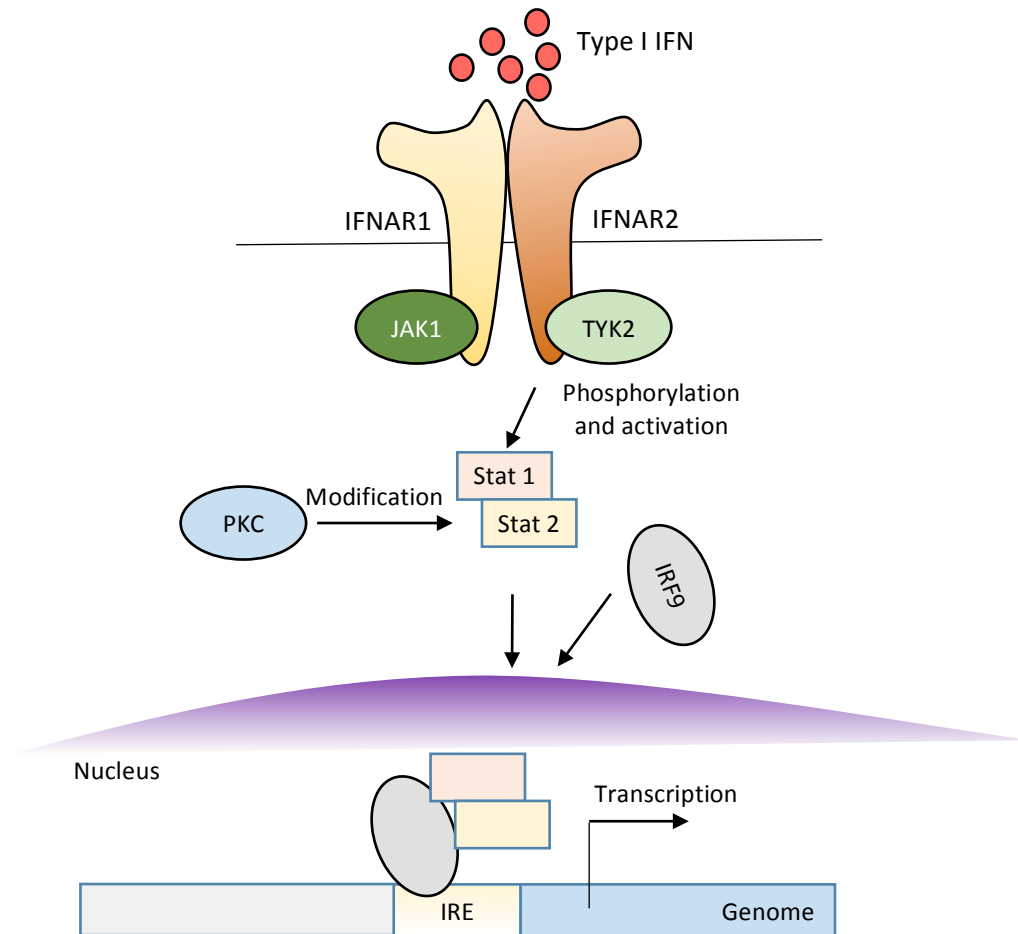


Figure 1-3. Type I Interferon signaling. Schematic representation of the main transcriptional complex induced by type I signaling. Type I IFN binds its receptor, which is a heterodimer consisting of the IFNAR1 and IFNAR2 chains. This activates two kinases (JAK1 and TYK2) associated with the receptor to phosphorylate the signal transducers STAT1 and STAT2, which bind each other. The STAT1 and STAT2 heterodimer subsequently binds IRF9 to form the transcriptionally active ISGF3 complex. ISGF3 is not fully active on its own, and requires additional phosphorylation from the protein kinase C (PKC) pathway. ISGF3 translocates to the nucleus and binds interferon responsive elements (IRE) within the genome, and recruits other enhancers and chromatin modulation complexes (not shown) to initiate interferon-induced transcriptional programs.

CHAPTER II: MATERIAL AND METHODS

Mice

C57BL/6 mice, TRIF-deficient, IFN α R1^{-/-} and C57BL/6 congenic CD45.1 mice (stock numbers, 000664, 005037, 032045 and 002014 respectively) were purchased from Jackson laboratory (Bar Harbor, ME). TEa CD4 TCR transgenic RAG^{-/-} mice specific to E α 52-68 peptide [259, 260] and TCR $\beta\delta$ ^{-/-} mice [261] were bred at Jax and in our laboratory. TEa Thy 1.1 mice were made by crossing TEa CD4 TCR transgenic mice with Thy 1.1 mice on C57BL/6 background from Jackson laboratory (stock number, 000406). All mice were maintained in the animal facility at the University of Connecticut Health Center under specific pathogen-free conditions and handled in accordance to National Institutes of Health federal guidelines.

Immunization and Tissue Processing

For IFN α R1 blockade experiments, $\sim 1 \times 10^5$ CD4 TEa cells isolated from pLN and spleen of TEa mice were i.v. transferred into C57BL/6 recipient mice. The next day mice were i.p. injected with 1mg/ml of anti-IFN α R1 mAb (MAR1-5A3 [262], BioXcell, West Lebanon, NH) or IgG control (I5381, Sigma-Aldrich, St. Louis, MO) and 4 h later immunized i.p with 100 μ g E α peptide (Invitrogen, Grand Island, NY or Genescript, Piscataway, NJ), 30 μ g LPS (*Salmonella typhimurium*, Sigma-Aldrich, St. Louis, MO) and 7 μ g of anti-CD134 mAb (clone OX86, BioXcell) as previously conducted [11, 19]. For simultaneous IFN- γ and IFN α R1 blockade experiments, mice that received TEa cell transfer as before were treated the next day with either anti-IFN- γ mAb (0.5 mg), anti-IFN α R1 mAb (1 mg), anti-IFN- γ +anti-IFN α R1 mAb (0.5 mg+1 mg) or mouse IgG+rat IgG control (0.5 mg+1 mg) Ab followed by immunization. For

CXCL9 blockade experiments, TEa cells were transferred into C57BL/6 recipients, as above following which they were immunized i.p. together with 100 µg of anti-CXCL9 mAb [195] or hamster IgG (0121-14, Southern Biotech, Birmingham, AL), and 8 h later a second treatment with anti-CXCL9 mAb or control was given. For the adoptive transfer competition experiment, CD4 T cells were isolated by negative isolation (EasySep™ Mouse CD4⁺ T Cell Isolation Kit, StemCell Technologies, Vancouver, Canada) from WT (CD45.1⁺) and IFNαR^{-/-} (CD45.2⁺) mice (both on C57BL/6J genetic background). The cells were mixed in a 1:1 ratio and 0.6x10⁵ cells of WT and IFNαR^{-/-} each were transferred into TCRβδ^{-/-} recipients, following which the mice were i.p immunized next day with 1 µg *S. aureus* enterotoxin A (SEA) (Toxin Tech, Sarasota, FL), 30 µg LPS and 25 µg anti-CD134 mAb similar to previous studies [19]. To study the memory response to Ag+LPS+anti-CD134 combination, ~1 x10⁵ CD4 TEa or Thy 1.1⁺ TEa cells were transferred into C57BL/6 mice, followed by the treatment with anti-IFNαR1 mAb or control and by immunization with Eα peptide, LPS and anti-CD134 mAb. Around day 28 to 31 mice were re-challenged with Eα peptide and LPS, following which on day 5 or 7, mice were sacrificed and TEa or Thy 1.1⁺ TEa cells from pLN and liver were analyzed.

To study the impact of poly I:C and CD134 in specific T cell expansion and cytokine differentiation, ~1x10⁶ of Thy 1.1⁺ TEa cells obtained from the pLN and spleen were transferred to C57BL/6 mice, followed by treatment with anti-IFNαR1 mAb or control and immunization with Eα+poly I:C, Eα+anti-CD134 or Eα+poly I:C+anti-CD134 (100 µg of Eα peptide, 40 µg of poly I:C (Sigma-Aldrich, P0913) and 7 µg of anti-CD134 mAb)). Whereas to study the memory response, mice were treated as above and around day 30 or 31 they were re-challenged with Eα and poly:IC, following which on day 5 or 7, they were sacrificed

and Thy 1.1⁺ TEa cells from spleen and liver were analyzed. To study if CD134 costimulation works together with IFN α R1 signaling to promote Ag-specific CD4 T cell response, $\sim 1 \times 10^6$ WT (Thy 1.1⁺ 1.2⁺ TEa) or CD134^{-/-} (Thy 1.1⁺ TEa) cells were separately transferred into C57BL/6 mice, followed by their treatment with anti-IFN α R1 mAb or control and immunization with E α +anti-CD134. On day 7 or 8 following immunization mice were sacrificed and WT and CD134^{-/-} Thy 1.1⁺ cells from spleen and liver were analyzed.

Lymphocytes obtained from pooled peripheral lymph nodes, pLN (inguinal, axillary, and brachial), spleen, liver and lung were isolated similar to our previous approaches [20]. Briefly, pLN and spleen cells were separately crushed through 100 mm nylon mesh strainer (BD Falcon, Franklin Lakes, NJ), and then spleen cells were further treated with 150 mM ammonium chloride to lyse red blood cells. For liver and lung cells, mice were first perfused with PBS containing heparin (Sigma-Aldrich), then the liver was minced through cell strainers and cells partitioned on a 35% Percoll (GE Health Care, Piscataway, NJ, USA) gradient. The lungs were cut into small pieces and incubated in 1.3 mM EDTA in basic salt solution (BSS) without Mg²⁺ and Ca²⁺ at 37°C for 30 min with agitation, followed by collagenase (Sigma-Aldrich) treatment at 37°C for 1 h with agitation. Lung cells were fractionated on a 44% and 67% Percoll gradient (GE Health Care) with lymphocytes partitioning at the interface.

Lymphocytes from all the tissues were suspended in BSS (balanced salt solution supplemented with HEPES, L-glutamine, penicillin, streptomycin, and gentamicin sulfate). For serum isolation, a few drops of blood was collected from the tail vein 3 h after immunization and added to the microtainer tube (BD Life Sciences, Franklin Lakes, NJ) with clot activator and serum gel separator. After 30 to 40 min at room temperature the tube was

spun at 8000 x g for 1.5 min and serum was stored at -80°C until analysis.

Flow cytometry

For surface staining ~ 0.5 to 1×10^6 cells were suspended in a staining buffer (BSS, 3% fetal bovine serum and 0.1% sodium azide) and nonspecific antibody binding was mitigated with Fc block (mouse serum (Sigma), human IgG (Sigma), and anti-mouse Fc mAb 2.4G2 [263] as we have routinely performed [11, 264]. Cells were stained on ice for 30 min in dark and then washed and suspended in the staining buffer or fixed with 2% paraformaldehyde in staining buffer for analysis the next day. For intracellular cytokine detection, $\sim 1 \times 10^6$ cells were restimulated *in vitro* with 5 μ g E α peptide or 50ng/ml phorbol 12-myristate 13-acetate (PMA) (Calbiochem) plus ionomycin (1 μ g/ml) (Invitrogen) at 37°C and 5% CO₂ in 0.2ml of complete tumor medium (CTM) (minimal essential medium (MEM) supplemented with 10% fetal bovine serum, dextrose, salts, amino acids, and antibiotics). After stimulation the cells were washed with staining buffer and stained for CD4, V α 2, V β 6. Cells were then fixed with 2% paraformaldehyde, permeabilized with 0.25% saponin (Sigma) and stained with anti-IFN- γ and anti-TNF in permeabilization buffer. For p-STAT1 intracellular staining, splenocytes were first treated with IgG or anti-IFN α R1 mAb (2.5 μ g) for 30 minutes, following which recombinant type I IFN (2000 U/ml) was added to the cells for 45 minutes. Cells were fixed with 1.5% paraformaldehyde, following which they were permeabilized with icecold methanol for 25 minutes (after this cells could be stored at -20° C and stained at a later time point). Before staining for p-STAT1 cells were washed with cold PBS to remove the methanol, and then stained for with CD4, V α 2, V β 6 and anti-p-STAT1 Abs in PBS containing, 0.5% BSA and 0.01% Na azide (phosphate buffer) for 30 minutes at room temperature. Following this, cells

were washed with phosphate buffer and samples were run on BD LSR II and data was analyzed using FlowJo 10.2 software (Tree Star, Ashland, OR). The mAbs were purchased from BD Pharmingen (CD3 AF700, V β 6 APC, V β 3 PE, CD45.1 FITC, TNF AF700), ebioscience (V α 2 FITC, V α 2 PEcy7, V β 6 PE, CD45.2 APC, Armenian Hamster IgG APC or PE, IFN- γ PerCpCy5.5 or APC), Biolegend (CXCR3 APC or PE), BD biosciences (CD4 V450, p-STAT1 AF647), Invitrogen (Live dead UV Blue) or Tonbo Biosciences (Ghost Dye Red 780).

In addition to above experiments we performed control experiments, where we analyzed the TEa cells in C57BL/6 mice at basal level without TEa cell transfer or immunization. Here, the average of percent TEa cells in spleen and liver of mice on day 0, were 1.19 ± 0.34 and 0.53 ± 0.10 respectively, whereas the average of total number of TEa cells in spleen and liver were 0.016 ± 0.005 and 0.001 ± 0.0003 ($\times 10^6$) respectively. In our second control, we analyzed TEa expansion in C57BL/6 mice that received TEa transfer as before, but no immunization. On day 5, the average percent TEa cells in spleen and liver of mice were 0.98 ± 0.04 and 0.54 ± 0.05 respectively, whereas the average of total number of TEa cells in spleen and liver were 0.015 ± 0.003 and $0.001 \pm 7.057^{e-005}$ ($\times 10^6$) respectively. Data in both these experiments is derived from 1 experiment, n = 3 mice represented as Mean \pm SEM. Overall this shows that without TEa transfer and immunization, TEa cells in lymphoid (spleen) and non-lymphoid tissue (liver) are very low in numbers and for optimal expansion, mice need to receive both TEa transfer and immunization.

ELISA, SDS-PAGE and Immunoblotting

Mouse IFN- β ELISA kit was from PBL assay science (Piscataway, NJ), mouse CXCL9/MIG

DuoSet kit (DY492) or CXCL10/IP-10/CRG-2 DuoSet ELISA kit (DY466) was from R&D Systems (Minneapolis, MN). All ELISA's were performed according to the manufacturer's instructions, and absorbance was determined at 450 nm using a Bio-Rad iMark (Bio-Rad, Hercules, CA) or Clariostar microplate reader (BMG Labtech, Cary, NC). The concentrations were calculated using a standard curve line of best fit on Microplate Manager Software (Bio-Rad) or MARS data analysis software (BMG Labtech). Briefly, for immunoblotting ~40 µg protein of liver tissue lysate suspended in denaturing SDS sample buffer were resolved on 4–15% SDS PAGE, transferred onto 0.2 µm nitrocellulose membrane using a semi-dry blotting device (Bio-Rad) and finally probed with anti-CXCL9 antibody (R&D Systems, AF-492-NA). The membrane was washed, incubated with secondary HRP-conjugated rabbit anti-goat antibody (Santa Cruz Biotechnology, Dallas, TX), washed and then band detection was performed using ECL plus (GE Healthcare Life Sciences). Recombinant CXCL9 (R&D Systems, 492-MM) was used as a positive control and anti-β-actin was used as the loading control (Abcam, Cambridge, MA).

Real Time-PCR

On day 5 the liver tissue was harvested and RNA was extracted from roughly 30 mg of liver tissue using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA was reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad) and a Real-Time quantitative PCR measurement of cDNA was then performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and a CFX96 real-time PCR instrument (Bio-Rad). For analysis, gene expression for both anti-IFNαR1 Ab and IgG treated group were normalized to β-actin and the fold change was calculated by subtracting the gene expression of IgG treated

group from anti-IFN α R1 group. PCR primers were generated by inputting the NCBI accession number for each gene in the primer bank website (https://pga.mgh.harvard.edu/cgi-bin/primerbank/new_search2.cgi) to get a validated forward and reverse primer sequence that was then purchased from Integrated DNA technologies, IDT (Coralville, Iowa) (Table 1).

Transwell Assay

Briefly, 24-well plate containing transwell inserts (6.5 mm diameter, 5 μ m pores; Corning, Lowell, MA) were coated with 50 μ l of 10 ng/ml murine fibronectin (Innovative research, Novi, MI) and incubated for 1 h at 37°C and 5% CO₂. The solution was removed and inserts were dried for 2 h at 37°C (without CO₂). The pLN lymphocytes were harvested from immunized mice on days 5 or 11 and suspended in CTM (without the FBS) supplemented with 0.5 % BSA. The lower chamber of the transwell was filled with 600 μ l of 1000 ng/ml of CXCL9, CXCL10 or CXCL1 in CTM (Peprotech, Rocky Hill, NJ). The lymphocyte suspension (~0.5 to 1x10⁶ cells) was then added to top of the insert and allowed to migrate for 30 min at 37°C and 5% CO₂. After migration the cells on the top and bottom of the insert were collected, washed with the staining buffer and labeled with fluorescently labeled antibodies to analyze the percent TEa cells by flow. The data is represented as ratio of percent CD4 TEa cells in the bottom of the well to percent CD4 TEa cells on top of the well.

Statistical analysis

The data between groups were compared by unpaired, two-tailed t-test by using GraphPad Prism (GraphPad Software, San Diego, CA, USA) where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

CHAPTER III:

**Optimal CD4 T cell priming after LPS-based adjuvanticity with CD134 costimulation
relies on CXCL9 production**

Abstract

Lipopolysaccharide is a powerful adjuvant, and while LPS mediated TLR4 signaling has been exquisitely delineated, the *in vivo* mechanism of how TLR4 responses impact T cell priming is far less clear. Besides costimulation, TNF and type 1 IFN are dominant cytokines released after TLR4 activation and can shape T cell responses but other downstream factors have not been extensively examined. Depending upon context, we show that IFN α R1 blockade resulted in minor to major effects on specific CD4 T cell clonal expansion and Th1 differentiation. To help explain these differences, it was hypothesized that IFN α R1 blockade would inhibit specific T cell migration by reducing chemokine receptor signaling, but specific CD4 T cells from IFN α R1 blocked mice were readily able to migrate in response to specific chemokines. Next, we examined downstream factors and found that type 1 IFN signaling was necessary for chemokine production when mice were immunized with specific Ag with LPS and CD134 costimulation. IFN α R signaling promoted CXCL9 and CXCL10 synthesis suggesting that these chemokines might be involved in the LPS and CD134 costimulation response. After immunization we show that CXCL9 blockade inhibited CD4 T cell accumulation in liver but also in lymph node even in the presence of elevated serum IFN- β levels. Thus, while type 1 IFN might have direct effects on primed CD4 T cells, the downstream chemokines that play a role during migration also impact T cell clonal expansion. In sum, CXCL9 production is a key benchmark for productive CD4 T cell vaccination strategies.

Introduction

Adjuvanted vaccines have promoted worldwide health through immunization programs [265]. One of the key ingredients in an adjuvanted vaccine is the adjuvant itself [266]. Typically adjuvants are ligands for pattern recognition receptors that stimulate appropriate levels of inflammation to activate antigen presenting cells, which prime and costimulate specific CD4⁺ T cells to help B cells synthesize specific antibodies or stimulate cytotoxic T cells to kill infected cells. Not surprisingly, most data from model systems show that immunization without an adjuvant fails to promote these characteristics of durable humoral and cellular immunity [267, 268]. Thus, a major area of investigation is centered on understanding the immune signals that emanate from an adjuvant or costimulatory responses that stimulate and/or sustain specific T cell longevity [269-271].

Many adjuvants are ligands for TLR and perhaps the most notable example is the TLR4 ligand bacterial LPS of which its derivative MPL in combination with alum is FDA approved in an adjuvanted HPV vaccine [272-274]. Seminal work has shown that TLR4 signaling relies on the MyD88 and TRIF adaptors [34], and the use of MPL was at least partially based on its tendency to signal through TRIF over MyD88 [4, 13]. In our model we used LPS since it promotes lasting memory CD4 T cell responses over that with MPL [275]. Important work is investigating less toxic variants of LPS manufactured by chemical degradation, synthetic assembly or genetically modification for their potential use as vaccine adjuvants [276], but a fundamental understanding of LPS-based CD4 T cell responses is critical for vaccine development as an approach to identify important pathways for T cell survival that might be characteristic of newly developed adjuvants.

Ultimately, TLR4 activation on antigen presenting cells, similar to the other TLRs, increases the expression of T cell costimulatory ligands [277], cell maturation [278], and also triggers release of a complex cytokine network [279]. Specifically, LPS adjuvanticity requires dendritic cells for optimal T cell priming [11], and the TLR4 adaptors largely play non-overlapping roles in the context of providing cues for T cell differentiation and survivability [280, 281]. The outcome of TLR4 stimulation or the use of LPS during immunization impacts humoral immunity [282], prevents peripheral T cell tolerance and promotes long-term T cell memory as found in different tissues of the body [10, 46, 283, 284]. Perhaps most striking is the addition of costimulation, which potentially increases the effectiveness of T cell priming. One example is CD134 (OX40) costimulation supplied in the form of enforced costimulation with an agonist mAb [19], and others such as CD40 and CD137 (4-1BB) also have similar effects [200, 285, 286]. Enforced costimulation is already in use for cancer immunotherapy validating its importance and under the right circumstance could be used to stimulate vaccine development for infectious disease or further improve cancer immunotherapy when combined with TLR ligands [18, 276, 287, 288]. Thus, triggering TLR together with enforced costimulation provides a powerful combination to prime T cell immunity, and the mechanism behind it is not only unclear but also vitally important to understand, so that the pathways leading to adverse events can be separated from the beneficial ones.

A major response to different adjuvant and enforced costimulation platforms are similar cytokine outputs. As an example, IL-6 and IL-1 β are typically produced and these factors regulate T cell responses but this commonality largely precludes them from explaining the differences in adjuvant responses. Therefore, probing downstream from the initial cytokine release to find factors that might be different between responses, and perhaps exert

specialized effects on T cell function, is critical to advance vaccine development. Our goal was to study this idea by examining type 1 IFN, which is not as widely produced as compared to TNF, but has been previously implicated in specific adjuvant responses [139, 289, 290]. Thus, we set out to determine if the potent LPS and anti-CD134 stimulation protocol relied on type 1 IFN or perhaps the downstream mediators in this complex cytokine network.

By tracing T cell and cytokine responses we found that specific antigen and low levels of LPS with anti-CD134 immunization exerted a strong survival signal on specific CD4 T cells in LN, liver and lung, while type 1 IFN release was rapid and robust. The role of type 1 IFN was context dependent, but nevertheless T cells without IFN α R were potentially handicapped when competing against WT T cells. Our data points to the downstream chemokines that play a role in maintaining specific T cell accumulation and suggests that testing for a precise cytokine and chemokine network might be an important benchmark for the effectiveness of immunization strategies and immunotherapy.

Results

Antigen, LPS and CD134 costimulation synergize to promote specific T cell clonal expansion and type 1 IFN production

The adjuvant LPS in combination with CD134 costimulation promotes robust CD4 and CD8 T cell expansion in response to *S. aureus* enterotoxin [200], as well CD4 T cell memory formation using a model Ag in place of enterotoxin [291]. To study this mechanism we transferred CD4 TCR transgenic T cells specific for E α 52-68 peptide (TEa) into C57BL/6 recipient mice and, similar to an immunotherapy strategy, immunized i.p. the next day (day 0) with LPS, anti-CD134 (agonist mAb, OX86) and E α 52-68 peptide followed by specific T cell detection (TCR V α 2 V β 6) on day 11 or 12 (Fig. 3-1A). By day 11 or 12 synergistic CD4 T cell clonal expansion and survival was detected in the liver, which was highest with the combination of peptide with LPS plus CD134 in comparison to peptide plus CD134 costimulation, or LPS (Fig. 3-1B). Since LPS induces type 1 IFN secretion [292], we tested if LPS and CD134 together increased type 1 IFN levels compared to either alone with peptide stimulation. Three hours after immunization, serum was collected and examined for IFN- β levels by ELISA (Fig. 3-1C). As predicted CD134 costimulation with LPS induced the highest IFN- β levels, suggesting that IFN- β may play a role in supporting the T cell response in our vaccine model.

In previous work, LPS was shown to promote effector CD8 and CD4 T cell accumulation in non-lymphoid tissues through the TRIF adaptor [20, 264], whereas Lipid-A-induced type 1 IFN signaling through TLR4-TRIF mediated CD4 T cell expansion in spleen [13, 47]. Similar to our approach, biologics are being combined with TLR agonists for immunotherapy of cancer and infectious disease vaccines [271, 276, 293, 294] and it was thus reasoned that a key cytokine

might be type 1 IFN. To determine if type 1 IFN was involved in the LPS and CD134 costimulation platform, the MAR15A3 mAb specific to the IFN α R1 subunit receptor [262] was used to block IFN α R signaling. Although IFN α R signaling is complex there is a substantial role for STAT1 phosphorylation [295], which in our study was inhibited in the presence of IFN α R1 blockade (Fig. 3-2A). Based on these data IFN α R1 was blocked *in vivo* using MAR15A3 mAb during the immunization approach described in Figure 1A. As expected the levels of liver IFN- β , measured by qRT-PCR (Fig. 3-2B, left panel), were decreased but surprisingly, serum IFN- β levels significantly increased in the blocked mice (Fig. 3-2B, right panel). Thus, blocking the type 1 IFN receptor uncouples a positive feedback loop previously described [107] as there is a significant reduction in IFN- β transcription, but IFN- β protein levels increase systemically due to blocked receptor mediated internalization. Hence, these data supports the notion that IFN α R1 blockage is effective and might play a role in the LPS plus anti-CD134 immunization approach.

To test this idea two groups of mice were immunized with LPS, anti-CD134 plus specific peptide; one group received Control IgG and the other anti-IFN α R1 mAb. On day 6 after immunization the peripheral LNs (pLN) were examined and showed a significant reduction in expansion, or survival, of the specific CD4 T cells when IFN α R1 was blocked (Fig. 3-2C, upper panels). This was the case for frequency and cell number, but in liver there was only a trend largely due to an outlier (Fig. 3-2C, lower panels). To test if host cells required IFN α R1 signaling, IFN α R1^{-/-} recipients were used in place of C57BL/6 wild type (WT) recipients. In this case, there was a significant reduction in percent of Ag-specific CD4 T cells in liver of IFN α R1^{-/-} mice on day 5 after immunization, but no difference was found in the pLN and lung (Fig. 3-2D). However, the total number of TEa cells were significantly reduced in both liver and lung of

IFN α R^{-/-} mice and reduced to almost 50 percent in the pLN (Table 2). To further understand how IFN α R signaling in specific-CD4 T cells impacts T cell expansion, we set up an experiment where WT and IFN α R^{-/-} specific CD4 T cells competed against each other for expansion in the same host. To increase the breadth and robustness of the T cell priming, *S. aureus* enterotoxin was used to model an oligoclonal response rather than a single TCR specific system. *S. aureus* enterotoxin A (SEA) potentially stimulates TCR V β 3 expressing CD4 T cells [43] and is regarded as a potential cancer therapeutic [296-298]. Thus, WT CD45.1⁺ mixed with IFN α R1^{-/-} CD4 T cells were transferred into TCR $\beta\delta$ ^{-/-} mice, which prevented competition from endogenous T cells, and then i.p. immunized with SEA, LPS and anti-CD134 (Fig. 3-3A). On days 7 or 8 after immunization, the IFN α R1^{-/-} cells were completely outcompeted by the WT V β 3 T cells (Fig. 3-3B). This was the case for pLN and liver and the same was also true for the V β 3 T cells, 15 days after immunization (Fig. 3-3C). Lastly, the frequency data mirrored total cell numbers (Table 3). The non-specific WT V β 14 T cells were unaffected in pLN, but they increased in the liver on day 15 significantly in comparison to IFN α R1^{-/-} T cells likely due to organ inflammation (data not shown. This is similar to other systems where unrelated T cells report to inflamed sites [299]. Thus, specific CD4 T cells deficient in IFN α R signaling were profoundly handicapped when competing against WT cells. Nevertheless, this dramatic dependency on IFN α R1 was smaller when the host was IFN α R1 deficient (Fig. 3-2D), or in the presence of IFN α R1 blockade by therapy (Fig. 3-2C). Overall these data suggests that, IFN α R1 signaling in both specific CD4 T cells (Fig 3-3) as well non-specific cells (Fig. 3-2D) could enhance specific T cell expansion. Further studies comparing the how IFN α R signaling in specific CD4 T cells versus non-specific cells of the host, impact T

cell expansion are needed, but clearly anti-IFN α R1 therapy could be used to suppress CD4 T cell responses.

We next assessed if IFN α R signaling can influence the effector function of specific CD4 T cells. For this we analyzed, intracellular levels of IFN- γ in lymphocytes isolated from the experiment done in Fig. 2-2C and Fig. 2-2D. As shown in Fig. 3-4A, left panel treatment with anti-IFN α R1 mAb reduced the percentage and total number of IFN- γ expressing Ag-specific (V α 2⁺ V β 6⁺ TEa) CD4 T cells in pLN after E α peptide stimulation in comparison to the control treated mice. Similar, but less significant impact was also seen in percentage of IFN- γ expressing non-specific (V α 2⁻ V β 6⁻ CD4) T cells after PMA+Ionomycin stimulation (Fig. 3-4A, right panel). However, the total number of IFN- γ expressing non-specific cells were significantly reduced in pLN with anti-IFN α R1 treatment. In liver and lung, IFN- γ production from specific or non-specific CD4 T cells was not much impacted with IFN α R1 blockade (data not shown). In comparison to IFN α R1 Ab blockade, analysis of cytokine expression from IFN α R^{-/-} mice from experiment done in Fig 2-2D, showed that the percentage of IFN- γ expressing specific CD4 T cells were significantly reduced in liver after E α peptide stimulation in comparison to WT mice (Fig. 3-4B, left upper panel). Similarly, the total number of IFN- γ expressing specific CD4 T cells were also significantly reduced in liver of IFN α R1^{-/-} mice (not shown). The percentage (Fig. 3-4B, left middle panel) and total number of IFN- γ expressing specific CD4 T cells (not shown) in pLN of IFN α R^{-/-} mice also trended towards reduction but did not reach significance. In comparison to pLN and liver, the percent (Fig. 3-4B, left lower panel) and total number of IFN- γ expressing specific CD4 T cells (data not shown) in lung of IFN α R1^{-/-} mice were not impacted. However, the percentage of IFN- γ expressing non-specific (V α 2⁻ V β 6⁻ CD4) T cells

were significantly reduced in pLN and lung after PMA+Ionomycin stimulation and the percent of IFN- γ ⁺ non-specific CD4 T cells also trended towards reduction in the liver (Fig. 3-4, right panels). The total number of IFN- γ expressing non-specific CD4 T cells were significantly reduced in pLN and lung of IFN α R^{-/-} mice after PMA+Ionomycin stimulation, whereas in liver, they were not significantly impacted (data not shown). Thus overall, the results from Fig. 3-3, indicate that IFN α R signaling in specific CD4 T cells is required to promote their expansion in pLN and liver, whereas results from Fig. 3-4 suggest that, IFN α R signaling in non-specific cells of host, such as APCs may impact Th1 differentiation in specific as well as non-specific CD4 T cells.

Downstream mediators of type 1 IFN and their function on effector CD4 T cells

The data above suggests that type 1 IFN blockade might inhibit a downstream pathway in CD4 T cells during this immunization approach. In fact, previous data from our lab showed that TRIF signaling in host cells enhances CXCR3 expression on specific CD4 T cells to presumably allow their migration to the peripheral tissue of liver [20].

To understand how IFN α R signaling impacts T cell accumulation, we examined if it could enhance the migration of CXCR3⁺ specific-CD4 T cells in pLN and liver. For this we analyzed CXCR3 expression on T cells from experiments done in Fig. 3-2C. We saw that, IFN α R signaling promoted the accumulation of CXCR3⁺ Ag-specific (V α 2⁺ V β 6⁺) as well as non specific (V α 2⁻ V β 6⁻) CD4 T cells in pLN (Fig. 3-5A, upper panel) and anti-IFN α R1 treatment reduced the percent of CXCR3⁺ specific CD4 T cells marginally, however it significantly reduced the percent of CXCR3⁺ non-specific CD4 T cells. Importantly, the total number of CXCR3⁺ specific and non-specific CD4 T cells were significantly reduced in pLN after anti-

IFN α R1 treatment (Fig 3-5A, lower panel). This suggests that IFN α R signaling may promote migration of CXCR3⁺ specific as well as non-specific CD4 T cells in pLN, however, our data in Fig. 3-2C shows that IFN α R1 blockade reduced the accumulation of specific CD4 T cells, but had no impact on non-specific CD4 T cells (data not shown). Moreover, IFN α R1 blockade did not influence the percent and total number of CXCR3⁺ specific or non-specific CD4 T cells in liver, suggesting that CXCR3 expression cannot not explain how IFN α R signaling promotes expansion or T cell accumulation to pLN and the liver.

We nevertheless sought to determine if the CXCR3 receptor on Ag-specific T cells was functional during our vaccine immunization. To study this, day 6 pLN cells from immunized mice were placed in the upper chamber of a transwell and various chemokines in the lower, and after 30 minutes we determined the percent of specific TEa CD4 T cell that migrated into the lower well (Fig. 3-6A). Based on a ratio of TEa CD4 T cell percentage in the bottom over the percent from the top well, it was clear that specific migration was minimal in media alone or with irrelevant chemokine CXCL1 (Fig. 3-6B). However, in response to CXCL9 or CXCL10 the ratios were about 3 whether or not the cells were from IFN α R1 blocked mice. Very similar data were generated using Day 11 pLN cells (Fig. 3-6C) suggesting that CXCR3 was functional and certainly the response to CXCL9 and CXCL10 was intact in the presence of IFN α R1 blockade. Since IFN- β driven chemokine receptor function was not impaired in our migration assay (Fig. 3-6), we hypothesized that IFN α R1 blockade inhibited chemokine production rather than receptor expression. Using qRT-PCR for CXCL9 and CXCL10 it is clearly shown that liver mRNA expression for both chemokines was significantly inhibited on days 2 and 5 after IFN α R1 blockade (Fig. 3-7A). CXCL11 is also a ligand for CXCR3, but C57BL/6 mice do not possess a functional CXCL11 gene [169, 170]. To further test this idea, CXCL9 protein levels

were determined by immunoblot using day 5 liver lysates and similar to the PCR data there was a consistent reduction in the CXCL9 protein levels after IFN α R1 blockade (Fig. 3-7B). Finally, by measuring serum CXCL9 and CXCL10 levels from the experiment in Fig. 3-2D, we showed a significant reduction in the IFN α R1^{-/-} mice, but importantly an increase in IFN- β (Fig. 6C). Secondly, we also found significantly reduced serum CXCL9 in immunized TRIF-deficient mice (Fig. 3-7D, left panel) further demonstrating the positive feed-forward loop of IFN- β driving CXCL9. Lastly, we assayed the remaining serum samples from Fig. 3-1C and found that LPS, but not CD134 costimulation, was critical for CXCL9 production when TEa CD4 T cells were activated with the cognate peptide (Fig. 3-7D, right panel).

In sum, these data suggest that the adjuvant response mediated by TLR ligands might provoke IFN- β to increase CXCL9 and CXCL10 synthesis thereby promoting adjuvanted T cell responses.

CXCL9 dominates over IFN- β to enhance specific T cell accumulation

To test the hypothesis that CXCL9 and/or CXCL10 play a role in adjuvanting T cell responses, CD4 TEa recipient mice were immunized with Ea peptide plus LPS and CD134 costimulation in the presence of CXCL9 and CXCL10 blockade. While the effects with CXCL10 blockade were trending perhaps due to incomplete inhibition (data not shown), there was nevertheless a statistically significant inhibition of specific CD4 T cell accumulation with CXCL9 blockade (Fig. 3-8A). Both the peripheral LN and the liver contained proportionally fewer specific CD4 T cells, and the lung was less impacted but certainly trended towards significant inhibition. Moreover the absolute number of TEa cells, were also significantly reduced in pLN after CXCL9 blockade (Table 4).

To understand how CXCL9 promotes T cell accumulation, we analyzed if CXCL9 enhances the specific CD4 T cell migration by recruiting CXCR3 expressing specific T cells. For this we analyzed the CXCR3 expression on specific CD4 T cells from experiments done in Fig. 3-8A and as shown in Fig. 3-8B, CXCL9 blockade significantly reduced the percent and total number of CXCR3⁺ specific CD4 T cells in pLN compared to the control treated mice. This suggests that CXCL9 may enhance specific CD4 T cell accumulation in pLN by promoting the migration of CXCR3⁺ specific T cells. However, if CXCL9 enhanced the migration of specific T cells to pLN, it would result in reduced T cell numbers to liver and lung tissues, but that was not the case (Fig. 3-8A). Moreover, CXCL9 did not impact percent or total number of CXCR3 expressing specific CD4 T cells in liver and lung tissue (data not shown) further indicating that mechanisms other than T cell migration may be responsible for CXCL9 mediated T cell accumulation.

We next analyzed if CXCL9 impacts T cell accumulation, by promoting T cell proliferation. To test this we analyzed Ki67 expression (a nuclear Ag used as a marker of cell proliferation) in specific T cells from experiments done in Fig. 3-8A. Our results indicate that CXCL9 does not influence the percent or total number of Ki67 expressing specific-CD4 T cells in pLN, liver or lung (data not shown). Thus, CXCL9 induced T cell accumulation is not a result of increased T cell proliferation. CXCL9 may also influence T cell accumulation by promoting T cell survival or preventing activation-induced cell death (AICD), which we have not analyzed. The mechanism of how CXCL9 promotes specific CD4 T cell accumulation to lymphoid (pLN) and non-lymphoid tissues (liver and lung) is important to understand, if one has to target CXCL9 in T cell vaccination strategies and we plan to address it in our future studies.

Collectively, these data shows that CXCL9 operates in multiple sites in the immune system and increases clonal expansion and accumulation of stimulated specific CD4 T cells. Mechanistically, it is possible that IFN- β induces CXCL9, and CXCL9 feeds back to increase IFN- β resulting in T cell accumulation through IFN α R1, which would be supported by the data in Figures 3-2 and 3-3. Thus, if this postulate were correct CXCL9 blockade should inhibit IFN- β production. As shown at the time point tested during T cell priming, IFN- β was not reduced as CXCL9 was blocked (Fig. 3-8C, left panel). Hence, specific CD4 T cell expansion was significantly inhibited when CXCL9 was blocked even when IFN- β was available. Lastly, CXCL9 blockade actually increased serum CXCL9 as measured by ELISA (Fig. 3-8C, right panel), which might be a function of antibody protection of CXCL9.

In sum, CXCL9 blockade significantly impaired the specific CD4 T cells from accumulating in lymphoid and non-lymphoid tissues demonstrating a role, not only during migration, but also in assisting clonal expansion or accumulation.

Discussion

LPS triggering of TLR4 is known to amplify antigen-specific CD4 and CD8 T cell responses [10], which is potentially enhanced by enforcing costimulation [19]. Although roles for pro-inflammatory cytokines have been suggested [11, 300-302], a mechanistic pathway leading to specific T cell survival remains unknown [303]. Here, we tested a role for type 1 IFN in a powerful vaccine and immunotherapy platform consisting of anti-CD134 agonist mAb and low levels of LPS mixed with specific antigenic peptide. Depending upon the context, Type 1 IFN was important, but mainly because it induced production and release of CXCL9 to support specific CD4 T cell accumulation in lymph node and liver after immunization. Regardless of the IFN- β levels, the optimal adjuvant effect of LPS depended upon CXCL9 even with added costimulation. It is proposed that CXCL9 production is an important benchmark for vaccine efficacy and perhaps immunotherapy.

In our immunization platform the TLR4 ligand LPS with CD134 costimulation induced type 1 IFN, but without LPS/CD134 activation the specific T cells did not survive which correlated with low levels of type 1 IFN and CXCL9 (Figs. 3-1 and 3-7). Related to our work a recent study demonstrated the importance of type 1 IFN signaling in lipid A induced T cell clonal expansion wherein providing exogenous IFN- β rescued diminished splenic Ag-specific T cell expansion in TRIF-deficient mice [47]. Similar to this study, previous results from our lab demonstrated that CD40 activation was capable of rescuing liver CD8 T cells in TRIF-deficient mice after LPS adjuvanticity [20]. Thus, we postulated that bypassing IFN α R signaling during immunization would impact T cell expansion but it was unclear if this would depend upon specific T cells or host cells such as APCs expressing IFN α R.

To study the mechanism of how type 1 IFN impacts T cell expansion we used several approaches. Analogous to a therapeutic setting, a blocking mAb resulted in a significant reduction in percent and number of specific pLN CD4 T cells, while a decrease was trending in the liver (Fig. 3-2C). In contrast, WT specific CD4 T cells in IFN α R1^{-/-} mice displayed a significant reduction in percent specific-CD4 T cells in liver, but no difference was seen in pLN and lung (Fig. 3-2D). However, the total number of specific CD4 T cells were significantly reduced in liver, lung and reduced to almost 50 percent in pLN of IFN α R1^{-/-} mice (Table 2). Thus, IFN α R signaling in both specific T cells as well as non-specific cells of the host could contribute to the specific CD4 T cell expansion. However, it is clear that IFN α R signaling in T cell is essential for specific CD4 T cell accumulation to pLN and liver, as seen in the competition model where WT specific T cells were far superior to IFN α R1^{-/-} specific T cells in expansion (Fig. 3-3B/C). One of the caveats here, is WT T cells could seed differently in tissues in comparison to IFN α R^{-/-} T cells resulting in some difference in their expansion. However, since we transferred only 0.6×10^5 cells each of WT and IFN α R1^{-/-} T cells, we may not be able to accurately assess if the cells seeded equivalently on day 0 after transfer. Secondly there is no way of finding if the T cells migrated equivalently to all organs or sites of the body.

We also show that IFN α R signaling impacted Th1 differentiation, where signaling in non-specific cells of the host was essential to promote IFN- γ production in specific CD4 T cells (pLN and liver) as well as non-specific CD4 T cells (pLN and lung) (Fig. 3-4B).

Thus, these three approaches exemplify how context is critical in determining the role of immune pathways. Specifically, the competition study showed an impressive difference, and while not as apparent in the other systems, there was some role for IFN α R during LPS and

CD134 immunization. Ultimately, these data speak to the importance of using multiple systems of neutralization to obtain the range of possibilities that can occur, versus those that occur during therapy. Much has been gained with the use of genetically altered murine models, but neutralization studies with blocking reagents might best approximate what occurs in patients treated with biologics.

To understand a mechanism of how type 1 IFN signaling influences T cell responses we examined several cytokine receptors downstream of TLR and CD134 activation. CXCR3 is induced by stimulation of TLR4 through TRIF signaling and might explain the lack of specific CD4 T cell accumulation in certain organs like liver since CXCR3 is known to mediate T cell migration into liver [161, 196, 304, 305] and recently shown to promote T cell migration across the tumor vasculature [304]. Our data show, that regardless of IFN α R blockade the specific CD4 T cells responded to the chemokines, CXCL9 and CXCL10 in a specific fashion (Fig. 3-6). Thus, faulty CXCR3 driven migration did not explain the basis for type 1 IFN mediated responses, suggesting that CXCR3 remained functional but perhaps its ligands were diminished after IFN α R neutralization. This is important since the ligands of CXCR3 are known to optimize Th1 responses in dLN by interacting with dendritic cells [197]. In our model, CXCL9 promoted T cell accumulation to pLN as well as in liver and lung, however it did not enhance Th1 differentiation (data not shown). Our vaccine model employs CD134 agonist, which as mentioned before, could induce Th1 differentiation [16, 17, 237, 239]; and possibly rescue it after CXCL9 blockade, therefore the results between these studies cannot be directly compared. Chemokine CXCL10 has also been shown to mediate positioning of CXCR3⁺ T cells in LN to assist in CD4 T cell priming during influenza vaccination [264], suggesting that even in a model of vaccine derived antigen CXCR3 ligands facilitate Th1 responses. Thus, it

was hypothesized that T cell function attributed to type 1 IFN in response to LPS and CD134 costimulation might actually occur through CXCR3 stimulation. IFN α R blockade significantly reduced CXCL9/10 transcript levels, and IFN α R^{-/-} hosts also contained significantly reduced CXCL9/10 serum levels. Nevertheless, IFN α R^{-/-} hosts contained high amounts of serum IFN- β (Fig. 3-7C). This suggests that the transferred specific T cells in the IFN α R^{-/-} hosts had ample access to IFN- β while the host APCs did not since they were IFN α R^{-/-} (Fig. 3-2D). Thus, an interesting question arises asking if the reduced T cell response in IFN α R^{-/-} mice was due to minimal levels of CXCL9/10 or because the APCs could not respond to IFN- β . To address this issue anti-CXCL9 mAb was used to block CXCL9 [195] during immunization, which demonstrated that specific CD4 T cell accumulation was significantly inhibited in LN and liver, even though IFN- β serum levels remained similar to an IgG control group (Fig. 3-8C).

On the other hand, when there are high IFN- β levels as seen in IFN α R^{-/-} hosts (Fig. 3-7C), the specific T cells have greater access to IFN- β , which functionally results in an increased dose of the cytokine despite modest amounts of CXCL9. Specifically, a higher than WT level of serum IFN- β might bypass the role of other downstream pathways making the cytokines appear redundant. Thus, high levels of type 1 IFN might promote T cell survival when other survival factors are limiting and IFN- β has been shown to directly induce T cell survival *in vitro* [48]. Similarly, IFN- β stimulates dendritic cells [306], but IFN α R was not required for dendritic cell maturation after LPS treatment, but rather a TLR4 TRIF-based MAP kinase intrinsic signal was critical [307]. Similarly, we previously observed a potent LPS adjuvant effect on LN T cells in TRIF-deficient mice, even though liver responses were impaired [20]. Thus, our platform of low levels of LPS administration can prime dendritic cells void of IFN α R signaling while enforcing CD134 costimulation on specific CD4 T cells provides a tool to enhance CD4 T cell priming.

In conclusion, shows that specific CD4 T cells were impaired in their ability to accumulate in LN or liver during CXCL9 blockade. A lone role in migration would have logically led to an increased number of the specific T cells in LN with reduced numbers in liver, but we observed a reduction in specific T cell numbers in both of the compartments. Thus, it will be important to test if CXCL9 breaks activation-induced cell death directly in T cells perhaps by enhancing interactions with dendritic cells [197]. Nevertheless, a major challenge in this regard will be to detect dead specific T cells *in vivo* that have not bound CXCL9. The other possibility is that CXCL9 enhances cell cycle progression leading to greater proliferation but we found no significant difference in Ki67 staining on day 6, with or without CXCL9 blockade (data not shown). Our study, however does demonstrate that CXCL9 promotes CD4 T cell accumulation substantially adding to the idea that CXCL9 facilitates memory responses by facilitating positioning of CM CD8 T cells with dendritic cells in LN [178, 198].

Therefore, CXCL9 has pleiotropic functions and understanding its cellular source, timing of release and the cells that it targets will add greatly to controlling cellular immunity for biomedical benefit. In particular, CXCL9 release might be especially important in vaccine adjuvant development and also in assessing enforced costimulation protocols for immunotherapy.

Figure 3-1

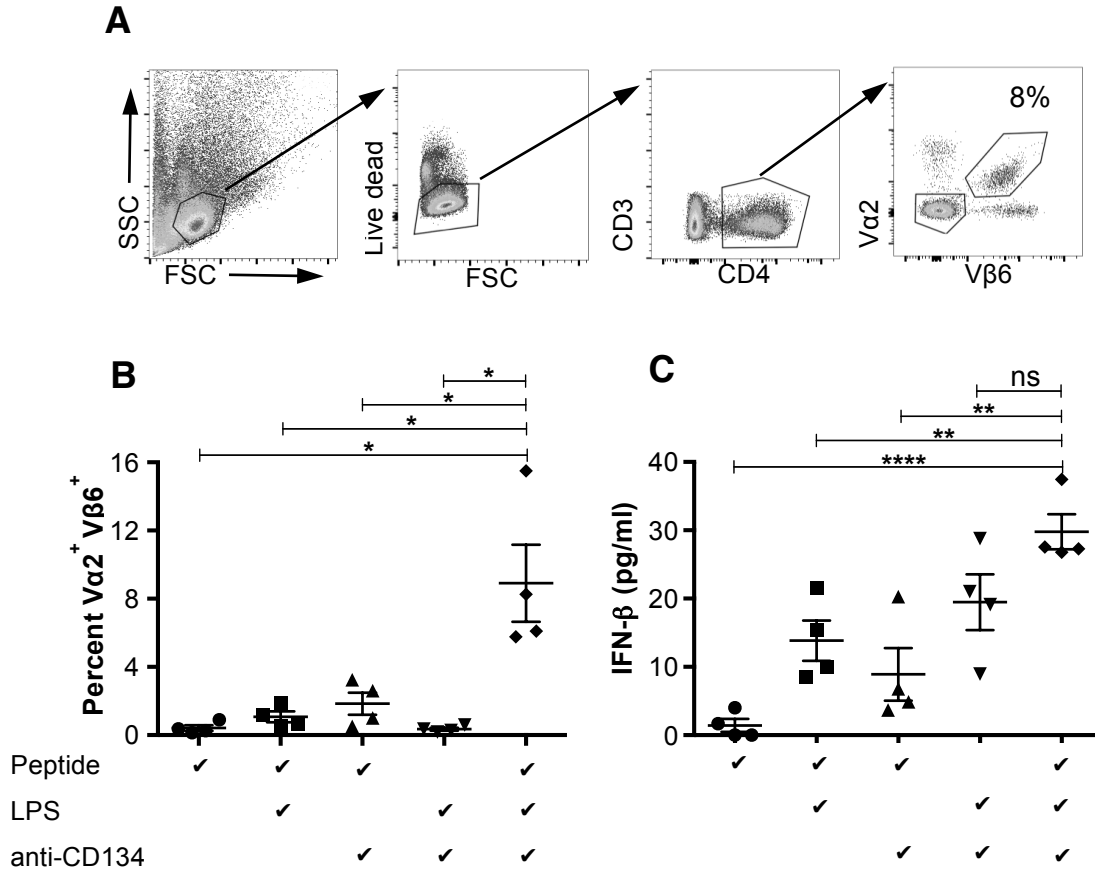


Figure 3-1. Antigenic peptide administered together with LPS and CD134 agonist promotes maximal T cell expansion and Type I IFN production.

Ag-specific T cells (TEa) were adoptively transferred to C57BL/6 mice that were immunized the next day with either Ea peptide, Ea+LPS, Ea+anti-CD134, LPS+anti-CD134 or Ea+LPS+anti-CD134. On days 11 or 12 the percentage of CD4 T cells expressing TCR Vα2⁺ Vβ6⁺ (Ag-specific T cells) in the liver were analyzed by gating on live cells. **(A)** Shows a representative gating strategy and **(B)** is the quantitated data. **(C)** Three hours after immunization the serum levels of type I IFN (IFN-β) were determined by ELISA. The data is pooled from 3 independent experiments. Fig. 1B. p value, * < 0.05. Fig 1C. p value, ** 0.01, **** < 0.0001. Error bars represent SEM of all experiments in that group.

Figure 3-2

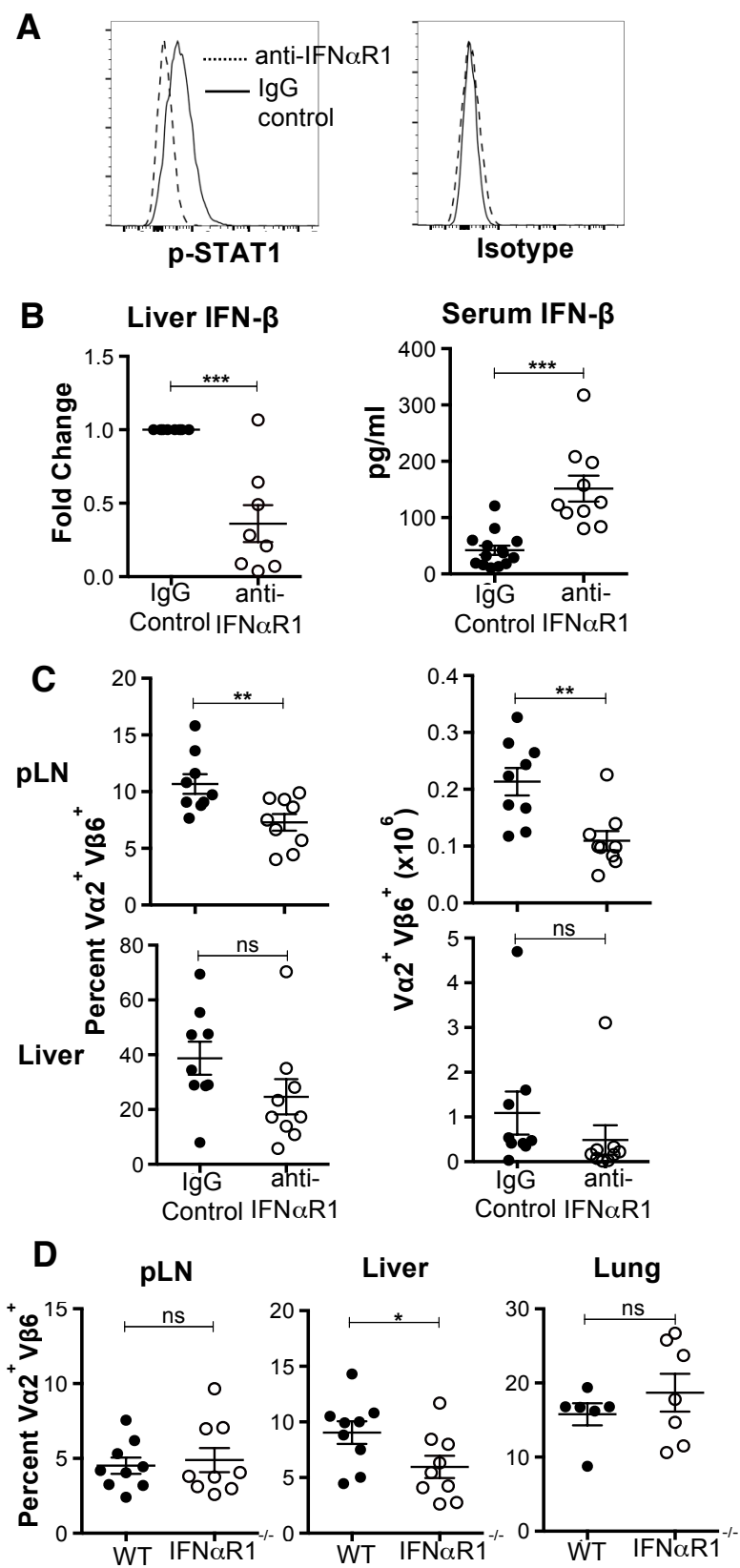


Figure 3-2. The impact of Type I IFN signaling on Ag-specific T cell expansion is context dependent. (A) Splenocytes isolated from a naive C57BL/6 mice were treated *in vitro* with anti-IFN α R1 mAb or IgG control (2.5 μ g mAb) for 30 min and stimulated with recombinant IFN- α (2000 U/ml) for 1 h 45 min. The cells then fixed and p-STAT1 expression in CD4 T cells was determined by flow cytometry. *Left*, overlay of histograms representing p-STAT1 in CD4 T cells from IgG (MFI: 600) and anti-IFN α R1 treated (MFI: 198) splenocytes and *Right*, overlay of isotype stained CD4 T cells from IgG (MFI: 159) and anti-IFN α R1 treated (MFI: 169) splenocytes. (B) *Left*, after transfer of Ag-specific T cells recipient mice were treated the next day with anti-IFN α R1 mAb or IgG control and immunized with E α +LPS+anti-CD134. On day 5 IFN- β expression in the liver was quantified by qRT-PCR. The data is representative of 4 independent studies with p value, *** < 0.001. *Right*, 3 h after immunization serum IFN- β was determined by ELISA, representative of 5 independent experiments with p value, **** < 0.0001. (C) Another group of mice treated as in 3-2B were analyzed for the; *Left*, percent and *Right*, total cell number of CD4⁺ V α 2⁺ V β 6⁺ T cells in pLN and liver by flow cytometry. The data is representative of 4 independent experiments with p value, ** < 0.01. (D) Ag-specific T cells were transferred into WT and IFN α R^{-/-} mice that were immunized with E α +LPS+anti-CD134. On day 5, the percent of CD4 T cells expressing TCR V α 2⁺ V β 6⁺ from pLN, liver and lung were quantitated by flow cytometry. The data is representative of 2-3 independent experiments with p value, * < 0.05. Error bars represent SEM of all experiments in that group.

Figure 3-3

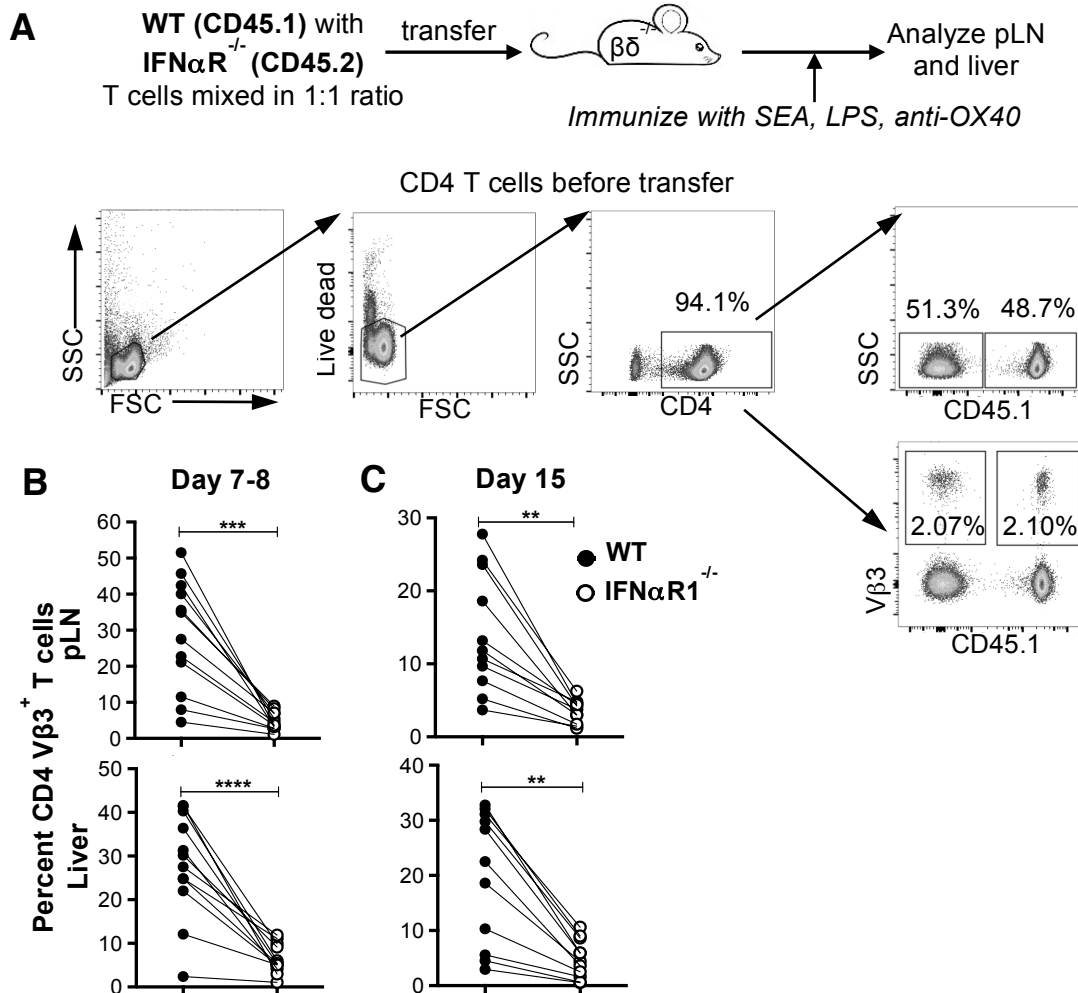


Figure 3-3. Absence of IFN α R signaling in specific T cells handicaps their expansion.

(A) CD4 T cells isolated from CD45.1⁺ WT mice and CD45.2⁺ IFN α R^{-/-} mice were mixed at approximately a 1:1 ratio to generate an equal percentage of CD45.1⁺ (WT) and CD45.1⁻ (IFN α R^{-/-}) T cells before transferring them into TCR $\beta\delta$ ^{-/-} recipients. The next day mice were i.p. Immunized with SEA, LPS and anti-CD134, and on days 7 or 8 (B) and 15 (C) the cells isolated from pLN (Top) and the liver (Bottom) were analyzed for the percent V β 3⁺ CD45.1⁺ (WT) and V β 3⁺ CD45.1⁻ (IFN α R^{-/-}) T cells by flow cytometry. The data is presented as average of percent WT (V β 3⁺ CD45.1⁺) and IFN α R^{-/-} (V β 3⁺ CD45.1⁻) T cells from all TCR $\beta\delta$ ^{-/-} mice where the connecting lines represent the WT and IFN α R^{-/-} T cells in an individual mouse. The data is representative of 4 independent studies with p value, ** < 0.01, *** < 0.05, **** < 0.001. Error bars represent SEM of all experiments in that group.

Figure 3-4

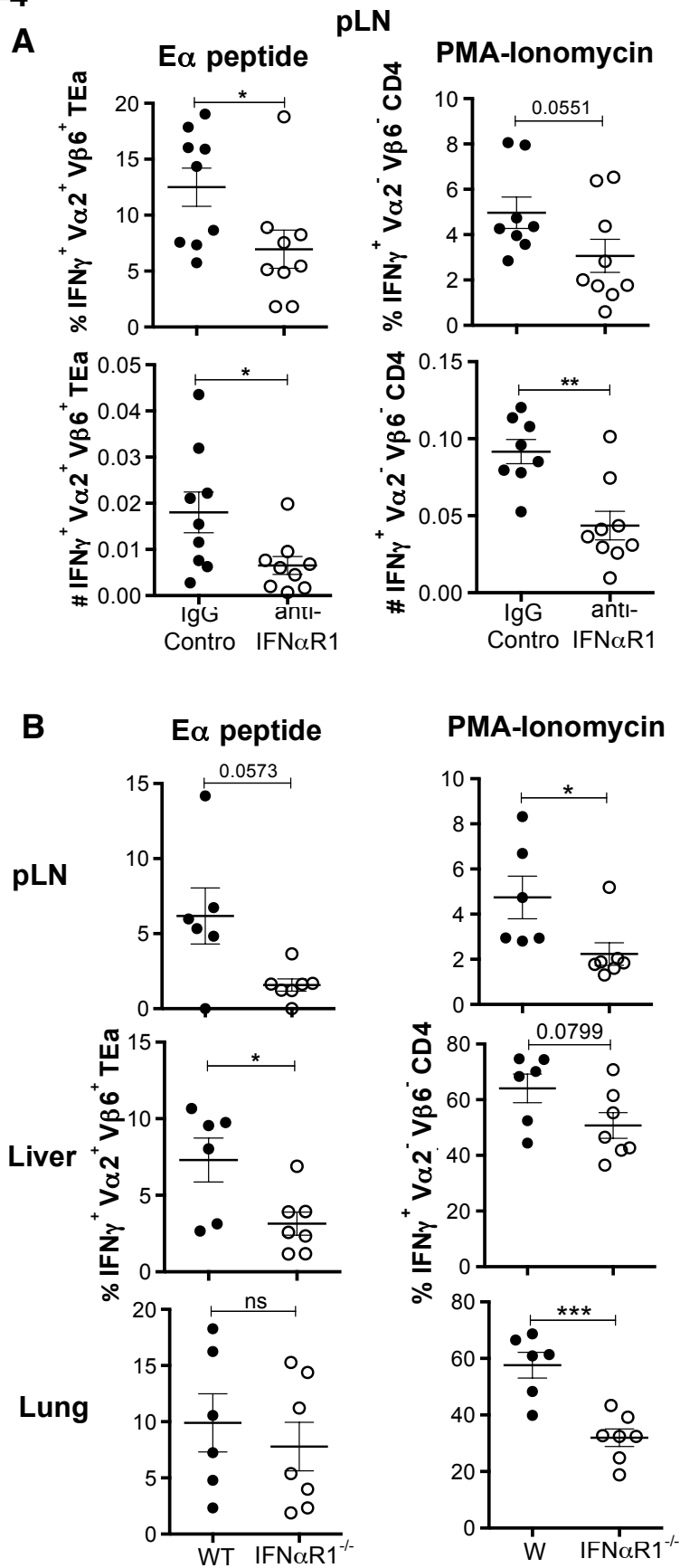


Figure 3-4. IFN α R signaling in non-specific cells of the host promotes IFN- γ production from specific as well as non-specific CD4 T cells. **A.** Mice treated in Fig. 3-2C, were used to analyze the percent (upper panels) and total number (lower panels) of IFN- γ expressing specific ($V\alpha 2^+ V\beta 6^+$) and non-specific ($V\alpha 2^- V\beta 6^-$) CD4 T, in pLN after with E α peptide or PMA+Ionomycin stimulation. **B.** Mice treated as in Fig. 3-2D were used to analyze the percent of IFN- γ expressing specific (left panels) and non-specific (right panels) CD4 T cells in pLN, liver and lung after E α peptide or PMA+Ionomycin stimulation. The data is representative of 2-3 independent experiments with p value, * < 0.05, ** < 0.01, *** < 0.001. Error bars represent SEM of all experiments in that group.

Figure 3-5

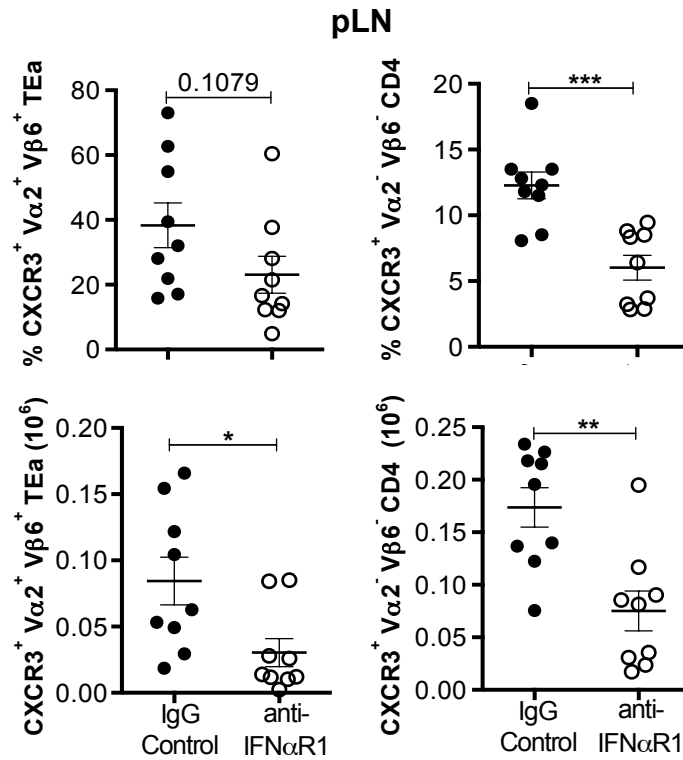


Figure 3-5. IFN α R signaling enhances the accumulation of CXCR3 expressing specific as well as non-specific CD4 T cells in pLN. Mice treated in Fig 3-2C, were used to calculate the percent and total number of CXCR3⁺ specific (Va2⁺ Vb6⁺) and non-specific (Va2⁻ Vb6⁻) CD4 T cells in pLN. The data is representative of 4 independent experiments with p value, * < 0.05, ** < 0.01, *** < 0.001. Error bars represent SEM of all experiments in that group

Figure 3-6 A

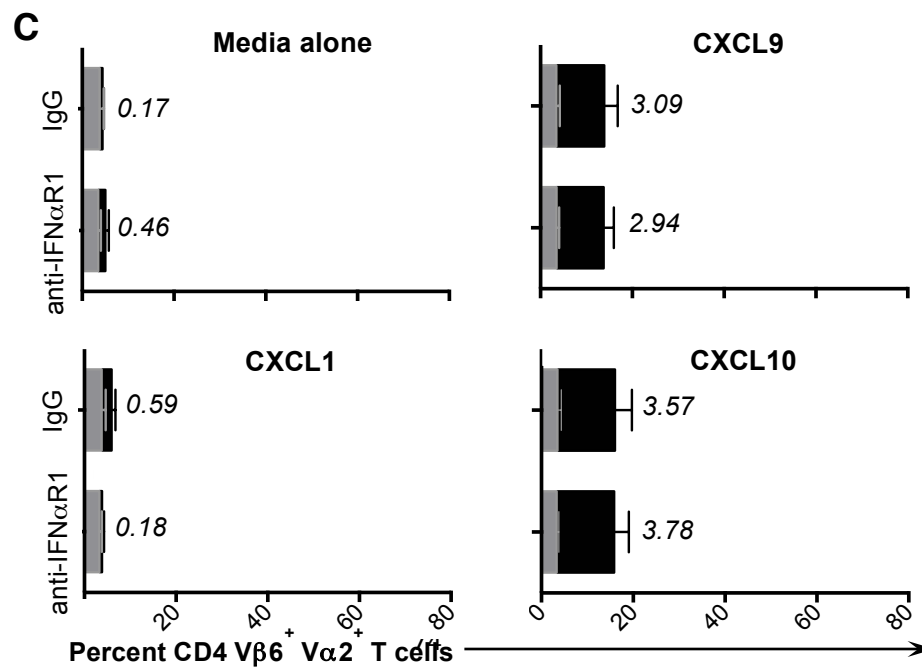
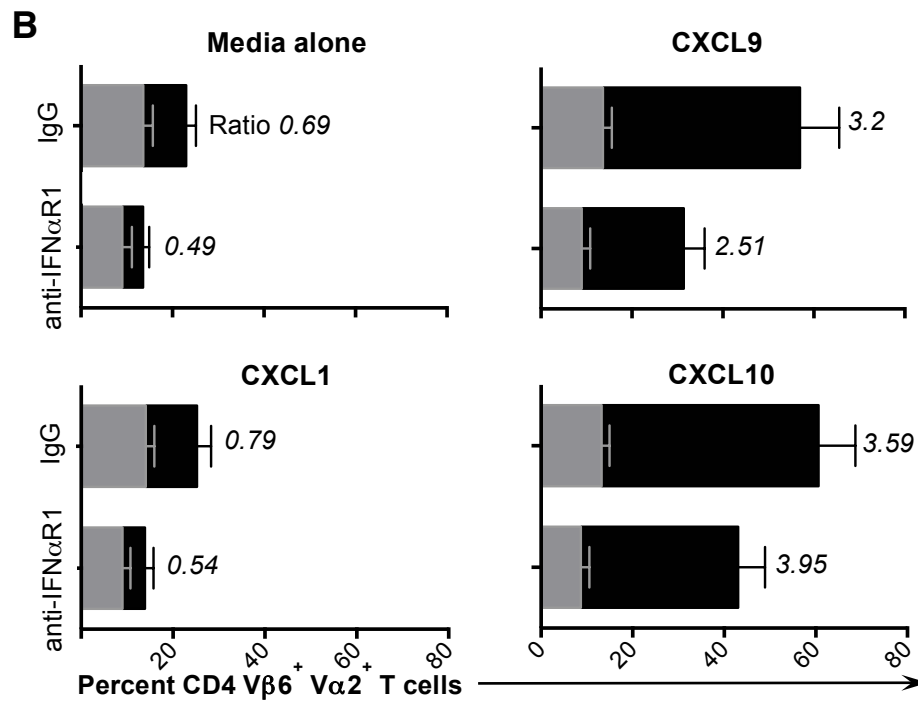
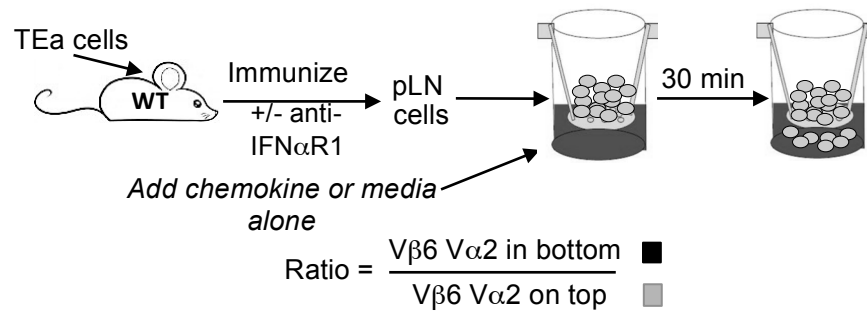


Figure 3-6. CXCL9 and CXCL10 promotes the migration of Ag-specific T cells in the absence of Type I IFN signaling. After transfer of Ag-specific T cells, the next day recipient mice were treated with anti-IFN α R1 or IgG control and immunized with E α +LPS+anti-CD134 and tested for migration potential **(A)**. On days 5 **(B)** and 11 **(C)** cells were harvested from the pLN, suspended in CTM and added into the transwell inserts. The bottom of the well was filled with CTM alone or with CXCL9, CXCL10 or CXCL1. At 30 min the cells on top versus bottom were stained and analyzed for the percent of CD4 T cells expressing TCR V α 2⁺ V β 6⁺. The graph shows the ratio of the percent of CD4 T cells expressing TCR V α 2⁺ V β 6⁺ in the bottom versus the top.

Figure 3-7

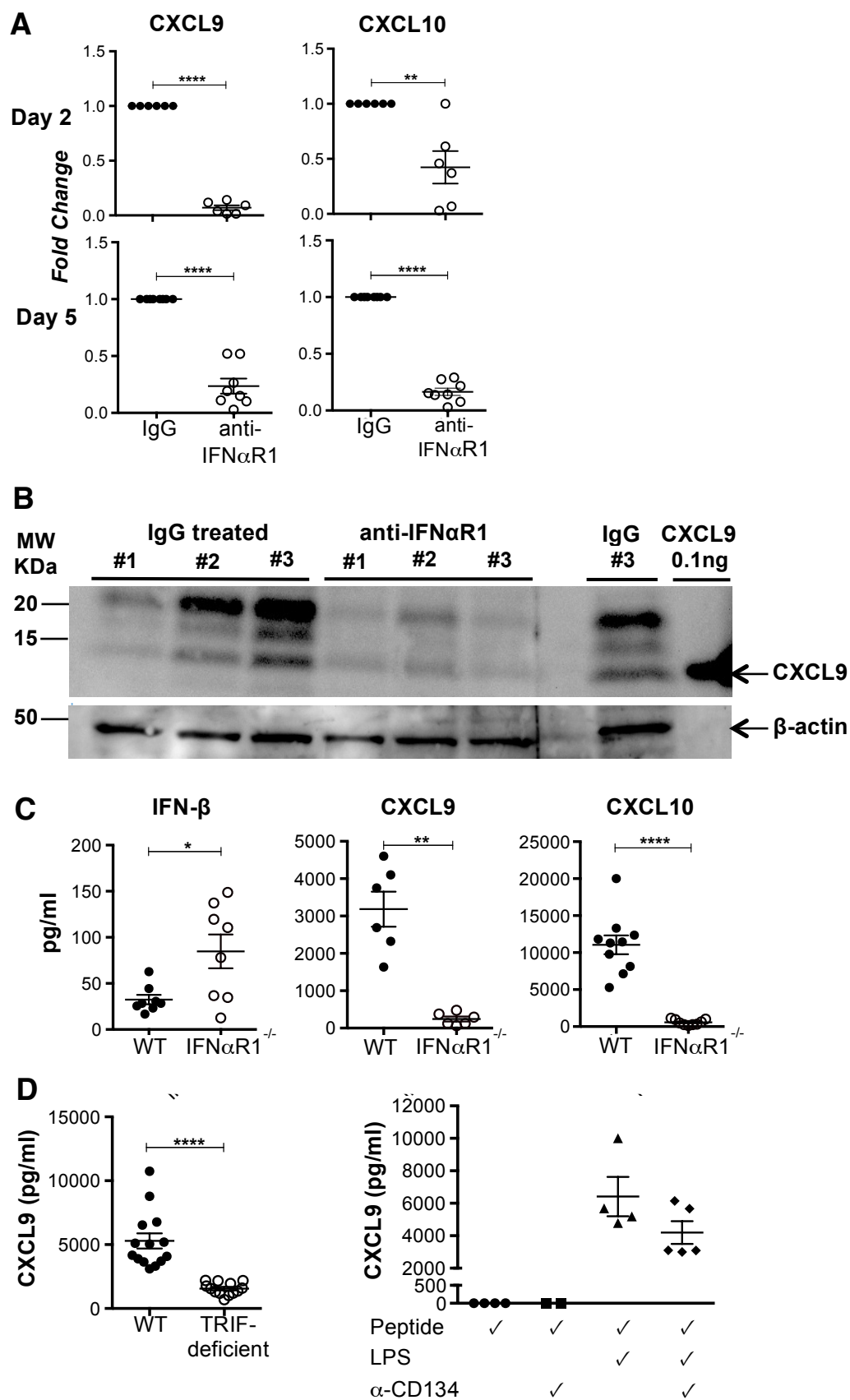


Figure 3-7. LPS induced Type I IFN signaling promotes the expression of chemokine CXCL9 and CXCL10. **(A)** The mice were treated as in Fig. 3-2C and liver tissue was harvested from the anti-IFN α R1 or IgG treated mice on day 2 (*Top*) and day 5 (*Bottom*) and CXCL9 (*Left*) and CXCL10 (*Right*) mRNA expression were analyzed by qRT-PCR. The gene expression is normalized to β -actin and represented as fold change of IgG treated over anti-IFN α R1. The data is representative of 3-4 studies with p value, **** < 0.0001; ** < 0.01. **(B)** CXCL9 protein expression in liver of IgG and anti-IFN α R1 treated mice was analyzed by immunoblot. Data represents 1 of 3 independent experiments, with 3 mice/group. **(C)** TEa cells were transferred into WT or IFN α R1^{-/-} mice that were immunized with E α +LPS+anti-CD134 (as in Fig. 3-2D) and 3 h later serum was collected followed by ELISA analysis of IFN- β , CXCL9 and CXCL10. The data is pooled from 3 independent experiments with p value, * < 0.05, ** < 0.01, **** < 0.0001. **(D)** *Left*, TEa recipient WT or TRIF-deficient mice were i.p. Immunized and 3 h later CXCL9 serum levels were determined by ELISA. The data is representative of 4 independent experiments with p value, **** < 0.0001. *Right*, The CXCL9 serum levels from the mice in Fig. 1 were analyzed by ELISA. The data is pooled from 2-3 experiments. Error bars represent SEM of all experiments in that group.

Figure 3-8

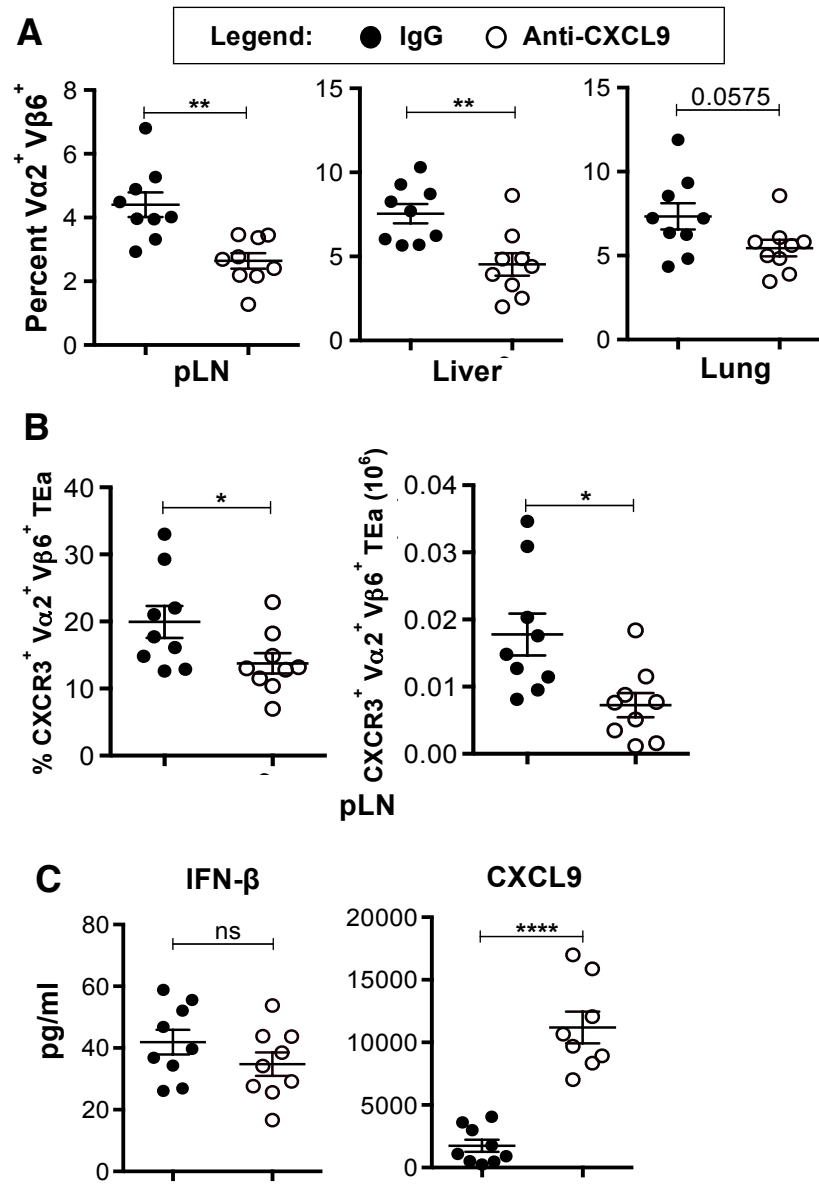


Figure 3-8. CXCL9 chemokine blockade interferes with Ag-specific T cell accumulation. **(A)** Recipient WT mice were immunized (E α +LPS+anti-CD134) the next day together with anti-CXCL9 or IgG control, and 8 h later anti-CXCL9 or IgG control was administered again. On day 6, the percent of CD4 T cells expressing TCR V α 2⁺ V β 6⁺ in pLN, liver and lung were analyzed by flow cytometry. The data is representative of 3 independent studies with p value, ** < 0.01. **(B)** Mice treated as in Fig. 3-8A were used to calculate the percent and total CXCR3 expressing (represented as 1×10^6) Ag-specific (V α 2⁺ V β 6⁺ TE α) CD4 T cells in pLN. The data is representative of 3 independent experiments with p value, * < 0.05. Error bars represent the SEM of all experiments in that group. **(C)** *Left*, The serum levels of IFN- β and *Right*, CXCL9 were determined by ELISA in 3 h (after immunization) serum from the mice in Fig. 3-8A. The data is representative of 4 independent experiments with p value, ***** < 0.0001. Error bars represent SEM of all experiments in that group.

Table 1. Oligonucleotide primers used for real-time RT-PCR

Gene	Accession No	Forward Primer	Reverse Primer	PMID
IFN β	NM_010510.1	5'-AGCTCCAAGAAAGGACGAACA-3'	5'-GCCCTGTAGGTGAGGTTGAT-3'	25822250; 24014881
CXCL9	NM_008599.4	5'-GGAGTTCGAGGAACCCTAGTG-3'	5'-GGGATTTGTAGTGGATCGTGC -3'	23520491
CXCL10	NM_021274.2	5'-CCAAGTGCTGCCGTCATTTTC -3'	5'-GGCTCGCAGGGATGATTTCAA-3'	22577359
β -Actin	NM_007393.3	5'-AAGGCCAACCGTGAAAAGAT-3'	5'-GTGGTACGACCAGAGGCATAC-3'	22981535; 23520491

PMID: PubMed reference

Table 2. IFN α R signaling impacts the accumulation of total number of Ag-specific T cells.[§]

Tissue	WT	IFN α R ^{-/-}
pLN	1.98 \pm 0.076	0.82 \pm 0.029
Liver	1.48 \pm 0.021 **	0.72 \pm 0.010
Lung	0.60 \pm 0.009 **	0.26 \pm 0.006

[§]The mice from Fig. 3-2D were used to calculate the total number of V α 2⁺ V β 6⁺ T cells (represented as $\times 10^5$ cells) in pLN, liver and lung on day 5. The data is representative of 2-3 independent experiments with p value, ** < 0.01, represented as Mean \pm SEM, analyzed using students unpaired t test.

Table 3. IFN α R signaling in T cells impacts the total number of SEA-specific T cells.[§]

pLN (V β 3 ⁺ T cells)	WT	IFN α R ^{-/-}
Day 7	0.2292 \pm 0.083 *	0.02816 \pm 0.008
Day 15	0.1128 \pm 0.057	0.02497 \pm 0.012
Liver (V β 3 ⁺ T cells)	WT	IFN α R ^{-/-}
Day 7	0.3953 \pm 0.094 **	0.07573 \pm 0.014
Day 15	0.7306 \pm 0.147 **	0.1675 \pm 0.034

[§] Mice from Fig 3-3B/C were used to calculate total number of V β 3⁺ CD45.1⁺ (WT) and V β 3⁺ CD45.1⁻ T (IFN α R^{-/-}) cells in each mouse. The table represents mean total number of SEA-specific WT and IFN α R^{-/-} T cells from 3-4 mice per group from 4 independent experiments. p value, * < 0.05 and ** < 0.01, represented as Mean \pm SEM using students unpaired t test.

Table 4. Chemokine CXCL9 impacts the total number of Ag-specific T cells in the pLN.[§]

	IgG	anti-CXCL9
pLN	0.089 ± 0.0098 **	0.048 ± 0.0083
Liver	0.090 ± 0.0158	0.061 ± 0.0105
Lung	0.065 ± 0.0081	0.046 ± 0.0077

[§] The mice from Fig. 3-8A were used to calculate the total number of V α 2⁺ V β 6⁺ TEa cells (represented as x10⁶ cells) in pLN, liver and lung on day 6. The data is representative of 3 independent experiments with p value ** < 0.01, represented as Mean ± SEM using students unpaired t test.

CHAPTER IV:

Combining Poly I:C with CD134 agonist promotes enhanced CD4 T cell expansion and Th1 differentiation that is dependent on type I IFN signaling

Abstract

Poly I:C is known to enhance both Th1 and CD8 T cell response, which makes it an ideal adjuvant in vaccines. Several reports have suggested that poly I:C mediates its adjuvant effect on CD4 T cells through induction of IFN α R signaling. Interestingly a recent study demonstrated, in a vaccine combining poly I:C with CD40 agonist that the enhanced CD4 T cell expansion and Th1 differentiation in response to this vaccine, relies on the CD134L expression on DC's. This suggested to us, that poly I:C could enhance the CD4 T cell response in poly I:C/anti-CD40 vaccine by indirectly triggering the CD134 costimulation. We therefore investigated, if poly I:C can be directly combined with CD134 costimulation to promote enhanced Ag-specific CD4 T cell expansion and Th1 differentiation. We show that, combining E α peptide with poly I:C and anti-CD134, promotes maximal Ag-specific CD4 T cell expansion and Th1 differentiation in comparison to peptide administered with poly I:C or anti-CD134 alone. Importantly, the enhanced T cell expansion and Th1 differentiation observed during poly I:C and anti-CD134 immunization was dependent on IFN α R signaling. Moreover, combining poly I:C with anti-CD134 promoted enhanced generation of effector memory CD4 T cells during a recall response and it was also dependent on IFN α R signaling. Thus, the ability of poly I:C and anti-CD134 to promote the generation of long-lived effector CD4 T cells, could be utilized in development of vaccines against diseases where Th1 responses are crucial. However, the more novel observation was that CD134 costimulation induced specific CD4 T cell expansion and Th1 differentiation also relied on IFN α R signaling. It is not known how CD134 costimulation triggers IFN α R signaling to promote effector CD4 response. Understanding the mechanism behind it is important for efficient targeting of CD134 in preclinical cancer trials.

Introduction

Several TLR agonists are currently being evaluated for their potential in inducing a potent CD4 Th1 response that is important in resistance to global infectious diseases such HIV, tuberculosis, and malaria. Among them, synthetic dsRNA, poly I:C is known to be a superior adjuvant in comparison to other TLR adjuvants in mediating enhanced CD4 T cell immunity to protein antigens [21, 22, 66]. Poly I:C, binds to endosomal TLR3 or cytosolic receptor MDA5 to induce strong interferon production [53-55, 308]. Poly I:C induced interferon has shown to promote the generation of multifunctional CD4 T cells with ability to induce high levels of IL-2, IFN- γ and TNF- α [21-23, 66].

Poly I:C and its derivative, Poly I:CLC, have been shown to enhance CD4 T cell and antibody immunity in vaccines against HIV, malaria, HPV [21, 23, 66, 69, 70], whereas Poly I:C₁₂U, is currently been investigated in certain cancers for its role in DC maturation, IL-12 induction and ability to enhance Th1-type and CTL responses [64, 65]. Notably, a few studies showed that combining poly I:C with costimulatory agonist CD40 further enhances the Ag-specific CD4 T cell expansion and Th1 differentiation [201, 202, 205, 206]. Following on this, subsequent studies demonstrated that, poly I:C and anti-CD40 mediated enhanced CD4 T response was dependent on IFN α R signaling induced OX40L/CD134L expression on DC's [27]. We therefore, decided to study if poly I:C can synergize with CD134 agonist directly to promote enhanced CD4 Th1-type immunity. Our results demonstrate that, administration of E α peptide with poly I:C and anti-CD134 promotes maximal specific CD4 T cell expansion and Th1 differentiation than peptide administered with poly I:C and anti-CD134 alone. The enhanced CD4 T cell response induced by combining poly I:C with anti-CD134 was dependent on IFN α R signaling, as the specific CD4 T cell expansion and Th1 differentiation were significantly

reduced in mice treated with anti-IFN α R1 mAb.

More importantly we observed that, CD134 costimulation induced CD4 T cell expansion and Th1 differentiation was also dependent on IFN α R signaling. The role of CD134 costimulation requiring IFN α R signaling to promote CD4 response is unknown. Is it important to identify the mechanism behind this in future, since CD134 is targeted in several advanced cancers to enhance the effector CD4 and CD8 T cell responses and promote anti-tumor immunity [248, 251, 252, 257, 309].

Finally, we also demonstrate that, combined administration of E α peptide with poly I:C and anti-CD134 programs specific-CD4 T cells during early immune response to promote their development into optimal effector memory CD4 T cells after a secondary challenge. The enhanced generation of effector memory CD4 T cells was also dependent on IFN α R signaling induced during early immune response. Thus, the combination of Ag, poly I:C and anti-CD134 should be explored in future for development of therapeutic vaccines against infectious diseases caused by intracellular bacteria and viruses, or in cancer where Th1 responses are crucial.

Results

Combined administration of poly I:C and CD134 agonist promotes enhanced CD4 T cell expansion and Th1 differentiation that is dependent on type I IFN signaling.

It was recently demonstrated that, poly I:C and CD40 agonist when combined together promotes enhanced CD4 T cell expansion and Th1 differentiation. This enhanced CD4 T cell response was dependent on IFN α R signaling induced CD134L expression on CD8⁺ DC's [27]. Hence, we analyzed if poly I:C could be combined with CD134 costimulation directly to promote enhanced CD4 T cell response. For this we transferred $\sim 1 \times 10^6$ Thy 1.1⁺ TEa cells to C57BL/6 mice, followed by their treatment with anti-IFN α R1 mAb or IgG control and immunization with E α +poly I:C, E α +anti-CD134 or E α +poly I:C+anti-CD134. We first confirmed, if poly I:C induces type I IFN in our vaccine model. As shown in Fig. 4-1A, immunization with poly I:C, induced ~ 180 -200 pg/ml of type I IFN (IFN- β) in serum, during both E α +poly I:C or E α +poly I:C+anti-CD134 immunization, whereas administration of E α +anti-CD134 induced minimal levels of type I IFN (~ 25 -30 pg/ml). Furthermore IFN α R1 blockade significantly increased the IFN- β levels in the serum during E α +poly I:C and E α +poly I:C+anti-CD134 immunization in comparison to control treated mice. This is likely due to unavailability of IFN α R1 to bind to IFN- β as seen previously with LPS and anti-CD134 vaccine. Overall, poly I:C induces significant levels of IFN- β and addition of anti-CD134 did not further influence it. Next we analyzed the expansion of Thy 1.1 cells on day 6 after immunization in mice treated as above and observed that both the percent (Fig. 4-1B, left panels) and total number of Thy 1.1 cells (Fig. 4-1B, right panels) were maximal in the spleen and liver of mice immunized with

E α , poly I:C and anti-CD134 in comparison to E α administered with poly I:C or anti-CD134 alone. In the liver, the expansion of Thy 1.1 cells were also enhanced with E α and anti-CD134 immunization, but the percent and total number of Thy 1.1 cells varied a lot between individual experiments (Fig. 4-1B, lower panels). Whereas, immunization with E α , poly I:C and anti-CD134 promoted consistent expansion of Thy 1.1 cells in liver, that were comparable between the experiments. Thus, combining poly I:C with anti-CD134 also promotes enhanced specific CD4 T cell expansion in the liver. Importantly, treatment with anti-IFN α R1 mAb significantly reduced the percent and total number of Thy 1.1 cells in spleen and liver during E α , poly I:C and anti-CD134 immunization, suggesting that IFN α R signaling is essential for the enhanced T cell expansion induced by poly I:C and anti-CD134 combination.

We next evaluated, if combined administration of E α peptide, poly I:C and anti-CD134 can promote enhanced Th1 differentiation in Thy 1.1 cells. For this, we analyzed the intracellular secretion of IFN- γ and TNF- α in Thy 1.1 cells from experiment done in Fig 4-1B. Our results demonstrate that, combining E α peptide with poly I:C and anti-CD134 increases the percentage of IFN- γ ⁺, TNF- α ⁺ and IFN- γ ⁺ TNF- α ⁺ Thy 1.1 cells in spleen after E α peptide stimulation in comparison to E α peptide given with poly I:C or anti-CD134 alone (Fig 4-2A, upper panels). The total numbers of IFN- γ ⁺, TNF- α ⁺ and IFN- γ ⁺ TNF- α ⁺ Thy 1.1 cells in spleen were also significantly increased with E α , poly I:C and anti-CD134 immunization (data not shown). In liver, however, immunization with E α and anti-CD134 promoted enhanced Th1 differentiation in comparison to E α +poly I:C or E α +poly I:C+anti-CD134 combination as seen by the increased percentage of IFN- γ ⁺, TNF- α ⁺ and IFN- γ ⁺ TNF- α ⁺ Thy 1.1 cells after E α peptide stimulation (Fig 4-2A, lower panels). Although, the percentage of IFN- γ ⁺ and TNF- α ⁺

Thy 1.1 cells were enhanced with E α and anti-CD134 immunization, the total number of IFN- γ^+ , TNF- α^+ and IFN- γ^+ TNF- α^+ Thy 1.1 cells were more significantly increased with E α , poly I:C and anti-CD134 immunization (Fig. 4-2B), suggesting that this vaccine also promotes enhanced Th1 differentiation in the liver. We next evaluated if treatment with anti-IFN α R1 Ab impacts the Th1 differentiation induced by E α , poly I:C and anti-CD134. As shown in the Fig 4-2, upper panels, IFN α R1 blockade significantly reduced the percentage of IFN- γ^+ Thy 1.1 cells, whereas the percentage of TNF- α^+ and IFN- γ^+ TNF- α^+ Thy 1.1 cells also trended towards reduction. Additionally, the total number of IFN- γ^+ , TNF- α^+ and IFN- γ^+ TNF- α^+ Thy 1.1 cells were significantly reduced in spleen after anti-IFN α R1 treatment (not shown). In liver, IFN α R1 blockade significantly reduced the percent of IFN- γ^+ and TNF- α^+ Thy 1.1 cells during E α peptide and anti-CD134 immunization, whereas the percent of IFN- γ^+ TNF- α^+ Thy 1.1 cells also trended towards reduction (Fig 4-2, upper panels). The total number of IFN- γ^+ , TNF- α^+ and IFN- γ^+ TNF- α^+ Thy 1.1 cells were however significantly reduced during E α , poly I:C and anti-CD134 immunization (Fig 4-2, lower panels).

Overall this result suggests, that combining Ag with poly I:C and anti-CD134 promotes enhanced specific CD4 T cell expansion in both spleen and liver. Whereas, Th1 differentiation was mainly enhanced in the spleen, except the total number of Th1 type CD4 cells were increased in the liver with this vaccine. Importantly, IFN α R signaling was required for Th1 differentiation during both E α +anti-CD134 and E α +poly I:C+anti-CD134 immunization. The role of CD134 costimulation relying on IFN α R signaling to promote effector CD4 T cell response is unknown and the mechanism behind it needs to be evaluated in future.

CD134 costimulation relies on type I IFN signaling to promote CD4 T cell expansion and Th1 differentiation.

As the results above, suggested that CD134 costimulation requires IFN α R signaling to promote Th1 differentiation, we next analyzed if specific CD4 T cells activated with CD134 agonist requires IFN α R signaling to induce Th1 differentiation. For this, we transferred $\sim 1 \times 10^6$ WT (Thy 1.1⁺ Thy 1.2⁺ TEa) or CD134^{-/-} (Thy 1.1⁺ Thy 1.2⁻ TEa⁺) CD4 T cells separately into C57BL/6 mice, followed by their treatment with anti-IFN α R1 Ab or control and immunization with E α and anti-CD134. Our results in Fig. 4-3A show that, the percent and total number of specific CD4 T cells on day 8 after immunization were significantly increased in the spleen and liver of mice that received WT T cells, in comparison to CD134^{-/-} T cells. The WT T cell expansion in the liver varied a lot between experiments after E α peptide and anti-CD134 immunization as seen previously in Fig. 4-1B. It is known that CD134 signaling in T cells enhances their expansion and survival [17, 212, 229], so this result was not surprising to us. More importantly, the treatment with anti-IFN α R1 mAb, resulted in significant reduction in the percentage (Fig. 4-3A, left panels), and the total number of specific WT T cells in spleen also trended towards reduction. In liver, anti-IFN α R1 treatment reduced the percent and total number of specific WT T cells, but it did not significantly do so (Fig. 4-3A, right panels). In mice that received CD134^{-/-} T cells, IFN α R1 blockade had no further impact on specific T cell expansion. This data is, however, different from the results shown in Fig. 4-1B, where the percent and total number of specific WT T cells, were not significantly reduced in spleen during E α peptide and anti-CD134 immunization after IFN α R1 blockade. This could be a result of IFN α R1 signaling impacting T cell survival during CD134 costimulation rather than expansion, as the data in Fig. 4-3 is analyzed on day 8 after expansion, in comparison to data in Fig 4.1,

which is representative of day 6 expansion. However, overall these results suggest that CD134 costimulation relies on IFN α R signaling to promote specific CD4 T cell expansion. We also analyzed the IFN- β levels in serum of mice that received WT or CD134 $^{-/-}$ T cells, followed by immunization. We saw in mice that received WT T cells, ~10 pg/ml of IFN- β were induced, which significantly increased in mice that received CD134 $^{-/-}$ T cells (Fig. 4-3B). It could indicate that CD134 costimulation in activated T cells enhances IFN α R1 expression on them, and in mice that receives CD134 $^{-/-}$ cells, the reduced IFN α R1 expression, results in increased IFN- β levels in the serum. However we haven't analyzed this possibility. Also in mice, that received WT T cells followed by anti-IFN α R1 treatment, the IFN- β levels were significantly increased in comparison to control treated mice, which could be due to unavailability of IFN α R1 to bind IFN- β . Whereas in mice that received CD134 $^{-/-}$ T cells, IFN α R1 blockade has no further impact on IFN- β levels. Overall even though we saw statistically significant differences in type I IFN levels in mice that received WT versus CD134 $^{-/-}$ T cells, we do not know if these differences are biologically significant.

We next evaluated, if CD134 costimulation in specific T cells requires IFN α R signaling to promote Th1 differentiation. For this, we analyzed intracellular IFN- γ , TNF- α expression in specific T cells from experiment done in Fig 4-3A. Our results shown in Fig. 4-4 demonstrate that, the percentage of IFN- γ $^{+}$, TNF- α $^{+}$ and IFN- γ $^{+}$ TNF- α $^{+}$ specific WT CD4 T cells were substantial in spleen after E α peptide (Fig. 4-4A) or PMA+Ionomycin (Fig. 4-4B) stimulation. In comparison, the percentage of IFN- γ $^{+}$, TNF- α $^{+}$ and IFN- γ $^{+}$ TNF- α $^{+}$ CD134 $^{-/-}$ T cells were significantly reduced. The total number of IFN- γ $^{+}$, TNF- α $^{+}$ and IFN- γ $^{+}$ TNF- α $^{+}$ T cells were also significantly reduced in mice that received CD134 $^{-/-}$ T cells (not shown). These results are in

agreement with the previous reports that show the role of CD134 signaling in promoting Th1 differentiation [17, 210, 239]. More importantly treatment with anti-IFN α R1 mAb significantly reduced the percent of IFN- γ ⁺, TNF- α ⁺, IFN- γ ⁺ TNF- α ⁺ specific WT T cells in spleen after E α peptide (Fig. 4-4A) and PMA+Ionomycin (Fig. 4-4B) stimulation. The total number of IFN- γ ⁺, TNF- α ⁺, IFN- γ ⁺ TNF- α ⁺ specific WT T cells in spleen were significantly reduced after E α peptide stimulation, whereas they trended towards reduction after PMA+Ionomycin stimulation, but did not reach significance (not shown). In liver, treatment with anti-IFN α R1 mAb did not impact the percentage of IFN- γ ⁺, TNF- α ⁺, IFN- γ ⁺ TNF- α ⁺ specific WT T cells during E α peptide stimulation (Fig. 4-4A). However, after PMA+Ionomycin stimulation the percentage (Fig. 4-4B) and total number of IFN- γ ⁺ and IFN- γ ⁺ TNF- α ⁺ specific WT T cells (not shown) trended towards reduction. Finally, there was IFN α R1 blockade did not influence the Th1 differentiation in on CD134^{-/-} CD4 T cells (not shown).

Overall, these results suggest that, CD134 signaling in specific CD4 T cells requires IFN α R signaling to promote optimal Th1 differentiation. This is a novel observation and the mechanism of how, CD134 and IFN α R signaling work together to promote CD4 T cell expansion and Th1 differentiation, needs to be explored in future studies.

Combined administration of poly I:C and CD134 agonist promotes enhanced generation of effector memory CD4 T cells.

Since combining poly I:C with CD134 agonist promoted enhanced CD4 T cell expansion and Th1 differentiation during primary immune response, we evaluated if this combination also promotes enhanced CD4 T cell expansion and cytokine differentiation during memory response. For this, we transferred $\sim 1 \times 10^6$ cells Thy 1.1⁺ TEa cells into C57BL/6 mice, followed

by their treatment with anti-IFN α R1 Ab or control and immunization with E α +poly I:C, E α +anti-CD134 and E α +poly I:C+anti-CD134 as before. On day 30 or 31 mice were re-challenged with E α and poly I:C and the specific memory CD4 T cells were analyzed in spleen and liver. As shown in the Fig. 4-5, immunization with E α , poly I:C and anti-CD134 resulted in significant increase in percent and total number of specific memory CD4 T cells in spleen in comparison to E α administered with poly I:C or anti-CD134 alone (Fig. 4-5, upper panels). Importantly, IFN α R1 blockade during early T cell priming reduced the percent of specific memory CD4 T cells in spleen, almost significantly, whereas, the total number of memory CD4 T cells, were significantly reduced (Fig. 4-5, upper panels). In liver, immunization with E α , poly I:C and anti-CD134 also promoted maximal expansion of specific memory CD4 T cells. However, unlike spleen, the expansion was not drastic and it did not significantly differ in comparison to the expansion induced with E α and anti-CD134 immunization (Fig. 4-5, lower panels).

Furthermore, IFN α R1 blockade did tend to reduce the percent and total number of specific memory CD4 T cells in liver, during E α , poly I:C and CD134 immunization, however, they were not significantly impacted.

Overall, this data suggests that, immunization with Ag, poly I:C and anti-CD134 programs the specific CD4 T cells in spleen during early phase of immune response to expand optimally after a secondary challenge.

We next determined, if immunization with E α , poly I:C and anti-CD134 promotes optimal Th1 differentiation of specific CD4 T cells during the recall response. For this we analyzed the intracellular IFN- γ , TNF- α expression in specific CD4 T cells from experiment done in Fig. 4-5. As shown in Fig 4-6, immunization with E α +poly I:C+anti-CD134 resulted in substantial increase in the percent of IFN- γ ⁺, IFN- γ ⁺ TNF- α ⁺ specific memory CD4 T cells in spleen, after

E α peptide (Fig. 4-6A) and PMA+Ionomycin stimulation (Fig. 4-6B). However, the percent of IFN- γ ⁺ and TNF- α ⁺ specific memory T cells were more significantly enhanced after PMA+Ionomycin stimulation, as there was a significant increase in percent of these cells with E α +poly:IC+anti-CD134 in comparison to E α +poly I:C and E α +anti-CD134 immunization (Fig. 4-6B, upper panels). Furthermore anti-IFN α R1 treatment significantly reduced the percent of IFN- γ ⁺ and IFN- γ ⁺ TNF- α ⁺ specific memory CD4 T cells in spleen, not only during E α +poly I:C+anti-CD134 immunization (after E α peptide stimulation, Fig. 4-6A, upper panels) but also during E α +poly I:C and E α +anti-CD134 immunization (with PMA+Ionomycin stimulation, Fig. 4-6B, upper panels). The total number of IFN- γ ⁺ and IFN- γ ⁺ TNF- α ⁺ specific memory T cells in spleen, also followed similar trend where they expanded more with E α +poly I:C+anti-CD134 immunization in comparison to E α given with poly I:C or anti-CD134 (not shown) and anti-IFN α R1 treatment reduced the total number of IFN- γ ⁺ and IFN- γ ⁺ TNF- α ⁺ memory T cells even though it didn't reach significance (not shown). In liver, immunization with E α +poly:IC+anti-CD134 increased the percent of IFN- γ ⁺ specific memory T cells after E α peptide stimulation (Fig. 4-6A, lower panels), but it was not significant in comparison to E α administered with poly I:C or anti-CD134. However, with PMA+Ionomycin stimulation, E α + poly I:C+anti-CD134 immunization increased the percent of IFN- γ ⁺ and IFN- γ ⁺ TNF- α ⁺ specific memory T cells significantly in comparison to E α +poly I:C or E α +anti-CD134 immunization (Fig 4-6B, lower panels). Furthermore, IFN α R1 blockade significantly reduced the percent of IFN- γ ⁺ specific memory T cells in liver after E α peptide stimulation (Fig. 4-6A, lower panels), and the percent of IFN- γ ⁺ and IFN- γ ⁺ TNF- α ⁺ specific memory T cells after PMA+Ionomycin stimulation (Fig. 4-6B, lower panels). Finally, immunization with E α +poly I:C+anti-CD134 also significantly

enhanced the Tbet expression (Th1 transcription factor Tbet, encoded by *TBX21*) on IFN- γ ⁺ specific memory T cells in spleen during E α peptide (Fig. 4-6A, upper right panel) and PMA+Ionomycin stimulation (Fig. 4-6B, upper right panel) and the enhanced Tbet expression was also dependent on IFN α R signaling. This suggests that IFN α R signaling induced with E α +poly I:C+anti-CD134 immunization during early immune response promotes Th1 lineage commitment in specific CD4 T cells, by enhancing the expression of Tbet.

Overall, these results highlights the importance of combining, Ag with poly I:C and CD134 agonist to promote long-lasting Th1-type CD4 T cell immunity, and this combination should be evaluated for its efficacy in vaccines targeting infectious diseases or cancer, where Th1 responses are crucial.

We next evaluated, if our previous vaccine combining of E α peptide, LPS and anti-CD134 requires IFN α R signaling to promote the generation of effector memory CD4 T cells, similar to poly I:C and anti-CD134 combination. To analyze this, TEa or Thy 1.1 cells were transferred to C57BL/6 mice, followed by their treatment with anti-IFN α R1 Ab or control and immunization with E α , LPS and anti-CD134. The mice were re-challenged with E α peptide and LPS on day 28 or 31, following which on day 5 or 7, the specific CD4 T cells were analyzed for expansion and cytokine secretion. As shown in Fig. 4-7A, IFN α R signaling induced by E α +LPS+anti-CD134 during early T cell priming, did not influence the expansion of specific memory CD4 T cells in pLN and liver, after a recall challenge. Furthermore, anti-IFN α R1 treatment did not impact the percent of IFN- γ ⁺ and TNF- α ⁺ specific memory CD4 T cells in pLN and liver after E α peptide stimulation (Fig 4-7B) or PMA+Ionomycin stimulation (not shown). Rather, the IFN α R1 blockade slightly enhanced the specific CD4 T cell expansion (Fig 4-7A) and Th1

differentiation (Fig 4-7B). Thus, even though the combination of peptide Ag, LPS and anti-CD134 is known to promote optimal CD4 T cell memory [19], LPS induced IFN α R signaling does not play a role in this. Higher dependence of poly I:C on IFN α R signaling to promote effector memory CD4 T cell response could be a result of it activating both TLR3 and MDA-5 signaling pathways, in comparison to LPS, which induces interferon only through TLR4-TRIF pathway. Therefore its possible that E α , LPS and anti-CD134 induced memory CD4 T cell response is dependent on MyD88 signaling, as a result of which the absence of IFN α R signaling has no impact on the LPS induced memory CD4 T cell response.

Discussion

Poly I:C and its derivatives have shown a great promise in promoting Th1-type immune response and therefore are used as adjuvants in vaccines against several infectious diseases such as HIV, HPV and malaria [21, 23, 66, 69, 70]. Moreover its ability to impact a variety of immune cells, such as CD4 [21, 23, 59, 66] and CD8 T cells [58, 310, 311], NK cells [112, 312] and DC's [22, 53, 57, 201] has compelled investigators to test its efficacy in cancer vaccines. Recently, few studies demonstrated that combining poly I:C with CD40 agonist promotes generation of multifunctional effector CD4 T and CD8 T cells that are able to protect against several viral infections (HIV, HCV, RSV) [201, 202, 204, 205, 313] and promote anti-tumor immunity in preclinical cancer models (cervical cancer, melanoma, lymphoma) [314-316]. Later studies, suggested that the enhanced effector T cell response induced by poly I:C and anti-CD40 combination relied on CD134L expression on CD8⁺ DC's [206], where IFN α R signaling enhanced the CD134L expression on DC's [27]. Hence we analyzed, if poly I:C can be directly combined with CD134 costimulation to promote enhanced CD4 T cell expansion and Th1 differentiation. Our results show that, combining peptide Ag with poly I:C and anti-CD134, promotes maximal CD4 T cell expansion and Th1 differentiation in lymphoid (spleen) and non-lymphoid tissue (liver) in comparison to Ag administered with poly I:C or anti-CD134 (Fig 4-1B). Furthermore, we saw that poly I:C and anti-CD134 combination enhanced the percentage (Fig 4-2A, upper panel) and total number (not shown) of Th1-type specific CD4 T cells in spleen after E α peptide stimulation. Whereas, in the liver, immunization with E α and anti-CD134 also increased the percentage of Th1-type CD4 T cells (Fig. 4-2A, lower panel) and E α and anti-CD134 induced Th1 differentiation relied on IFN α R signaling (Fig. 4-2A). However,

the total number of Th1-type CD4 cells, were more significantly increased with E α +poly I:C+anti-CD134 immunization (Fig. 4-2B). Importantly, similar to poly I:C and anti-CD40 combination, poly I:C and anti-CD134 induced Th1 differentiation in spleen also relied on IFN α R signaling (Fig 4-2A, upper panel).

The primary source of type I IFN in our vaccine model is poly I:C, whereas anti-CD134 stimulation induced minimal levels of type I IFN (Fig 4-1A). The high molecular weight poly I:C used in our study could trigger type I IFN through both TLR3 and MDA5 pathways. Previous study from our lab demonstrated that, poly I:C induced effector CD8 T cell expansion during an acute response was independent on TLR3 signaling, whereas generation of effector memory CD8 T cells required TLR3 signaling [311]. In line with this, poly I:C induced long-term effector CD4 cell response to a DC-targeted HIV-gag vaccine was shown to be dependent on both TLR3 and MDA-5 pathways [21, 22]. Understanding, which signaling pathway is triggered during poly I:C and anti-CD134 immunization, could help evaluate the mechanism of how this combination enhances effector CD4 T cell response.

In sum, results above suggest that combining Ag with poly I:C and CD134 agonist promotes enhanced CD4 T cell expansion and Th1 differentiation. Therefore, this combination should be evaluated for its effectiveness in therapeutic vaccines against infectious disease or tumor models where Th1 responses are crucial.

As, immunization with E α and anti-CD134 relied on IFN α R signaling to promote optimal Th1 differentiation in liver (Fig 4-2A, lower panel), we further evaluated, if CD134 costimulation in T cells requires IFN α R signaling to promote Th1 differentiation. Our results show that specific WT CD4 T cells expanded significantly more in comparison to CD134^{-/-} T cells in spleen during E α and anti-CD134 immunization and this expansion was dependent on IFN α R signaling (Fig.

4-3A). In liver, WT T cell expansion varied a lot between the experiments, but it was still dependent on IFN α R signaling. Furthermore, WT T cells stimulated with E α and anti-CD134 induced optimal Th1 differentiation after E α peptide (in spleen, Fig. 4-4A, upper panel) and PMA+Ionomycin (in spleen and liver, Fig. 4-4B) stimulation in comparison to CD134^{-/-} T cells. Importantly, the CD134 costimulation induced Th1 differentiation in WT CD4 T cells of spleen, required IFN α R signaling (Fig. 4-4A/B). Thus even though administration of Ag with CD134 agonist induced minimal levels of type I IFN (IFN- β) (Fig. 4-3B), it was somehow sufficient to promote specific CD4 T cell expansion and Th1 differentiation.

Antibodies targeting CD134 are currently used in several preclinical cancer trials to promote generation of tumor specific CD4 and CD8 T cells with optimal effector function [18, 244, 248, 257, 309]. Therefore, understanding how CD134 agonist triggers interferon induction to promote CD4 T cell expansion and Th1 differentiation is necessary for further use of the therapeutics targeting CD134.

Finally, as combining poly I:C and anti-CD134 promoted enhanced CD4 T cell expansion and Th1 differentiation during primary immune response, we analyzed if this combination could promote enhanced generation of effector memory CD4 T cells during a recall response. Our results in Fig. 4-5A show that, immunization with E α , poly I:C and anti-CD134 resulted in substantial increase in the percent and total number of specific memory CD4 T cells in spleen in comparison to E α administered with poly I:C or anti-CD134 (Fig. 4-5A, upper panel).

Whereas, in liver, E α +poly I:C+anti-CD134 immunization significantly enhanced the specific CD4 T cell expansion in comparison to E α and poly I:C, but not in comparison to E α and anti-CD134 immunization (Fig. 4-5A, lower panel). Importantly, the absence of IFN α R signaling during E α +poly I:C+anti-CD134 caused significant reduction in total number of specific

memory CD4 T cells in spleen, whereas in liver, the total number of memory CD4 T cells were reduced, but were not significantly impacted. Overall, immunization with Ag, poly I:C and anti-CD134 promotes enhanced generation of specific memory CD4 T cells during a recall challenge and it is dependent on IFN α R signaling induced early during the immune response. Moreover, immunization with E α , poly I:C and anti-CD134 also promoted enhanced Th1 differentiation of specific CD4 T cells in spleen after a recall challenge (Fig. 4-6A, upper panel). In liver, the total number of specific CD4 T cells after E α +poly I:C+anti-CD134 immunization were less in comparison to spleen (not shown), therefore E α peptide stimulation did not activate the CD4 T cells enough (Fig. 4-6A, lower panel). However, after PMA+Ionomycin stimulation the Th1 differentiation of specific CD4 T cells was enhanced in liver with E α +poly I:C+anti-CD134 immunization (Fig. 4-6B, lower panel). Importantly the enhanced Th1 differentiation seen in spleen and liver after E α +poly I:C+anti-CD134 immunization was dependent on IFN α R signaling induced early during T cell priming (Fig. 4-6A/B). Additionally, immunization with E α , poly I:C and anti-CD134 also enhanced the Tbet expression on IFN- γ ⁺ specific CD4 T cells in spleen after E α peptide and PMA+Ionomycin stimulation (Fig 4-6A/B, upper right panel), however it did not impact Tbet expression in liver (not shown). The enhanced Tbet expression on IFN- γ ⁺ specific CD4 T cells (in spleen) was also dependent on IFN α R signaling. Tbet is known to regulate IFN- γ production by binding to the IFN- γ gene and up regulating the IL-12R β 2 expression [317, 318]. Thus, immunization with E α +poly I:C+anti-CD134 seems to promote the Th1 lineage commitment in specific CD4 T cells through type I IFN, mediated induction of Tbet.

Above mentioned results, are similar to a study published this year by Esteban Celis group,

where authors show, that adding anti-CD134 mAb to trivax vaccine (that combines peptide Ag, with poly I:C and anti-CD40) during a prime and boost immunization promotes the generation of long-lasting effector CD4 T cells [315]. However, their data shows that addition of anti-CD134 to Trivax, does not enhance the effector CD4 T cell response on day 7, in comparison to Trivax given alone, whereas in our model, adding anti-CD134 to poly I:C, enhances the effector CD4 T response during early immune response (day 6) as well. It is possible that the addition of anti-CD134 to poly I:C and anti-CD40 does not impact early CD4 T cell response differently than poly I:C and anti-CD40 combination, but rather enhances the effector CD4 T cell response after a prime and boost immunization. Thus, it is important to compare CD4 T cell response induced by poly I:C and anti-CD134 and poly I:C, anti-CD134 and anti-CD40 combination in the same experiment to see which combination is better at promoting short-term and long term CD4 T cell response. Moreover, their results do not show that Ag+poly I:C+anti-CD40+anti-CD134 induced CD4 T cell response was dependent on IFN α R signaling; they rather showed using gardiquimod, GDQ (TLR7 agonist) that Ag+GDQ+anti-CD40+anti-CD134 mediated effector CD4 T cell response was dependent on IFN α R signaling. Overall, our results show that combining poly I:C with CD134 agonist promotes enhanced generation of Th1-type CD4 T cells during both primary and memory phase of immune response. Additionally, the observation that CD134 costimulation requires IFN α R signaling to promote CD4 T cell expansion and Th1 differentiation is important and the mechanism behind this needs to be evaluated in the future studies.

Figure 4-1

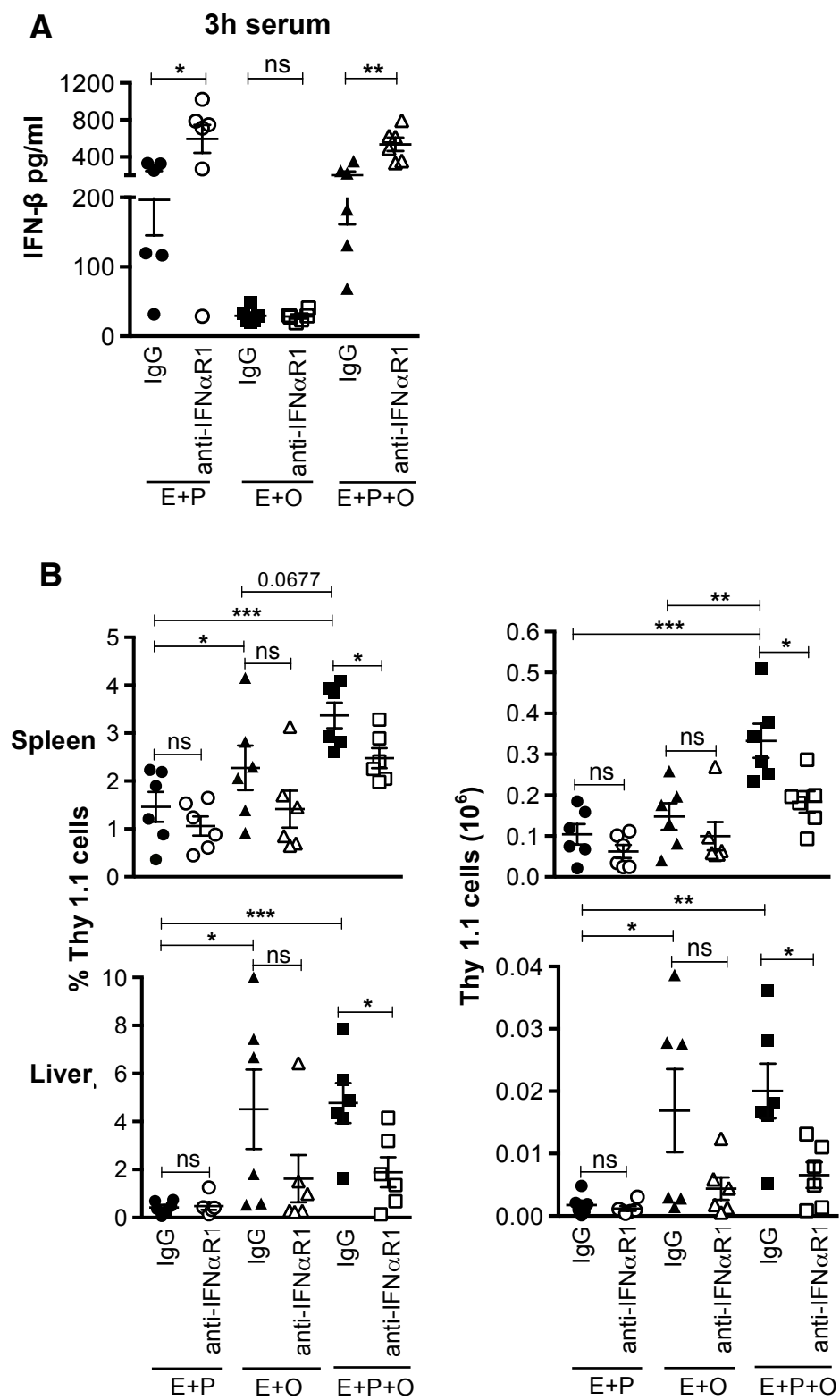


Figure 4-1. Administration of Ag together with poly I:C and anti-CD134 promotes maximal expansion of specific CD4 T cells. About 1×10^6 of TEa⁺ Thy 1.1⁺ cells were transferred into C57BL/6 mice. Next day mice were treated with anti-IFN α R1 mAb or control, followed by immunization with E α +poly I:C, E α +anti-CD134 or E α +poly I:C+anti-CD134. **A.** The data represents the Elisa analysis of IFN- β levels in serum at 3 hr from mice treated as above. **B.** The graph depicts the percent and total number of Thy 1.1 cells in spleen and liver of mice on day 6 after immunization. Data is representative of 3 independent experiments with p value, * < 0.05, ** < 0.01. Error bars represent SEM of all experiments in that group.

Figure 4-2

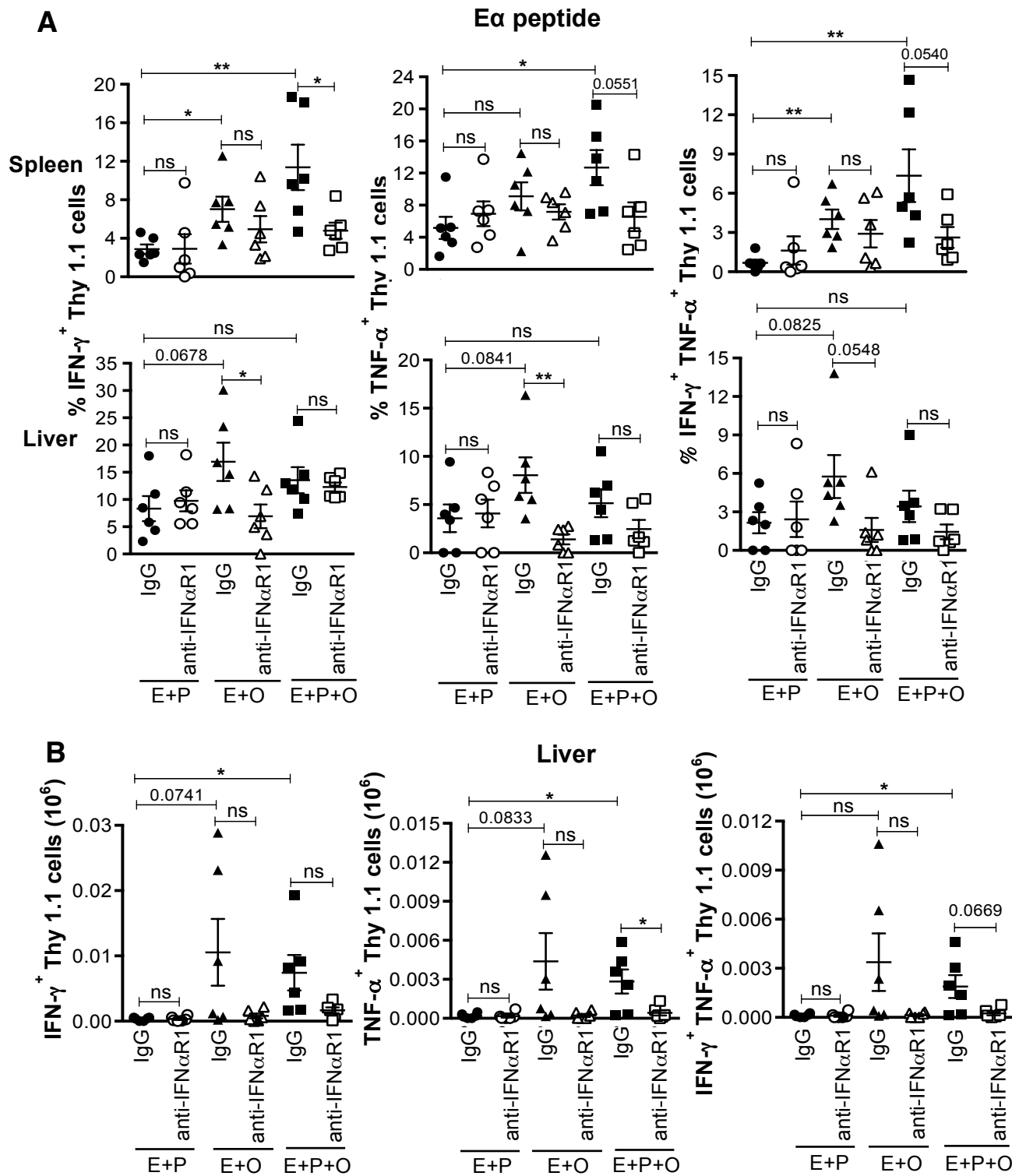


Figure 4-2. Administration of Ag together with poly I:C and anti-CD134 promotes enhanced Th1 differentiation of specific CD4 T cells. Mice treated as in Fig. 4-2 were analyzed for the percent and total number of IFN- γ^+ , TNF- α^+ and IFN- γ^+ TNF- α^+ expressing Thy 1.1 cells in spleen and liver after *in vitro* stimulation with E α peptide. Data is representative of 3 independent experiments with p value, * < 0.05, ** < 0.01. Error bars represent SEM of all experiments in that group.

Figure 4-3

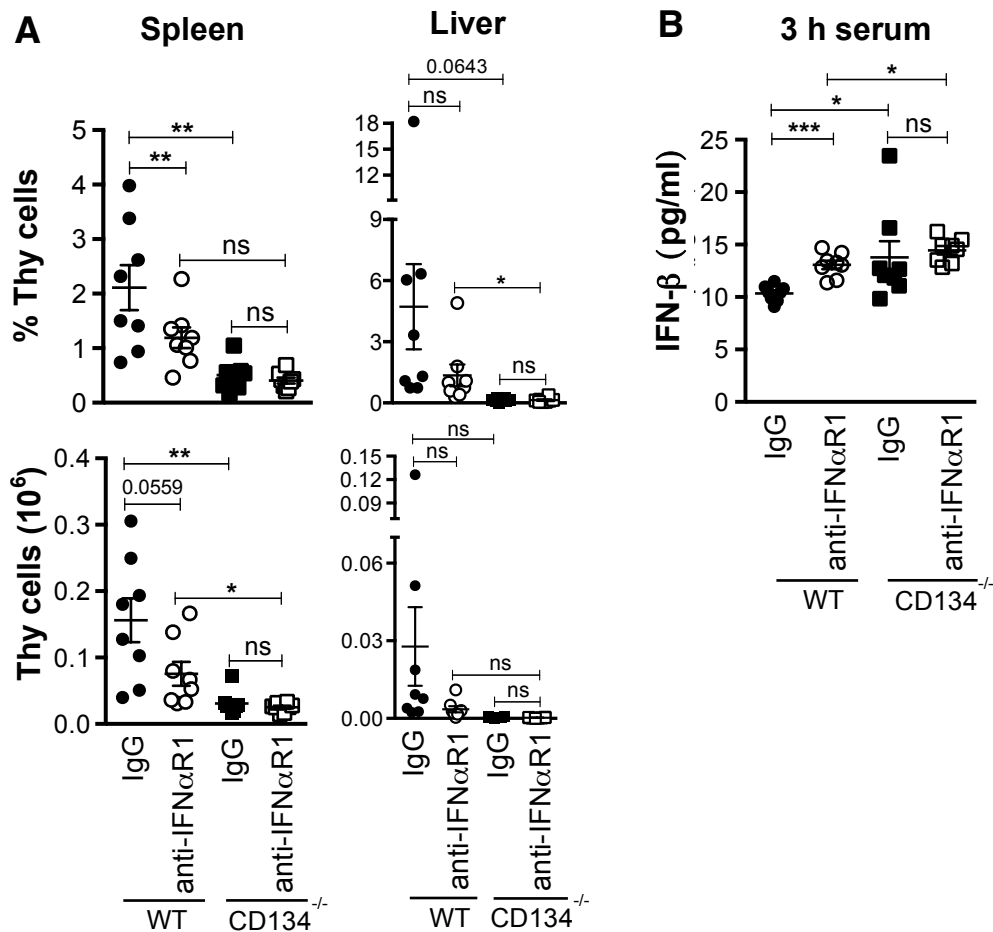


Figure 4-3. CD134 costimulation requires IFN α R signaling to promote specific CD4 T cell expansion. About 1×10^6 WT or CD134^{-/-} Thy 1.1 TEa cells were separately transferred into C57Bl/6 mice followed by treatment with anti-IFN α R1 mAb or IgG control and immunization with E α peptide and anti-CD134. The data represents **A**. The percent and total number of WT (TEa⁺ Thy 1.1⁺ Thy 1.2⁺) and CD134^{-/-} (TEa⁺ Thy 1.1⁺ Thy 1.2⁻) cells in the spleen and liver of mice on day 8 following immunization. **B**. Elisa analysis of IFN- β levels in serum, 3 h after immunization in mice treated as above. The data is representative of 4 independent experiments with p value, * < 0.05, ** < 0.01, *** < 0.001. Error bars represent SEM of all experiments in that group.

Figure 4-4 A

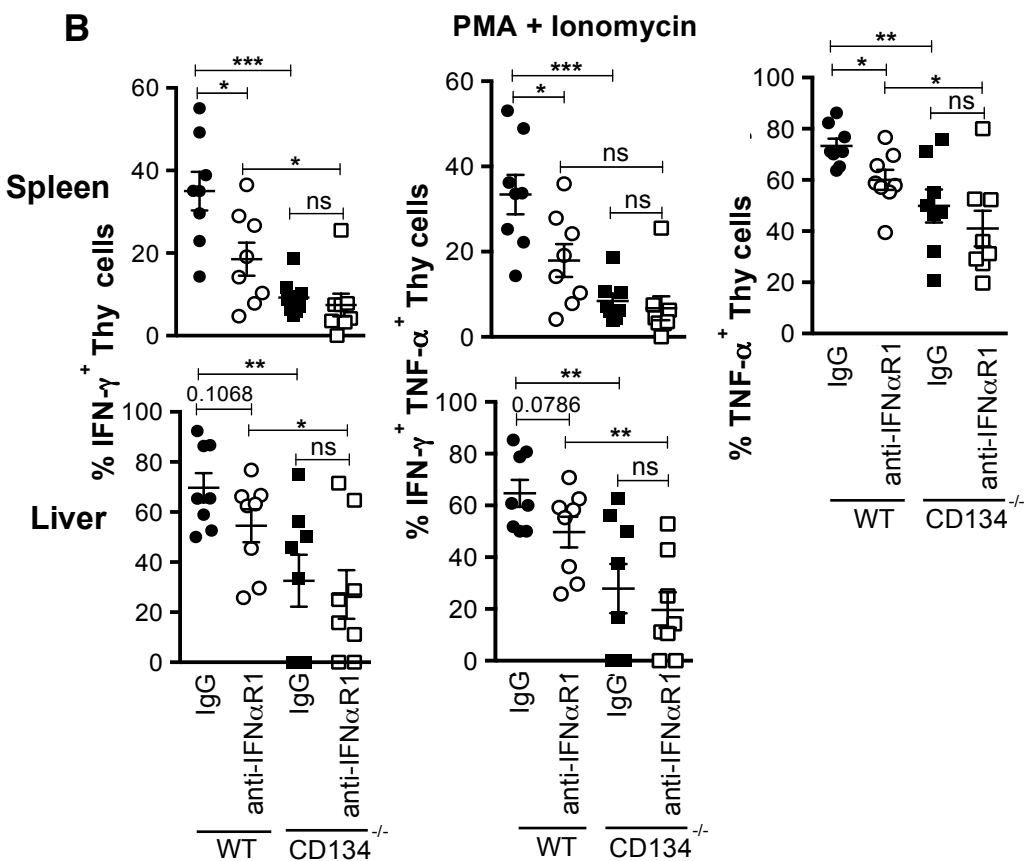
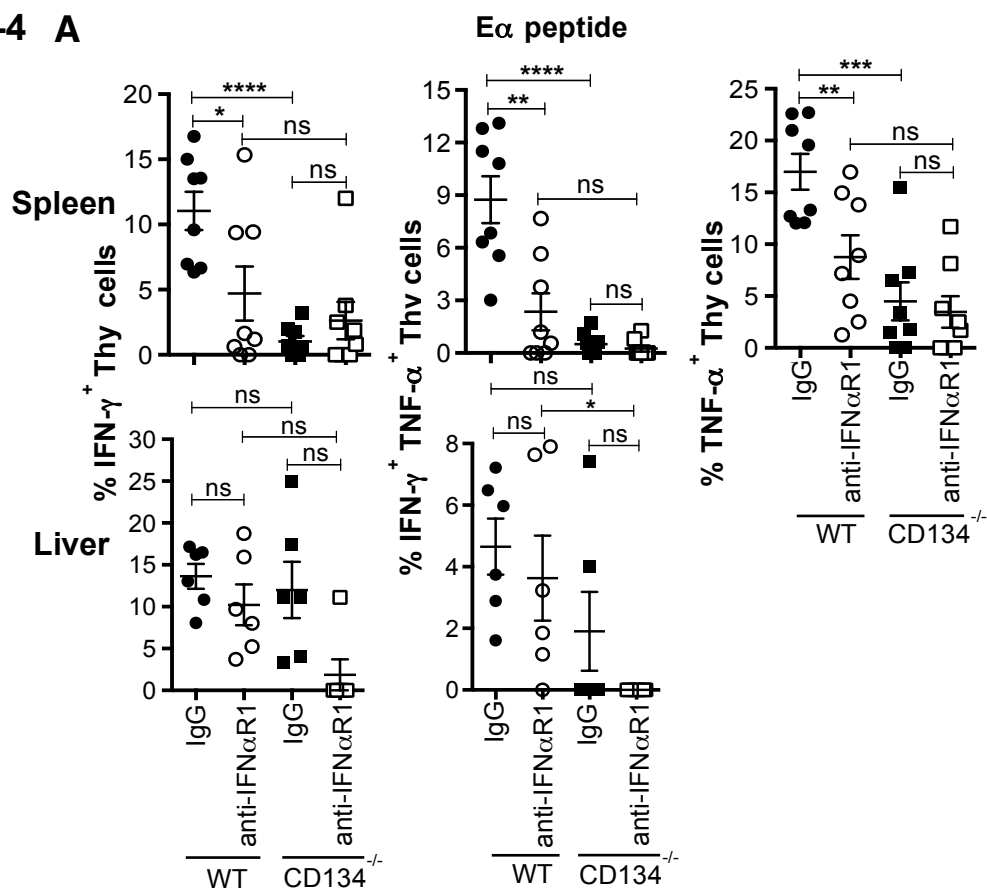


Figure 4-4. CD134 costimulation requires IFN α R signaling to promote optimal Th1 differentiation. Mice treated in Fig 4-3, were analyzed for the percent of IFN- γ ⁺, TNF- α ⁺, IFN- γ ⁺ TNF- α ⁺ expressing WT and CD134^{-/-} Thy 1.1 cells in spleen and liver after *in vitro* stimulation with **A.** E α peptide and **B.** PMA+Ionomycin. The data is representative of 4 independent experiments with p value, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. Error bars represent SEM of all experiments in that group.

Figure 4-5

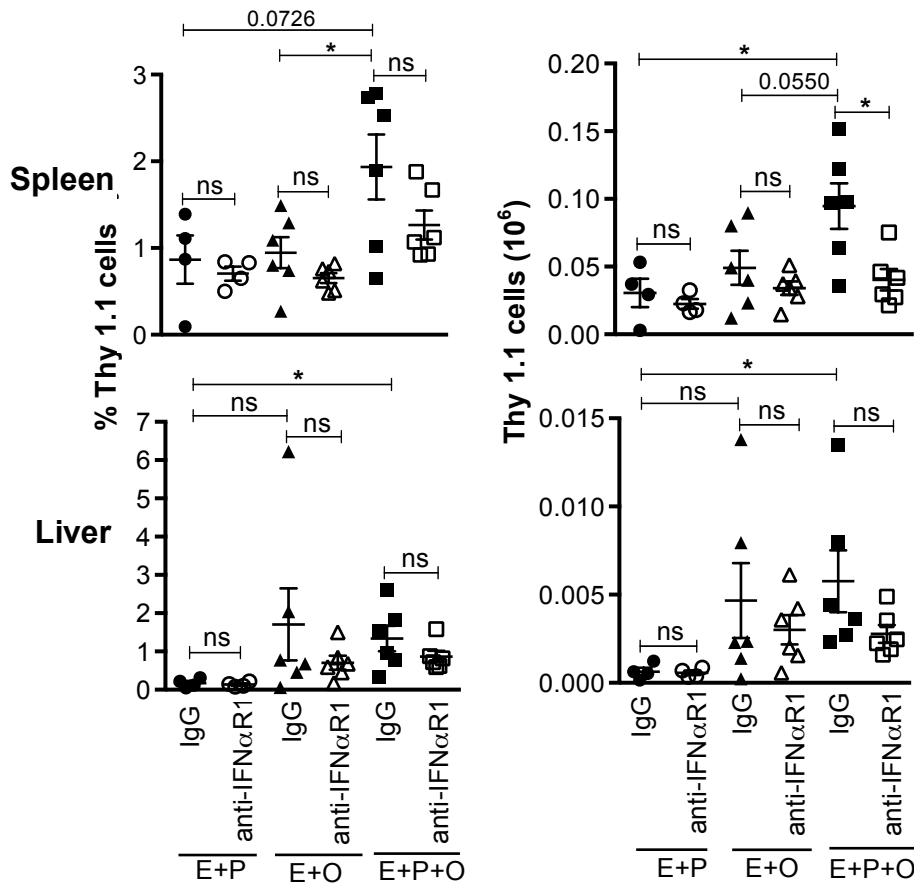


Figure 4-5. Administration of Ag with poly I:C and anti-CD134 promotes maximal expansion of specific CD4 T cells during memory response. About 1×10^6 Thy 1.1 TEa cells were transferred into C57BL/6 mice followed by their treatment with anti-IFN α R1 mAb or IgG control and immunization with E α +poly I:C, E α +anti-CD134 or E α +poly I:C+anti-CD134. Mice were re-challenged on day 30 or 31 with E α + poly I:C, following which the percent (left panel) and total number of Thy 1.1 cells ($\times 10^6$) (right panel) were analyzed on day 6 in spleen and liver. The data is representative of 2 independent experiments with p value, * < 0.05. Error bars represent SEM of all experiments in that group.

Figure 4-6

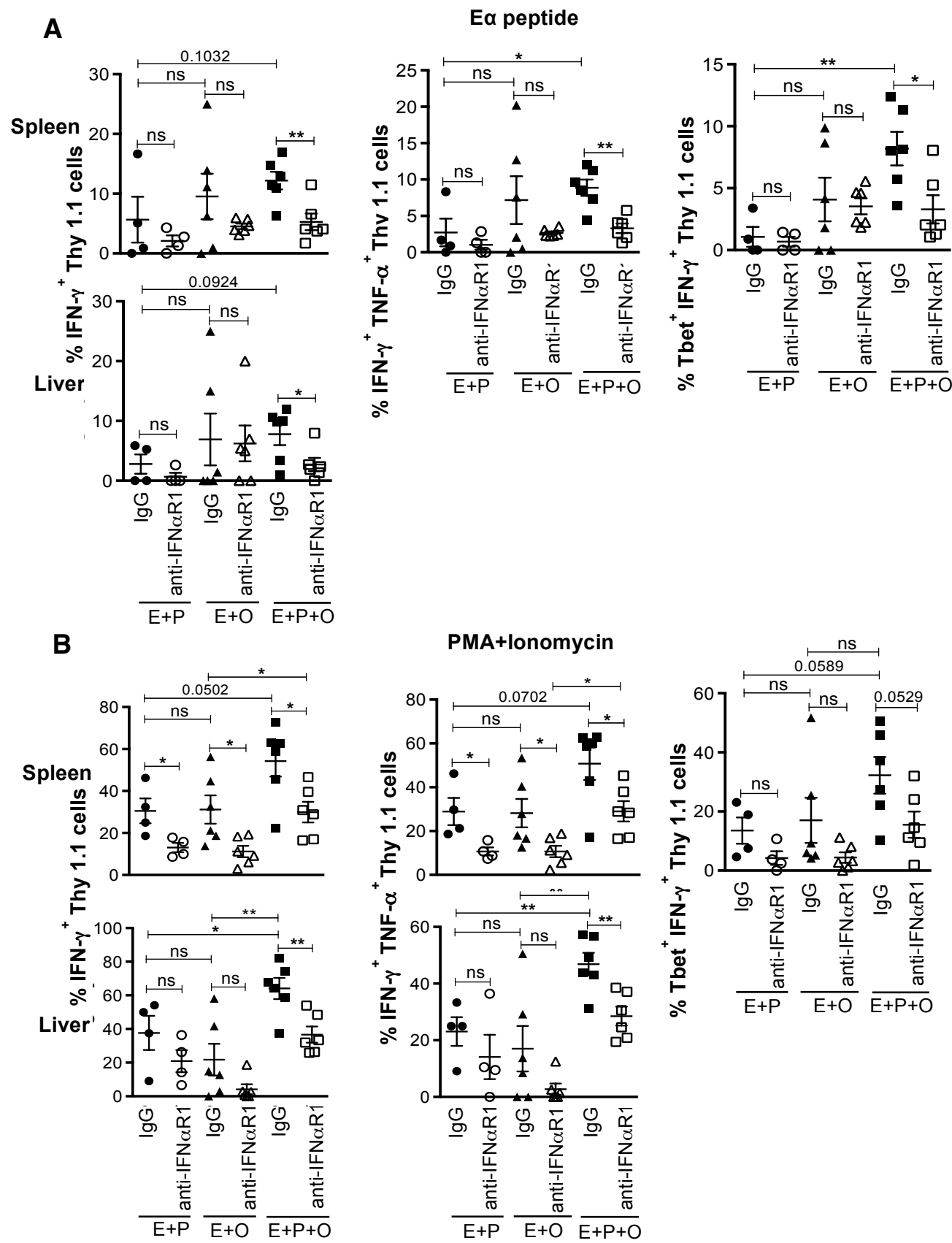


Figure 4-6. Administration of Ag with poly I:C and anti-CD134 promotes enhanced Th1 differentiation of specific T cells during memory response. Mice treated in Fig. 4-5 were analyzed for the percent and total number of IFN- γ ⁺, TNF- α ⁺ and IFN- γ ⁺ TNF- α ⁺ expressing Thy 1.1 cells in spleen and liver after *in vitro* stimulation with **A.** E α peptide and **B.** PMA+Ionomycin stimulation. The data is representative of 4 independent experiments with p value, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. Error bars represent SEM of all experiments in that group.

Figure 4-7

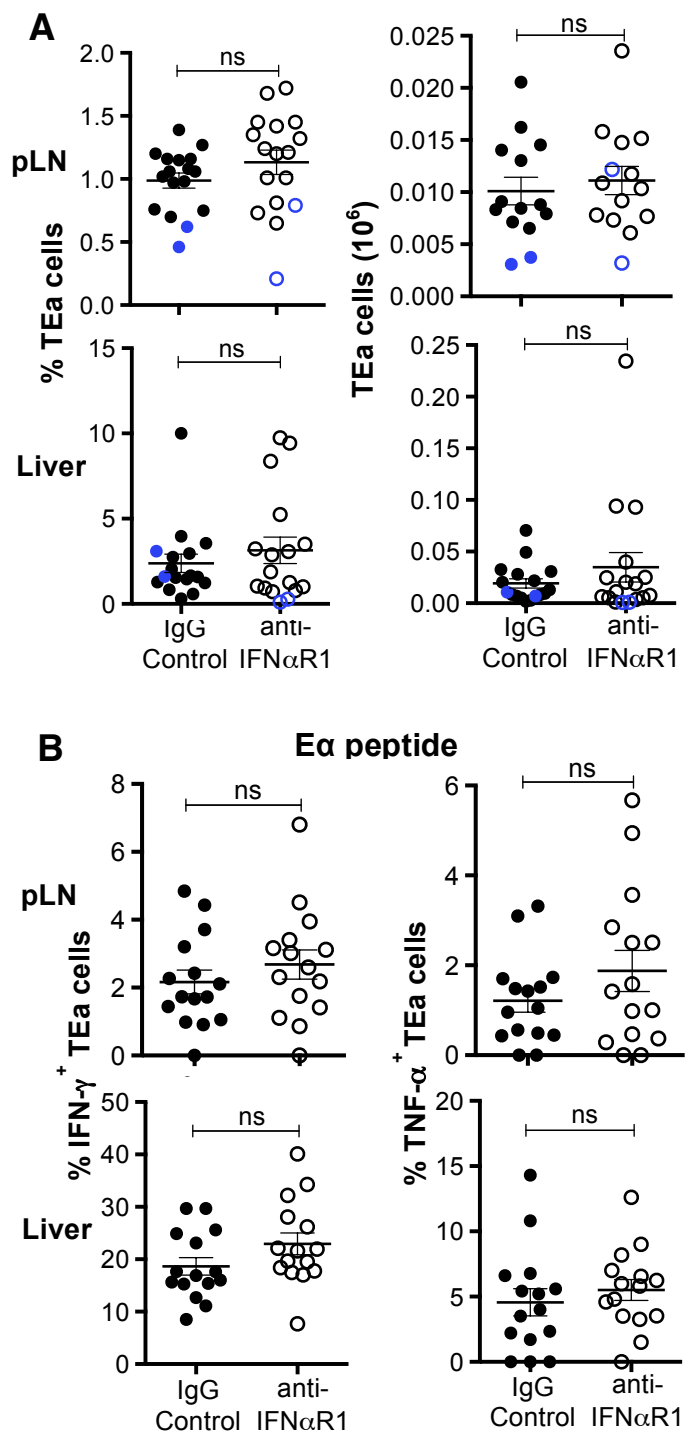


Figure 4-7. LPS induced IFN α R signaling is not required for specific CD4 T cell expansion and Th1 differentiation during memory response. Mice were treated as in Fig. 3-2, briefly $\sim 1 \times 10^5$ CD4 TEa or Thy 1.1 cells were transferred into C57BL/6 mice followed by their treatment with anti-IFN α R1 mAb or control and immunization with E α peptide, LPS and anti-CD134 mAb. On day 28 to day 31, mice were re-challenged with E α peptide and LPS, following which on day 5 or 7, memory CD4 T cells in pLN and liver were analyzed for **A.** Percent and total number ($\times 10^6$) of TEa or Thy 1.1 CD4 T cells. Black colored circles represent TEa cells whereas blue colored circles represent Thy 1.1 cells. **B.** Percent of IFN- γ^+ and TNF- α^+ expressing TEa cells after *in vitro* stimulation with E α peptide. Data is representative of 5-6 individual experiments, where error bars represent SEM of all experiments in that group.

Chapter V: DISCUSSION AND FUTURE DIRECTIONS

(I) Understanding role of LPS induced signaling pathways in promoting optimal CD4 T cell immunity

There are still no fully effective vaccines against many widespread infectious diseases, such as HIV, tuberculosis, and malaria. Although, humoral immunity has an important role in protecting against HIV infection [319] and influences certain stages of malaria infection [320], CD4-Th1 and CD8 T cell immunity also play a critical role in controlling these infections [321-323]. Moreover, accumulating evidence suggests that vaccines targeting CD4 T cells can be effective in generating potent anti-tumor responses [324-327]. In line with this, there have been major advances in the last decade in development of adjuvants, which enhance the magnitude of T cell response in a vaccine. However, much less work is done in understanding how these adjuvants work at molecular level and how signaling mechanisms induced by these adjuvants enhance the CD4, Th1 response. Therefore, our aim is to understand how adjuvants, LPS and poly I:C induced responses impact the CD4 T cell expansion and Th1 differentiation.

LPS, is a potent Th1 response inducing adjuvant, and injecting it systemically within a day after antigen exposure increases the level of T cell clonal expansion, long-term survival and T cell migration to the non-lymphoid tissues [328]. Even though MPL and not LPS, is used in approved human vaccines against HPV and HBV [14], due to its reduced toxicity, we decided to use LPS for few reasons (1) It promotes lasting CD4 T cell responses over MPL, which enhances short term CD4 T cell response [15] (2) To understand, if LPS induced IFN α R signaling could explain how LPS and anti-CD134 combination promotes enhanced CD4 T cell

expansion, survival and memory. (3) To analyze, if LPS-TRIF induced CD4 T cell migration and Th1 differentiation in non-lymphoid tissues of liver and lung, depends on downstream induction of type I IFN.

(II) LPS induced IFN α R signaling in both specific T cells and non-specific cells contribute to CD4 T cell expansion and Th1 differentiation

As mentioned above, one of our goals is to understand the mechanism of how LPS and CD134 agonist together promote enhanced CD4 T cell expansion and survival. Previous studies done in our lab have evaluated the role of different LPS induced pathways in enhancing the T cell response. LPS induced MyD88 pathway was shown to enhance T cell accumulation in both lymphoid and non-lymphoid tissues and much has been done in this area [11, 329]. Whereas, LPS induced TRIF signaling is known to promote initial T cell clonal expansion, up regulate the expression of costimulatory molecules on APCs and induce Th1 differentiation in CD4 T cells [47, 277]. Subsequent studies in our lab showed the importance of TRIF signaling in promoting CD4 T cell accumulation to peripheral tissues of liver and lung [20], but the mechanism behind this is unknown. TRIF is known to induce type I interferon production [40], and type I IFN is known to directly act on T cells to enhance their expansion and survival [48, 133]. Therefore, we hypothesized that, LPS-TRIF induced type I interferon, could enhance the CD4 T cell expansion in the vaccine combination of LPS and CD134 agonist. Our rationale behind this, was the enhanced production of type I IFN (IFN- β) (Fig. 3-1B), seen during E α peptide, LPS and anti-CD134 immunization, that corresponded with the synergistic T cell expansion this vaccine induced (Fig. 3-1C). Recently lipid A, included IFN α R signaling was shown to promote CD4 and CD8 T cell clonal expansion, during immunization

with lipid A and ovalbumin peptides (for MHC I and MHC II) [47]. However, they did not analyze if IFN α R signaling enhances CD4 T cell response in a vaccine model combining LPS and anti-CD134. They also did not evaluate the role of IFN α R signaling in promoting T cell expansion in tissues other than spleen.

Our results show that depending on the model used, IFN α R signaling in both specific CD4 T cells and non-specific cells of the host impact the T cell expansion. Using a competition model, we saw that IFN α R signaling in specific T cells was required for their expansion in pLN and liver (Fig. 3-3). However, analyzing specific T cell expansion in IFN α R1^{-/-} mice that received IFN α R sufficient T cells, suggested that IFN α R signaling in non-specific cells of the host also contribute to T cell expansion mainly in the liver and lung (Fig. 3-2D). Thus overall, IFN α R signaling in both specific T cells and non-specific cells promote CD4 T cell expansion.

However, unlike the report mentioned above, where lipid A induced IFN α R signaling was required for T cell clonal expansion in spleen [47], we did not see a difference in CD4 T cell expansion in spleen. It is possible that additional inflammatory cytokines induced by LPS in comparison to lipid A, may support some level of T cell expansion in spleen after IFN α R blockade. Additionally, addition of CD134 agonist to LPS, may impact the type I interferon mediated T cell expansion differently in comparison to lipid A alone.

In terms of Th1 differentiation, we saw that IFN α R signaling in non-specific cells of the host was predominantly required to promote Th1 differentiation from both specific and non-specific CD4 T cells, since IFN- γ production by specific T cells after E α peptide stimulation, was significantly reduced in liver and also trended towards significant reduction in pLN of IFN α R1^{-/-} mice (Fig. 3-4B, left panels). Moreover, IFN- γ production was also significantly reduced in non-

specific CD4 T cells in pLN and lung of IFN α R1^{-/-} mice after PMA+Ionomycin stimulation (Fig. 3-4B, right panels).

The mechanism of how IFN α R signaling in non-specific cells impact CD4 T cell expansion or Th1 differentiation could be evaluated in future using a model, where Rag^{-/-} bone marrow is mixed with IFN α R1^{-/-} bone marrow and transferred to lethally-irradiated IFN α R1^{-/-} hosts. In this case, T cells will be exclusively IFN α R-deficient, but APCs will be both WT and IFN α R deficient. Mice could then be immunized with SEA, LPS and anti-CD134, followed by the analysis of V β 3⁺ T cell expansion and Th1 differentiation. This will tell us, if IFN α R signaling from Rag^{-/-} bone marrow could rescue the T cell expansion and Th1 differentiation in IFN α R1^{-/-} hosts.

Additional studies will be required to understand, if IFN α R signaling promotes CD4 T cell expansion by impacting T cell proliferation [142] or preventing T cell death to increase their survival [48]. IFN α R signaling could also impact T cell expansion indirectly by promoting the maturation of DCs; by enhancing the expression of MHC-II and costimulatory molecules [277] to prime the T cells optimally. It is important to note that, IFN- γ production in specific CD4 T cells was reduced in pLN and liver of IFN α R1^{-/-} mice (Fig. 3-4B) that correlates with reduced CXCL9 and CXCL10 levels in serum of these mice (Fig. 3-7C). This could mean that, IFN α R signaling impacts specific CD4 T cell expansion indirectly by inducing chemokine's CXCL9 and CXCL10 through IFN- γ production.

(III) Chemokine CXCL9 plays a significant role in promoting CD4 T cell accumulation in lymphoid as well as non-lymphoid tissues

Before evaluating, if IFN α R signaling promotes T cell accumulation through induction of chemokines, CXCL9 and CXCL10, we analyzed if it enhances the expression of chemokine receptor CXCR3 on specific CD4 T cells. Previous study from our lab showed that, LPS induced TRIF signaling enhanced the CXCR3 expression on specific CD4 T cells in pLN and liver, which could explain the T cell migration to peripheral tissue of liver [20]. Our results show that, IFN α R1 blockade reduced the accumulation of CXCR3⁺ specific (Fig. 3-5, left panel) as well as non-specific CD4 T cells (Fig. 3-5, right panel) in pLN, however IFN α R signaling influenced the accumulation of only specific CD4 T cells in pLN (Fig. 3-2C), but not of non-specific CD4 T cells (not shown). IFN α R blockade also did not influence the accumulation of CXCR3⁺ specific T cells in liver and lung. Therefore, increased migration of CXCR3⁺ CD4 T cells in pLN, could not explain how IFN α R signaling promotes CD4 T cell expansion in liver and lung. In line with this, we saw that CXCR3 receptor was functional on specific CD4 T cells after IFN α R blockade, since chemokines, CXCL9 and CXCL10 were able to promote their migration in a chemotaxis assay (Fig. 3-6). This suggested that, IFN α R signaling might impact the expression CXCR3 ligands rather than CXCR3 expression. Indeed, we saw that CXCL9 and CXCL10 expression were significantly reduced in the absence of IFN α R signaling (Fig. 3-7C). Moreover, LPS induced TRIF signaling was required for CXCL9 production (Fig. 3-7D, left panel), suggesting that LPS-TRIF mediated CD4 T cell accumulation to liver and lung in previous studies [20], could be a result of enhanced CXCL9 production in this tissues.

When we evaluated, the role of these chemokine's in promoting T cell accumulation, we saw that CXCL9 promoted significant T cell accumulation in the pLN and liver (Fig. 3-8A), whereas

CXCL10 did not significantly impact the T cell accumulation (not shown). This could have been due to incomplete blockade of CXCL10, as IFN α R signaling induced higher levels of CXCL10 in our vaccine model (Fig. 3-7C, rightmost panel). Perhaps, blocking CXCL10 with higher concentration of mAb in future could help ascertain if CXCL10 impacts the T cell accumulation similar to CXCL9. It will also be interesting to analyze if combined blockade of CXCL9 and CXCL10 could impact T cell accumulation more profoundly, since it was recently shown that collaboration between CXCL9 and CXCL10 is required to promote T cell-DC interaction and localization of T cells to LN periphery near the pathogen infected cells to allow their Th1 differentiation [197]. However, in our vaccine model, CXCL9 or CXCL10 did not impact Th1 differentiation (not shown) probably since our vaccine employs CD134 agonist, which under appropriate conditions is known induce Th1 differentiation [17, 239], and could rescue the cytokine secretion after CXCL9 or CXCL10 blockade.

To further evaluate the mechanism of how CXCL9 promotes T cell accumulation, we analyzed if it enhances the migration of CXCR3⁺ specific CD4 T cells and saw that CXCL9 did enhance accumulation of CXCR3⁺ specific T cells in pLN (Fig. 3-8B), however it did not do so in liver and lung (not shown). If CXCL9 would have impacted T cell migration from pLN (where T cells initially mount an immune response) to the liver and lung, reduced number of T cells in liver and lung after CXCL9 blockade, would have resulted in increased T cell numbers in the pLN, but that was not the case. Thus CXCL9 does not seem to impact CD4 T cell accumulation through increased T cell migration. It is possible that CXCL9 recruits other CXCR3 expressing cells such as NK cells [189] and pDC's [330] which may produce IFN- γ in response to type I IFN to further enhance CXCL9 production, creating a feed forward loop to promote CD4 T cell

accumulation. We haven't analyzed if CXCL9 impacts accumulation of these cells in our model and it could be evaluated in the future studies.

Another possibility is that, CXCL9 directly acts on T cells to enhance their proliferation, however, we did not find any significant difference in Ki67 expression of specific CD4 T cells, with or without CXCL9 blockade (not shown). CXCL9 could work by inhibiting the T cell death and promoting their survival, however the challenge in this regard is to detect dead specific T cells *in vivo* that have not bound CXCL9. However, it could be analyzed in future by staining specific CD4 T cells with annexin V or analyzing the expression of Bcl-xL and Bcl-2 (anti-apoptotic proteins) in specific T cells after treatment with anti-CXCL9 or control Ab.

Overall our results show, that CXCL9 plays a dominant role in promoting T cell accumulation in lymphoid (pLN) and non-lymphoid tissue (liver and lung) and this was seen in spite of no difference in the surrounding levels of type I IFN. Our future studies will focus on understanding the mechanism of how CXCL9 impacts T cell accumulation.

(IV) Combining poly I:C with CD134 agonist promotes long lasting Th1 immunity

As discussed before, for fighting against infectious diseases such as HIV, TB and malaria as well as in cancer, both Th1 and CD8 T cell immunity is important. Poly I:C's ability to induce both Th1 and CD8 T cell response makes it an attractive vaccine adjuvant [21, 22, 58, 331]. Recently, few studies using combinatorial approaches showed that, combining poly I:C with a potent costimulatory molecule CD40 promotes enhanced effector CD4 and CD8 T cell responses which provide protection in several infectious diseases [201, 202, 204, 205, 313] as well as tumor models [314-316]. Subsequent reports indicated that the synergistic CD4 T cell response induced by poly I:C and anti-CD40 is dependent on the expression of CD134L and

CD70 on CD8⁺ DC's [206]. However, CD134L was found more crucial in comparison to CD70 in enhancing the CD4 T cell response to poly I:C and anti-CD40 immunization. It was shown later in subsequent studies that poly I:C induced IFN α R signaling enhances CD134L expression on CD8⁺ DC's [27]. Therefore, we evaluated if poly I:C can be directly combined with CD134 agonist to promote enhanced CD4 T cell expansion and Th1 differentiation similar to poly I:C and anti-CD40 combination. Our results show that combining Ag with poly I:C and anti-CD134 promotes enhanced CD4 T cell expansion (Fig. 4-1B) and Th1 differentiation (Fig. 4-2B) during primary immune response and promotes the generation of long lasting effector memory CD4 T cells (Fig. 4-5 and 4-6). Importantly, the enhanced CD4 T expansion and Th1 differentiation induced by poly I:C and anti-CD134 combination was dependent on IFN α R signaling. Poly I:C was the main source of type I IFN in this combination (Fig. 4-1A), whereas anti-CD134 induced minimal levels of type I IFN. We however do not know the cellular source of type I IFN and whether poly I:C acts through TLR3 or MDA5 signaling pathway. It will be interesting to analyze the specific role of these signaling pathways in enhancing CD4 T cell immunity during poly I:C and anti-CD134 vaccination.

Similar to our results, a recent study showed that addition of CD134 agonist to trivax vaccine, (a vaccine combining peptide Ag's with poly I:C and anti-CD40) enhances the long-lasting effector CD4 T cell response [315]. However, unlike our study, their results show that addition of anti-CD134 to trivax, does not enhance the primary CD4 T cell response, but rather prime and boost immunization with anti-CD134 and trivax enhances the long-term effector CD4 T cell response. The anti-CD134 mAb they have used in their study, is derived from rat (as used in our model) and could generate antibodies in mouse with repeated prime and boost immunization, however they did not address this issue. They also have used higher dose of

anti-CD134 Ab (200 µg) to generate long-term effector CD4 T cell response, whereas our results show that, as less as of 7 µg of anti-CD134 Ab could be combined with poly I:C to enhance both short-term and long-term CD4 T cell response. It will be important in future, to compare within the same experiment, how single immunization with Ag+poly I:C+anti-CD134 and Ag+poly I:C+anti-CD40+anti-CD134 impacts primary and memory CD4 T cell response, to accurately ascertain if the addition of anti-CD40 to poly I:C and anti-CD134 used in our model can be beneficial in providing optimal CD4 T cell immunity.

It is important to note that, unlike poly I:C and anti-CD134 combination, LPS induced IFN α R signaling was not essential for generation of effector memory CD4 T cells in Ag, LPS and anti-CD134 vaccine combination (Fig. 4-7). Rather inhibition of IFN α R signaling during early T cell priming resulted in slight increase in CD4 T cell expansion and Th1 differentiation during the recall response. The dispensable role of type I IFN during LPS and anti-CD134 immunization, whereas poly I:C's dependence on it for generation of CD4 Th1-type cells could be a result of higher levels of type I IFN induced by poly I:C, through activation of both TLR3 and MDA-5 pathways, in comparison to LPS, which activates only TLR4-TRIF pathway.

(VI) CD134 stimulation relies on IFN α R signaling to promotes optimal Th1 differentiation

Our results, demonstrate that CD134 costimulation in specific T cells, requires IFN α R signaling to promote CD4 T cell expansion and Th1 differentiation in the spleen (Fig 4-3 and 4-4).

CD134 signaling is been targeted in several preclinical cancer trials to promote enhanced generation of effector CD4 and CD8 T cells. Hence, understanding the mechanism of how CD134 costimulation induces IFN α R signaling to promote CD4 T cell expansion and Th1 differentiation is important. There are few possibilities that could be evaluated. (1) CD134

agonist mAb targeted to CD4 T cells could bind through its other end to the Fc γ Rs expressed on innate cells such as APCs to promote release of type I IFN. It has been shown before with monocyte derived DC's that simultaneous binding of antibodies targeting inhibitory Fc γ Rs and binding of IgGs from human plasma to the activating Fc γ Rs promote the induction of genes downstream of type I IFN [332]. To understand if anti-CD134 mAb binds to the Fc receptor to trigger type I IFN release, we could evaluate if Fc less anti-CD134 mAb results in reduced type I IFN secretion or impacts CD4 T cell responses *in vivo* distinctly in comparison to Fc receptor bearing anti-CD134 mAb (2) Additionally, CD134 expression on T cells could also regulate IFN α R expression on them, so that the minimal levels of type I IFN, induced during E α and anti-CD134 immunization bind to the enhanced levels of IFN α R1 expressed on WT T cells and activate the signaling.

Understanding, how CD134 costimulation triggers IFN α R signaling could help evaluate in future if targeting CD134 in cancer models results in optimal effector CD4 T cell response and anti-tumor immunity or would result in unwanted side effects due to excessive induction of type I Interferon induced Th1-type effector cytokines.

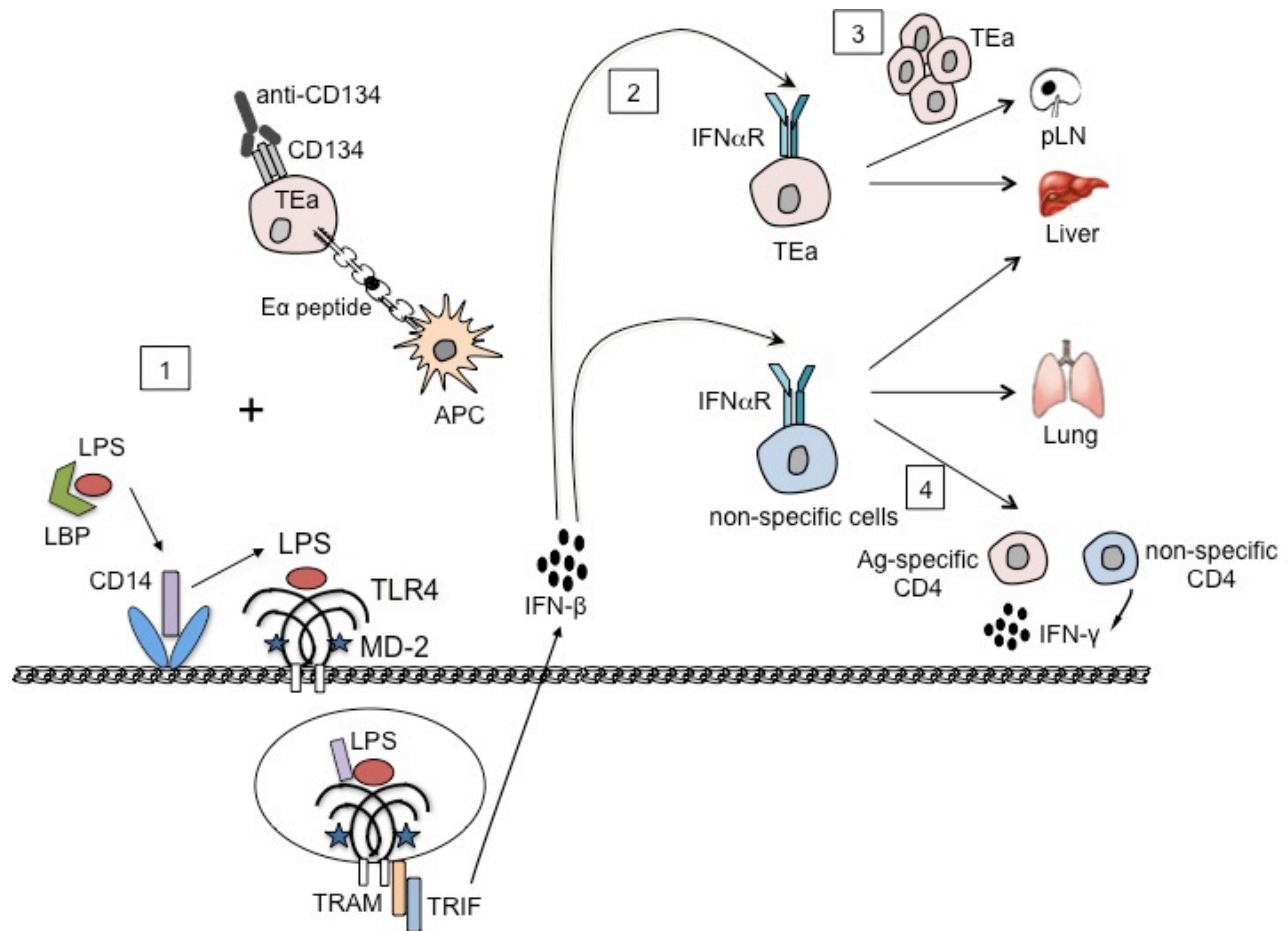


Fig 5-1. IFNαR1 signaling in both Ag-specific and non-specific cells is required to promote specific CD4 T cell response during Ag, LPS and anti-CD134 immunization. (1) During immunization with LPS and anti-CD134, LPS activates TLR4-TRIF pathway to induce type I IFN (IFN-β). (2) Type I IFN activates the IFNαR signaling in specific CD4 T cells as well as non-specific cells of the host (unknown). (3) IFNαR signaling in specific CD4 T cells promotes Ag-specific CD4 T cell expansion in pLN and liver, whereas signaling in non-specific cells of host is required for specific T cell expansion in liver and lung (4). Furthermore, IFNαR signaling in non-specific cells of the host, triggers IFN-γ production from specific (pLN, liver) as well as non-specific CD4 T cells (pLN, lung). Ours is the first study evaluating how LPS induced IFNαR signaling in Ag-specific T cells versus non-specific cells impact the CD4 T cell response.

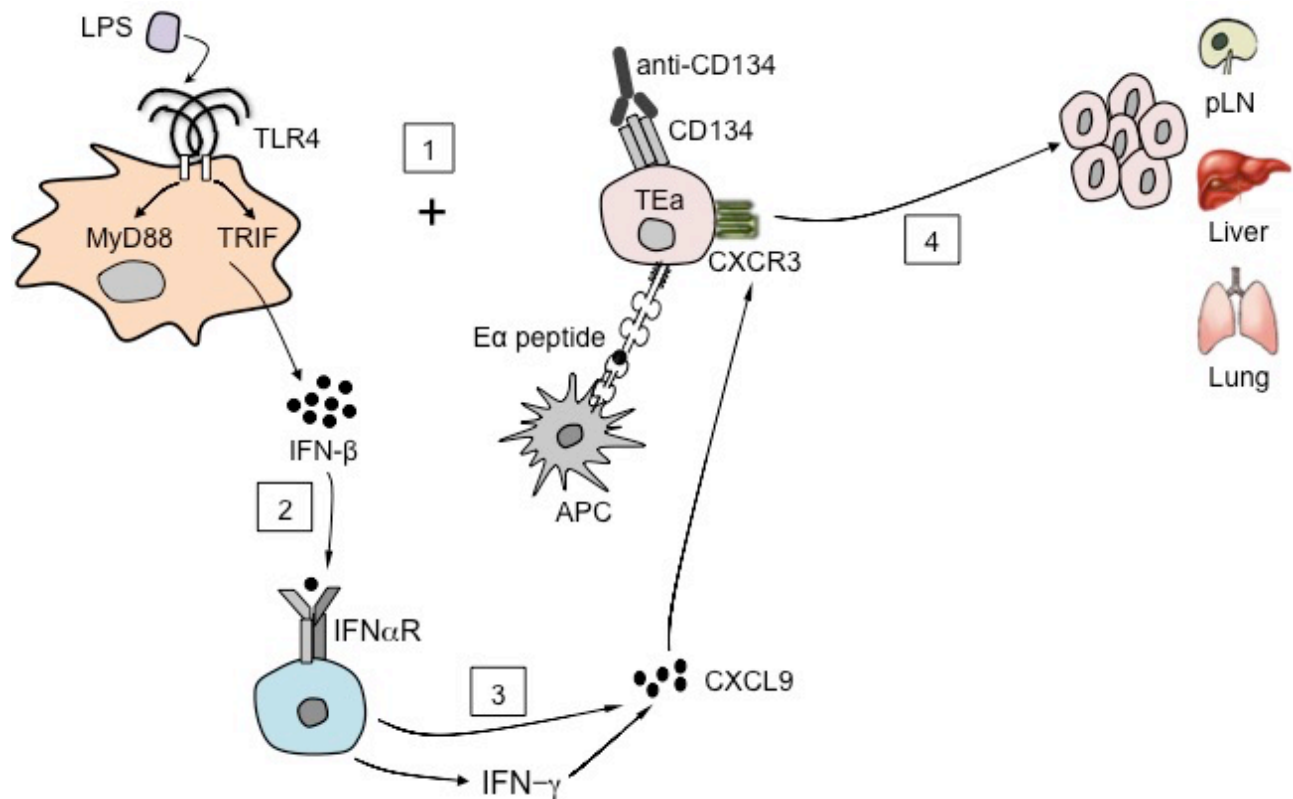


Figure 5-2. LPS induced IFN α R1 signaling triggers CXCL9 production to promote Ag-specific CD4 T cell accumulation in lymphoid as well as non-lymphoid tissues.

(1) During immunization with Ag, LPS and anti-CD134, LPS activates TLR4-TRIF pathway to promote type I IFN (IFN- β) production. (2) IFN- β binds to the IFN α R and activates IFN α R signaling (3) IFN α R signaling promotes the release of chemokine CXCL9, which could also be induced indirectly through IFN- γ induced during LPS and anti-CD134 immunization (data not shown). (4) Following this, CXCL9 may bind CXCR3 on activated CD4 T cells and promote their accumulation in pLN, liver and lung. The mechanism behind this is unknown. However, it suggests that CXCL9 could be targeted in T cell vaccination strategies to promote Ag-specific CD4 T cell accumulation in both lymphoid and non-lymphoid tissues.

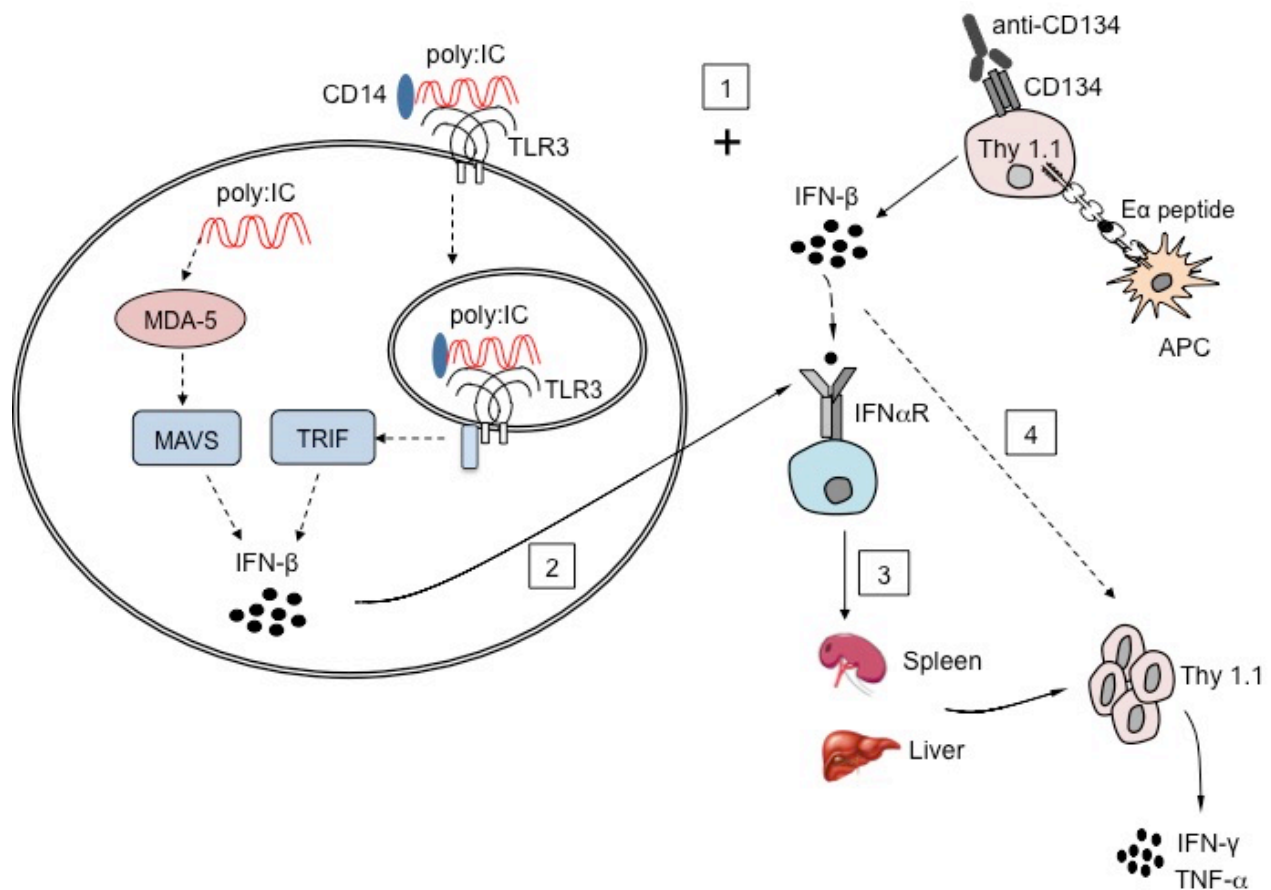


Figure 5-3. Ag, poly:I:C and anti-CD134 induced maximal CD4 T cell expansion and Th1 differentiation is dependent on IFN α R1 signaling, however CD134 costimulation also requires IFN α R1 signaling to promote CD4 T cell response. (1) During poly I:C and anti-CD134 immunization poly I:C could trigger type I IFN, (IFN- β) induction through either TLR3 or MDA-5 signaling pathways. (2) Released type I IFN, then activates IFN α R signaling, which together with CD134 costimulation promotes maximal CD4 T cell expansion and Th1 differentiation in spleen and liver during both primary and recall response. (4) CD134 costimulation in T cells also induces minimal levels of type I IFN (IFN- β) which promotes CD4 T cell expansion and Th1 differentiation through an unknown mechanism. The mechanism of how CD134 costimulation triggers IFN α R signaling needs to be evaluated in future, for efficient targeting of CD134 in preclinical tumor models.

References

1. De Gregorio, E., Caproni, E., Ulmer, J. B. (2013) Vaccine adjuvants: mode of action. *Front Immunol* 4, 214.
2. Lindblad, E. B. (2004) Aluminium compounds for use in vaccines. *Immunol Cell Biol* 82, 497-505.
3. Petrovsky, N. and Aguilar, J. C. (2004) Vaccine adjuvants: current state and future trends. *Immunol Cell Biol* 82, 488-96.
4. Didierlaurent, A. M., Morel, S., Lockman, L., Giannini, S. L., Bisteau, M., Carlsen, H., Kielland, A., Vosters, O., Vanderheyde, N., Schiavetti, F., Larocque, D., Van Mechelen, M., Garcon, N. (2009) AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J Immunol* 183, 6186-97.
5. Pantaleo, G. and Koup, R. A. (2004) Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nat Med* 10, 806-10.
6. Hoft, D. F. (2008) Tuberculosis vaccine development: goals, immunological design, and evaluation. *Lancet* 372, 164-75.
7. Reyes-Sandoval, A., Pearson, F. E., Todryk, S., Ewer, K. (2009) Potency assays for novel T-cell-inducing vaccines against malaria. *Curr Opin Mol Ther* 11, 72-80.
8. Johnson, A. G., Gaines, S., Landy, M. (1956) Studies on the O antigen of *Salmonella typhosa*. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide. *The Journal of experimental medicine* 103, 225-46.
9. Condie, R. M., Zak, S. J., Good, R. A. (1955) Effect of meningococcal endotoxin on the immune response. *Proc Soc Exp Biol Med* 90, 355-60.
10. Vella, A. T., McCormack, J. E., Linsley, P. S., Kappler, J. W., Marrack, P. (1995) Lipopolysaccharide interferes with the induction of peripheral T cell death. *Immunity* 2, 261-70.
11. McAleer, J. P., Zammit, D. J., Lefrancois, L., Rossi, R. J., Vella, A. T. (2007) The lipopolysaccharide adjuvant effect on T cells relies on nonoverlapping contributions from the MyD88 pathway and CD11c⁺ cells. *J Immunol* 179, 6524-35.

12. Qureshi, N., Takayama, K., Ribi, E. (1982) Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of *Salmonella typhimurium*. *J Biol Chem* 257, 11808-15.
13. Mata-Haro, V., Cekic, C., Martin, M., Chilton, P. M., Casella, C. R., Mitchell, T. C. (2007) The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* 316, 1628-32.
14. Casella, C. R. and Mitchell, T. C. (2008) Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cell Mol Life Sci* 65, 3231-40.
15. Thompson, B. S., Chilton, P. M., Ward, J. R., Evans, J. T., Mitchell, T. C. (2005) The low-toxicity versions of LPS, MPL adjuvant and RC529, are efficient adjuvants for CD4+ T cells. *J Leukoc Biol* 78, 1273-80.
16. Kaleeba, J. A., Offner, H., Vandenbark, A. A., Lublinski, A., Weinberg, A. D. (1998) The OX-40 receptor provides a potent co-stimulatory signal capable of inducing encephalitogenicity in myelin-specific CD4+ T cells. *Int Immunol* 10, 453-61.
17. Gramaglia, I., Weinberg, A. D., Lemon, M., Croft, M. (1998) Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol* 161, 6510-7.
18. Weinberg, A. D., Morris, N. P., Kovacsovics-Bankowski, M., Urba, W. J., Curti, B. D. (2011) Science gone translational: the OX40 agonist story. *Immunol Rev* 244, 218-31.
19. Maxwell, J. R., Weinberg, A., Prell, R. A., Vella, A. T. (2000) Danger and OX40 receptor signaling synergize to enhance memory T cell survival by inhibiting peripheral deletion. *J Immunol* 164, 107-12.
20. McAleer, J. P., Rossi, R. J., Vella, A. T. (2009) Lipopolysaccharide potentiates effector T cell accumulation into nonlymphoid tissues through TRIF. *J Immunol* 182, 5322-30.
21. Trumpfheller, C., Caskey, M., Nchinda, G., Longhi, M. P., Mizenina, O., Huang, Y., Schlesinger, S. J., Colonna, M., Steinman, R. M. (2008) The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. *Proc Natl Acad Sci U S A* 105, 2574-9.
22. Longhi, M. P., Trumpfheller, C., Idoyaga, J., Caskey, M., Matos, I., Kluger, C., Salazar, A. M., Colonna, M., Steinman, R. M. (2009) Dendritic cells require a systemic type I

interferon response to mature and induce CD4⁺ Th1 immunity with poly IC as adjuvant. *The Journal of experimental medicine* 206, 1589-602.

23. Tewari, K., Flynn, B. J., Boscardin, S. B., Kastenmueller, K., Salazar, A. M., Anderson, C. A., Soundarapandian, V., Ahumada, A., Keler, T., Hoffman, S. L., Nussenzweig, M. C., Steinman, R. M., Seder, R. A. (2010) Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4⁺ T cell responses to *Plasmodium falciparum* circumsporozoite protein (CSP) and alphaDEC-CSP in non human primates. *Vaccine* 28, 7256-66.
24. Ohlfest, J. R., Andersen, B. M., Litterman, A. J., Xia, J., Pennell, C. A., Swier, L. E., Salazar, A. M., Olin, M. R. (2013) Vaccine injection site matters: qualitative and quantitative defects in CD8 T cells primed as a function of proximity to the tumor in a murine glioma model. *J Immunol* 190, 613-20.
25. Cho, H. I. and Celis, E. (2009) Optimized peptide vaccines eliciting extensive CD8 T-cell responses with therapeutic antitumor effects. *Cancer Res* 69, 9012-9.
26. Nagato, T., Lee, Y. R., Harabuchi, Y., Celis, E. (2014) Combinatorial immunotherapy of polyinosinic-polycytidylic acid and blockade of programmed death-ligand 1 induce effective CD8 T-cell responses against established tumors. *Clin Cancer Res* 20, 1223-34.
27. Kurche, J. S., Haluszczak, C., McWilliams, J. A., Sanchez, P. J., Kedl, R. M. (2012) Type I IFN-dependent T cell activation is mediated by IFN-dependent dendritic cell OX40 ligand expression and is independent of T cell IFNR expression. *J Immunol* 188, 585-93.
28. Palsson-McDermott, E. M. and O'Neill, L. A. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113, 153-62.
29. Caroff, M. and Karibian, D. (2003) Structure of bacterial lipopolysaccharides. *Carbohydr Res* 338, 2431-47.
30. Alexander, C. and Rietschel, E. T. (2001) Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res* 7, 167-202.
31. Antonopoulos, C., El Sanadi, C., Kaiser, W. J., Mocarski, E. S., Dubyak, G. R. (2013) Proapoptotic chemotherapeutic drugs induce noncanonical processing and release of IL-1 β via caspase-8 in dendritic cells. *J Immunol* 191, 4789-803.

32. Viriyakosol, S., Tobias, P. S., Kitchens, R. L., Kirkland, T. N. (2001) MD-2 binds to bacterial lipopolysaccharide. *J Biol Chem* 276, 38044-51.
33. Ogata, H., Su, I., Miyake, K., Nagai, Y., Akashi, S., Mecklenbrauker, I., Rajewsky, K., Kimoto, M., Tarakhovsky, A. (2000) The toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. *The Journal of experimental medicine* 192, 23-9.
34. Kawai, T. and Akira, S. (2007) TLR signaling. *Semin Immunol* 19, 24-32.
35. Kagan, J. C. and Medzhitov, R. (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* 125, 943-55.
36. Bonham, K. S., Orzalli, M. H., Hayashi, K., Wolf, A. I., Glanemann, C., Weninger, W., Iwasaki, A., Knipe, D. M., Kagan, J. C. (2014) A promiscuous lipid-binding protein diversifies the subcellular sites of toll-like receptor signal transduction. *Cell* 156, 705-16.
37. Akira, S. and Hoshino, K. (2003) Myeloid differentiation factor 88-dependent and -independent pathways in toll-like receptor signaling. *J Infect Dis* 187 Suppl 2, S356-63.
38. Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., Takeda, K., Akira, S. (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* 420, 324-9.
39. Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S., Medzhitov, R. (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nature immunology* 9, 361-8.
40. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., Akira, S. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301, 640-3.
41. Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., Beutler, B. (2003) Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 424, 743-8.
42. Marrack, P. and Kappler, J. (1990) The staphylococcal enterotoxins and their relatives. *Science* 248, 705-11.
43. McCormack, J. E., Callahan, J. E., Kappler, J., Marrack, P. C. (1993) Profound deletion of mature T cells in vivo by chronic exposure to exogenous superantigen. *J Immunol* 150, 3785-92.

44. Pape, K. A., Khoruts, A., Mondino, A., Jenkins, M. K. (1997) Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigen-activated CD4⁺ T cells. *J Immunol* 159, 591-8.
45. Reinhardt, R. L., Khoruts, A., Merica, R., Zell, T., Jenkins, M. K. (2001) Visualizing the generation of memory CD4 T cells in the whole body. *Nature* 410, 101-5.
46. Vella, A. T., Mitchell, T., Groth, B., Linsley, P. S., Green, J. M., Thompson, C. B., Kappler, J. W., Marrack, P. (1997) CD28 engagement and proinflammatory cytokines contribute to T cell expansion and long-term survival in vivo. *J Immunol* 158, 4714-20.
47. Gandhapudi, S. K., Chilton, P. M., Mitchell, T. C. (2013) TRIF is required for TLR4 mediated adjuvant effects on T cell clonal expansion. *PLoS One* 8, e56855.
48. Marrack, P., Kappler, J., Mitchell, T. (1999) Type I interferons keep activated T cells alive. *The Journal of experimental medicine* 189, 521-30.
49. Pilling, D., Akbar, A. N., Girdlestone, J., Orteu, C. H., Borthwick, N. J., Amft, N., Scheel-Toellner, D., Buckley, C. D., Salmon, M. (1999) Interferon-beta mediates stromal cell rescue of T cells from apoptosis. *Eur J Immunol* 29, 1041-50.
50. Radvanyi, L. G., Banerjee, A., Weir, M., Messner, H. (1999) Low levels of interferon-alpha induce CD86 (B7.2) expression and accelerates dendritic cell maturation from human peripheral blood mononuclear cells. *Scand J Immunol* 50, 499-509.
51. Ito, T., Amakawa, R., Inaba, M., Ikehara, S., Inaba, K., Fukuhara, S. (2001) Differential regulation of human blood dendritic cell subsets by IFNs. *J Immunol* 166, 2961-9.
52. Montoya, M., Schiavoni, G., Mattei, F., Gresser, I., Belardelli, F., Borrow, P., Tough, D. F. (2002) Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* 99, 3263-71.
53. Alexopoulou, L., Holt, A. C., Medzhitov, R., Flavell, R. A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413, 732-8.
54. Matsumoto, M., Kikkawa, S., Kohase, M., Miyake, K., Seya, T. (2002) Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem Biophys Res Commun* 293, 1364-9.
55. Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C. S.,

- Reis e Sousa, C., Matsuura, Y., Fujita, T., Akira, S. (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441, 101-5.
56. Okahira, S., Nishikawa, F., Nishikawa, S., Akazawa, T., Seya, T., Matsumoto, M. (2005) Interferon-beta induction through toll-like receptor 3 depends on double-stranded RNA structure. *DNA Cell Biol* 24, 614-23.
 57. Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R. A., Diamond, M. S., Colonna, M. (2006) Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc Natl Acad Sci U S A* 103, 8459-64.
 58. Fujimoto, C., Nakagawa, Y., Ohara, K., Takahashi, H. (2004) Polyriboinosinic polyribocytidylic acid [poly(I:C)]/TLR3 signaling allows class I processing of exogenous protein and induction of HIV-specific CD8⁺ cytotoxic T lymphocytes. *Int Immunol* 16, 55-63.
 59. McNally, B., Willette, M., Ye, F., Partida-Sanchez, S., Flano, E. (2012) Intranasal administration of dsRNA analog poly(I:C) induces interferon-alpha receptor-dependent accumulation of antigen experienced T cells in the airways. *PLoS One* 7, e51351.
 60. Pulko, V., Liu, X., Krco, C. J., Harris, K. J., Frigola, X., Kwon, E. D., Dong, H. (2009) TLR3-stimulated dendritic cells up-regulate B7-H1 expression and influence the magnitude of CD8 T cell responses to tumor vaccination. *J Immunol* 183, 3634-41.
 61. Nordlund, J. J., Wolff, S. M., Levy, H. B. (1970) Inhibition of biologic activity of poly I: poly C by human plasma. *Proc Soc Exp Biol Med* 133, 439-44.
 62. Brodsky, I., Strayer, D. R., Krueger, L. J., Carter, W. A. (1985) Clinical studies with ampligen (mismatched double-stranded RNA). *J Biol Response Mod* 4, 669-75.
 63. Ichinohe, T., Kawaguchi, A., Tamura, S., Takahashi, H., Sawa, H., Ninomiya, A., Imai, M., Itamura, S., Odagiri, T., Tashiro, M., Chiba, J., Sata, T., Kurata, T., Hasegawa, H. (2007) Intranasal immunization with H5N1 vaccine plus Poly I:Poly C12U, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge. *Microbes Infect* 9, 1333-40.
 64. Adams, M., Navabi, H., Jasani, B., Man, S., Fiander, A., Evans, A. S., Donninger, C., Mason, M. (2003) Dendritic cell (DC) based therapy for cervical cancer: use of DC

- pulsed with tumour lysate and matured with a novel synthetic clinically non-toxic double stranded RNA analogue poly [I]:poly [C(12)U] (Ampligen R). *Vaccine* 21, 787-90.
65. Jasani, B., Navabi, H., Adams, M. (2009) Ampligen: a potential toll-like 3 receptor adjuvant for immunotherapy of cancer. *Vaccine* 27, 3401-4.
 66. Stahl-Hennig, C., Eisenblatter, M., Jasny, E., Rzehak, T., Tenner-Racz, K., Trumpfheller, C., Salazar, A. M., Uberla, K., Nieto, K., Kleinschmidt, J., Schulte, R., Gissmann, L., Muller, M., Sacher, A., Racz, P., Steinman, R. M., Uguccioni, M., Ignatius, R. (2009) Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. *PLoS Pathog* 5, e1000373.
 67. Levy, H. B., Baer, G., Baron, S., Buckler, C. E., Gibbs, C. J., Iadarola, M. J., London, W. T., Rice, J. (1975) A modified polyribonucleosinic-polyribocytidylic acid complex that induces interferon in primates. *J Infect Dis* 132, 434-9.
 68. Martins, K. A., Steffens, J. T., van Tongeren, S. A., Wells, J. B., Bergeron, A. A., Dickson, S. P., Dye, J. M., Salazar, A. M., Bavari, S. (2014) Toll-like receptor agonist augments virus-like particle-mediated protection from Ebola virus with transient immune activation. *PLoS One* 9, e89735.
 69. Kastenmuller, K., Espinosa, D. A., Trager, L., Stoyanov, C., Salazar, A. M., Pokalwar, S., Singh, S., Dutta, S., Ockenhouse, C. F., Zavala, F., Seder, R. A. (2013) Full-length *Plasmodium falciparum* circumsporozoite protein administered with long-chain poly(I:C) or the Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion elicits potent antibody and CD4⁺ T cell immunity and protection in mice. *Infect Immun* 81, 789-800.
 70. Flynn, B. J., Kastenmuller, K., Wille-Reece, U., Tomaras, G. D., Alam, M., Lindsay, R. W., Salazar, A. M., Perdiguero, B., Gomez, C. E., Wagner, R., Esteban, M., Park, C. G., Trumpfheller, C., Keler, T., Pantaleo, G., Steinman, R. M., Seder, R. (2011) Immunization with HIV Gag targeted to dendritic cells followed by recombinant New York vaccinia virus induces robust T-cell immunity in nonhuman primates. *Proc Natl Acad Sci U S A* 108, 7131-6.
 71. Sabbatini, P., Tsuji, T., Ferran, L., Ritter, E., Sedrak, C., Tuballes, K., Jungbluth, A. A., Ritter, G., Aghajanian, C., Bell-McGuinn, K., Hensley, M. L., Konner, J., Tew, W.,

- Spriggs, D. R., Hoffman, E. W., Venhaus, R., Pan, L., Salazar, A. M., Diefenbach, C. M., Old, L. J., Gnjjatic, S. (2012) Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients. *Clin Cancer Res* 18, 6497-508.
72. Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A., Seya, T. (2003) Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J Immunol* 171, 3154-62.
 73. Funami, K., Matsumoto, M., Oshiumi, H., Akazawa, T., Yamamoto, A., Seya, T. (2004) The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling. *Int Immunol* 16, 1143-54.
 74. Cario, E. and Podolsky, D. K. (2000) Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* 68, 7010-7.
 75. Heinz, S., Haehnel, V., Karaghiosoff, M., Schwarzfischer, L., Muller, M., Krause, S. W., Rehli, M. (2003) Species-specific regulation of Toll-like receptor 3 genes in men and mice. *J Biol Chem* 278, 21502-9.
 76. Kim, J. I., Lee, C. J., Jin, M. S., Lee, C. H., Paik, S. G., Lee, H., Lee, J. O. (2005) Crystal structure of CD14 and its implications for lipopolysaccharide signaling. *J Biol Chem* 280, 11347-51.
 77. Kawai, T. and Akira, S. (2007) Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med* 13, 460-9.
 78. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., Seya, T. (2003) TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 4, 161-7.
 79. Meylan, E., Burns, K., Hofmann, K., Blancheteau, V., Martinon, F., Kelliher, M., Tschopp, J. (2004) RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nat Immunol* 5, 503-7.
 80. Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R., Hiscott, J. (2003) Triggering the interferon antiviral response through an IKK-related pathway. *Science* 300, 1148-51.

81. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., Maniatis, T. (2003) IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4, 491-6.
82. Sarkar, S. N., Peters, K. L., Elco, C. P., Sakamoto, S., Pal, S., Sen, G. C. (2004) Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. *Nat Struct Mol Biol* 11, 1060-7.
83. Kawai, T. and Akira, S. (2008) Toll-like receptor and RIG-I-like receptor signaling. *Ann N Y Acad Sci* 1143, 1-20.
84. Yoneyama, M. and Fujita, T. (2007) Function of RIG-I-like receptors in antiviral innate immunity. *J Biol Chem* 282, 15315-8.
85. Cui, Y., Li, M., Walton, K. D., Sun, K., Hanover, J. A., Furth, P. A., Hennighausen, L. (2001) The Stat3/5 locus encodes novel endoplasmic reticulum and helicase-like proteins that are preferentially expressed in normal and neoplastic mammary tissue. *Genomics* 78, 129-34.
86. Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., Fujita, T. (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5, 730-7.
87. Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S., Hartmann, G. (2006) 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314, 994-7.
88. Takahashi, K., Yoneyama, M., Nishihori, T., Hirai, R., Kumeta, H., Narita, R., Gale, M., Jr., Inagaki, F., Fujita, T. (2008) Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. *Mol Cell* 29, 428-40.
89. Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., Dermody, T. S., Fujita, T., Akira, S. (2008) Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *The Journal of experimental medicine* 205, 1601-10.
90. Rothenfusser, S., Goutagny, N., DiPerna, G., Gong, M., Monks, B. G., Schoenemeyer, A., Yamamoto, M., Akira, S., Fitzgerald, K. A. (2005) The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* 175, 5260-8.

91. Murali, A., Li, X., Ranjith-Kumar, C. T., Bhardwaj, K., Holzenburg, A., Li, P., Kao, C. C. (2008) Structure and function of LGP2, a DEX(D/H) helicase that regulates the innate immunity response. *J Biol Chem* 283, 15825-33.
92. Venkataraman, T., Valdes, M., Elsby, R., Kakuta, S., Caceres, G., Saijo, S., Iwakura, Y., Barber, G. N. (2007) Loss of DEX(D/H) box RNA helicase LGP2 manifests disparate antiviral responses. *J Immunol* 178, 6444-55.
93. Satoh, T., Kato, H., Kumagai, Y., Yoneyama, M., Sato, S., Matsushita, K., Tsujimura, T., Fujita, T., Akira, S., Takeuchi, O. (2010) LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc Natl Acad Sci U S A* 107, 1512-7.
94. Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O., Akira, S. (2005) IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 6, 981-8.
95. Kumar, H., Kawai, T., Kato, H., Sato, S., Takahashi, K., Coban, C., Yamamoto, M., Uematsu, S., Ishii, K. J., Takeuchi, O., Akira, S. (2006) Essential role of IPS-1 in innate immune responses against RNA viruses. *The Journal of experimental medicine* 203, 1795-803.
96. Takeuchi, O. and Akira, S. (2008) MDA5/RIG-I and virus recognition. *Curr Opin Immunol* 20, 17-22.
97. Takahashi, K., Kawai, T., Kumar, H., Sato, S., Yonehara, S., Akira, S. (2006) Roles of caspase-8 and caspase-10 in innate immune responses to double-stranded RNA. *J Immunol* 176, 4520-4.
98. Isaacs, A. and Lindenmann, J. (2015) Pillars Article: Virus Interference. I. The Interferon. *Proc R Soc Lond B Biol Sci.* 1957. 147: 258-267. *J Immunol* 195, 1911-20.
99. De Maeyer, E. and De Maeyer-Guignard, J. (1998) Type I interferons. *Int Rev Immunol* 17, 53-73.
100. Uze, G., Lutfalla, G., Mogensen, K. E. (1995) Alpha and beta interferons and their receptor and their friends and relations. *J Interferon Cytokine Res* 15, 3-26.
101. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., Schreiber, R. D. (1998) How cells respond to interferons. *Annu Rev Biochem* 67, 227-64.
102. Pinto, A. K., Daffis, S., Brien, J. D., Gainey, M. D., Yokoyama, W. M., Sheehan, K. C., Murphy, K. M., Schreiber, R. D., Diamond, M. S. (2011) A temporal role of type I

interferon signaling in CD8+ T cell maturation during acute West Nile virus infection. PLoS Pathog 7, e1002407.

103. Marshall, H. D., Urban, S. L., Welsh, R. M. (2011) Virus-induced transient immune suppression and the inhibition of T cell proliferation by type I interferon. J Virol 85, 5929-39.
104. Sadler, A. J. and Williams, B. R. (2008) Interferon-inducible antiviral effectors. Nat Rev Immunol 8, 559-68.
105. Yan, N. and Chen, Z. J. (2012) Intrinsic antiviral immunity. Nat Immunol 13, 214-22.
106. McNab, F. W., Rajsbaum, R., Stoye, J. P., O'Garra, A. (2011) Tripartite-motif proteins and innate immune regulation. Curr Opin Immunol 23, 46-56.
107. Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M., Aguet, M. (1994) Functional role of type I and type II interferons in antiviral defense. Science 264, 1918-21.
108. Ralph, P., Nakoinz, I., Rennick, D. (1988) Role of interleukin 2, interleukin 4, and alpha, beta, and gamma interferon in stimulating macrophage antibody-dependent tumoricidal activity. The Journal of experimental medicine 167, 712-7.
109. Taylor, J. L. and Grossberg, S. E. (1998) The effects of interferon-alpha on the production and action of other cytokines. Semin Oncol 25, 23-9.
110. Rothfuchs, A. G., Gigliotti, D., Palmblad, K., Andersson, U., Wigzell, H., Rottenberg, M. E. (2001) IFN-alpha beta-dependent, IFN-gamma secretion by bone marrow-derived macrophages controls an intracellular bacterial infection. J Immunol 167, 6453-61.
111. Biron, C. A., Sonnenfeld, G., Welsh, R. M. (1984) Interferon induces natural killer cell blastogenesis in vivo. J Leukoc Biol 35, 31-7.
112. Gidlund, M., Orn, A., Wigzell, H., Senik, A., Gresser, I. (1978) Enhanced NK cell activity in mice injected with interferon and interferon inducers. Nature 273, 759-61.
113. Tough, D. F., Borrow, P., Sprent, J. (1996) Induction of bystander T cell proliferation by viruses and type I interferon in vivo. Science 272, 1947-50.
114. Santini, S. M., Di Pucchio, T., Lapenta, C., Parlato, S., Logozzi, M., Belardelli, F. (2002) The natural alliance between type I interferon and dendritic cells and its role in linking innate and adaptive immunity. J Interferon Cytokine Res 22, 1071-80.

115. Le Bon, A., Etchart, N., Rossmann, C., Ashton, M., Hou, S., Gewert, D., Borrow, P., Tough, D. F. (2003) Cross-priming of CD8⁺ T cells stimulated by virus-induced type I interferon. *Nat Immunol* 4, 1009-15.
116. Velazquez, L., Fellous, M., Stark, G. R., Pellegrini, S. (1992) A protein tyrosine kinase in the interferon alpha/beta signaling pathway. *Cell* 70, 313-22.
117. Silvennoinen, O., Ihle, J. N., Schlessinger, J., Levy, D. E. (1993) Interferon-induced nuclear signalling by Jak protein tyrosine kinases. *Nature* 366, 583-5.
118. Platanias, L. C. (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* 5, 375-86.
119. van Boxel-Dezaire, A. H., Rani, M. R., Stark, G. R. (2006) Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity* 25, 361-72.
120. Gimeno, R., Lee, C. K., Schindler, C., Levy, D. E. (2005) Stat1 and Stat2 but not Stat3 arbitrate contradictory growth signals elicited by alpha/beta interferon in T lymphocytes. *Mol Cell Biol* 25, 5456-65.
121. Tanabe, Y., Nishibori, T., Su, L., Arduini, R. M., Baker, D. P., David, M. (2005) Cutting edge: role of STAT1, STAT3, and STAT5 in IFN-alpha beta responses in T lymphocytes. *J Immunol* 174, 609-13.
122. Nguyen, K. B., Cousens, L. P., Doughty, L. A., Pien, G. C., Durbin, J. E., Biron, C. A. (2000) Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: STAT1 resolves a paradox. *Nat Immunol* 1, 70-6.
123. Nguyen, K. B., Watford, W. T., Salomon, R., Hofmann, S. R., Pien, G. C., Morinobu, A., Gadina, M., O'Shea, J. J., Biron, C. A. (2002) Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science* 297, 2063-6.
124. Hahm, B., Trifilo, M. J., Zuniga, E. I., Oldstone, M. B. (2005) Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. *Immunity* 22, 247-57.
125. Le Bon, A., Durand, V., Kamphuis, E., Thompson, C., Bulfone-Paus, S., Rossmann, C., Kalinke, U., Tough, D. F. (2006) Direct stimulation of T cells by type I IFN enhances the CD8⁺ T cell response during cross-priming. *J Immunol* 176, 4682-9.
126. Parlato, S., Santini, S. M., Lapenta, C., Di Pucchio, T., Logozzi, M., Spada, M., Giammarioli, A. M., Malorni, W., Fais, S., Belardelli, F. (2001) Expression of CCR-7,

MIP-3 β , and Th-1 chemokines in type I IFN-induced monocyte-derived dendritic cells: importance for the rapid acquisition of potent migratory and functional activities. *Blood* 98, 3022-9.

127. Rouzaut, A., Garasa, S., Teijeira, A., Gonzalez, I., Martinez-Forero, I., Suarez, N., Larrea, E., Alfaro, C., Palazon, A., Dubrot, J., Hervas-Stubbs, S., Melero, I. (2010) Dendritic cells adhere to and transmigrate across lymphatic endothelium in response to IFN- α . *Eur J Immunol* 40, 3054-63.
128. Gautier, G., Humbert, M., Deauvieu, F., Scuiller, M., Hiscott, J., Bates, E. E., Trinchieri, G., Caux, C., Garrone, P. (2005) A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *The Journal of experimental medicine* 201, 1435-46.
129. Cousens, L. P., Orange, J. S., Su, H. C., Biron, C. A. (1997) Interferon- α/β inhibition of interleukin 12 and interferon- γ production in vitro and endogenously during viral infection. *Proc Natl Acad Sci U S A* 94, 634-9.
130. Dalod, M., Salazar-Mather, T. P., Malmgaard, L., Lewis, C., Asselin-Paturel, C., Briere, F., Trinchieri, G., Biron, C. A. (2002) Interferon α/β and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. *The Journal of experimental medicine* 195, 517-28.
131. Orange, J. S., Wolf, S. F., Biron, C. A. (1994) Effects of IL-12 on the response and susceptibility to experimental viral infections. *J Immunol* 152, 1253-64.
132. Tough, D. F. (2004) Type I interferon as a link between innate and adaptive immunity through dendritic cell stimulation. *Leuk Lymphoma* 45, 257-64.
133. Havenar-Daughton, C., Kolumam, G. A., Murali-Krishna, K. (2006) Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection. *J Immunol* 176, 3315-9.
134. Kolumam, G. A., Thomas, S., Thompson, L. J., Sprent, J., Murali-Krishna, K. (2005) Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *The Journal of experimental medicine* 202, 637-50.

135. Aichele, P., Unsoeld, H., Koschella, M., Schweier, O., Kalinke, U., Vucikuja, S. (2006) CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. *J Immunol* 176, 4525-9.
136. Wiesel, M., Kratky, W., Oxenius, A. (2011) Type I IFN substitutes for T cell help during viral infections. *J Immunol* 186, 754-63.
137. Kaser, A., Nagata, S., Tilg, H. (1999) Interferon alpha augments activation-induced T cell death by upregulation of Fas (CD95/APO-1) and Fas ligand expression. *Cytokine* 11, 736-43.
138. Terawaki, S., Chikuma, S., Shibayama, S., Hayashi, T., Yoshida, T., Okazaki, T., Honjo, T. (2011) IFN-alpha directly promotes programmed cell death-1 transcription and limits the duration of T cell-mediated immunity. *J Immunol* 186, 2772-9.
139. Sun, S., Zhang, X., Tough, D. F., Sprent, J. (1998) Type I interferon-mediated stimulation of T cells by CpG DNA. *The Journal of experimental medicine* 188, 2335-42.
140. Zella, D., Romerio, F., Curreli, S., Secchiero, P., Cicala, C., Zagury, D., Gallo, R. C. (2000) IFN-alpha 2b reduces IL-2 production and IL-2 receptor function in primary CD4+ T cells. *J Immunol* 164, 2296-302.
141. Dondi, E., Rogge, L., Lutfalla, G., Uze, G., Pellegrini, S. (2003) Down-modulation of responses to type I IFN upon T cell activation. *J Immunol* 170, 749-56.
142. Matikainen, S., Sareneva, T., Ronni, T., Lehtonen, A., Koskinen, P. J., Julkunen, I. (1999) Interferon-alpha activates multiple STAT proteins and upregulates proliferation-associated IL-2Ralpha, c-myc, and pim-1 genes in human T cells. *Blood* 93, 1980-91.
143. Gil, M. P., Salomon, R., Louten, J., Biron, C. A. (2006) Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses in vivo. *Blood* 107, 987-93.
144. Curtsinger, J. M., Valenzuela, J. O., Agarwal, P., Lins, D., Mescher, M. F. (2005) Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J Immunol* 174, 4465-9.
145. Hervas-Stubbs, S., Riezu-Boj, J. I., Gonzalez, I., Mancheno, U., Dubrot, J., Azpilicueta, A., Gabari, I., Palazon, A., Aranguren, A., Ruiz, J., Prieto, J., Larrea, E., Melero, I. (2010) Effects of IFN-alpha as a signal-3 cytokine on human naive and antigen-experienced CD8(+) T cells. *Eur J Immunol* 40, 3389-402.

146. Shibuya, H., Nagai, T., Ishii, A., Yamamoto, K., Hirohata, S. (2003) Differential regulation of Th1 responses and CD154 expression in human CD4⁺ T cells by IFN- α . *Clin Exp Immunol* 132, 216-24.
147. Sareneva, T., Julkunen, I., Matikainen, S. (2000) IFN- α and IL-12 induce IL-18 receptor gene expression in human NK and T cells. *J Immunol* 165, 1933-8.
148. Strengell, M., Julkunen, I., Matikainen, S. (2004) IFN- α regulates IL-21 and IL-21R expression in human NK and T cells. *J Leukoc Biol* 76, 416-22.
149. Strengell, M., Matikainen, S., Siren, J., Lehtonen, A., Foster, D., Julkunen, I., Sareneva, T. (2003) IL-21 in synergy with IL-15 or IL-18 enhances IFN- γ production in human NK and T cells. *J Immunol* 170, 5464-9.
150. Huber, J. P., Ramos, H. J., Gill, M. A., Farrar, J. D. (2010) Cutting edge: Type I IFN reverses human Th2 commitment and stability by suppressing GATA3. *J Immunol* 185, 813-7.
151. Schandene, L., Del Prete, G. F., Cogan, E., Stordeur, P., Crusiaux, A., Kennes, B., Romagnani, S., Goldman, M. (1996) Recombinant interferon- α selectively inhibits the production of interleukin-5 by human CD4⁺ T cells. *J Clin Invest* 97, 309-15.
152. Shibuya, H. and Hirohata, S. (2005) Differential effects of IFN- α on the expression of various TH2 cytokines in human CD4⁺ T cells. *J Allergy Clin Immunol* 116, 205-12.
153. Eriksen, K. W., Sommer, V. H., Woetmann, A., Rasmussen, A. B., Brender, C., Svejgaard, A., Skov, S., Geisler, C., Odum, N. (2004) Bi-phasic effect of interferon (IFN)- α : IFN- α up- and down-regulates interleukin-4 signaling in human T cells. *J Biol Chem* 279, 169-76.
154. Moschen, A. R., Geiger, S., Krehan, I., Kaser, A., Tilg, H. (2008) Interferon- α controls IL-17 expression in vitro and in vivo. *Immunobiology* 213, 779-87.
155. Wiesel, M., Crouse, J., Bedenikovic, G., Sutherland, A., Joller, N., Oxenius, A. (2012) Type-I IFN drives the differentiation of short-lived effector CD8⁺ T cells in vivo. *Eur J Immunol* 42, 320-9.
156. Xiao, Z., Casey, K. A., Jameson, S. C., Curtsinger, J. M., Mescher, M. F. (2009) Programming for CD8 T cell memory development requires IL-12 or type I IFN. *J Immunol* 182, 2786-94.

157. Ramos, H. J., Davis, A. M., Cole, A. G., Schatzle, J. D., Forman, J., Farrar, J. D. (2009) Reciprocal responsiveness to interleukin-12 and interferon-alpha specifies human CD8+ effector versus central memory T-cell fates. *Blood* 113, 5516-25.
158. Davis, A. M., Ramos, H. J., Davis, L. S., Farrar, J. D. (2008) Cutting edge: a T-bet-independent role for IFN-alpha/beta in regulating IL-2 secretion in human CD4+ central memory T cells. *J Immunol* 181, 8204-8.
159. Loetscher, M., Loetscher, P., Brass, N., Meese, E., Moser, B. (1998) Lymphocyte-specific chemokine receptor CXCR3: regulation, chemokine binding and gene localization. *Eur J Immunol* 28, 3696-705.
160. Qin, S., Rottman, J. B., Myers, P., Kassam, N., Weinblatt, M., Loetscher, M., Koch, A. E., Moser, B., Mackay, C. R. (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* 101, 746-54.
161. Shields, P. L., Morland, C. M., Salmon, M., Qin, S., Hubscher, S. G., Adams, D. H. (1999) Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. *J Immunol* 163, 6236-43.
162. Yamamoto, J., Adachi, Y., Onoue, Y., Adachi, Y. S., Okabe, Y., Itazawa, T., Toyoda, M., Seki, T., Morohashi, M., Matsushima, K., Miyawaki, T. (2000) Differential expression of the chemokine receptors by the Th1- and Th2-type effector populations within circulating CD4+ T cells. *J Leukoc Biol* 68, 568-74.
163. Kim, C. H., Rott, L., Kunkel, E. J., Genovese, M. C., Andrew, D. P., Wu, L., Butcher, E. C. (2001) Rules of chemokine receptor association with T cell polarization in vivo. *J Clin Invest* 108, 1331-9.
164. Clark-Lewis, I., Mattioli, I., Gong, J. H., Loetscher, P. (2003) Structure-function relationship between the human chemokine receptor CXCR3 and its ligands. *J Biol Chem* 278, 289-95.
165. Loetscher, M., Gerber, B., Loetscher, P., Jones, S. A., Piali, L., Clark-Lewis, I., Baggiolini, M., Moser, B. (1996) Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. *The Journal of experimental medicine* 184, 963-9.

166. Lasagni, L., Francalanci, M., Annunziato, F., Lazzeri, E., Giannini, S., Cosmi, L., Sagrinati, C., Mazzinghi, B., Orlando, C., Maggi, E., Marra, F., Romagnani, S., Serio, M., Romagnani, P. (2003) An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *The Journal of experimental medicine* 197, 1537-49.
167. Ehlert, J. E., Addison, C. A., Burdick, M. D., Kunkel, S. L., Strieter, R. M. (2004) Identification and partial characterization of a variant of human CXCR3 generated by posttranscriptional exon skipping. *J Immunol* 173, 6234-40.
168. Cole, K. E., Strick, C. A., Paradis, T. J., Ogborne, K. T., Loetscher, M., Gladue, R. P., Lin, W., Boyd, J. G., Moser, B., Wood, D. E., Sahagan, B. G., Neote, K. (1998) Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *The Journal of experimental medicine* 187, 2009-21.
169. Wuest, T. R. and Carr, D. J. (2008) Dysregulation of CXCR3 signaling due to CXCL10 deficiency impairs the antiviral response to herpes simplex virus 1 infection. *J Immunol* 181, 7985-93.
170. Carr, D. J., Wuest, T., Ash, J. (2008) An increase in herpes simplex virus type 1 in the anterior segment of the eye is linked to a deficiency in NK cell infiltration in mice deficient in CXCR3. *J Interferon Cytokine Res* 28, 245-51.
171. Farber, J. M. (1997) Mig and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol* 61, 246-57.
172. Kastenmuller, W., Torabi-Parizi, P., Subramanian, N., Lammermann, T., Germain, R. N. (2012) A spatially-organized multicellular innate immune response in lymph nodes limits systemic pathogen spread. *Cell* 150, 1235-48.
173. Ohmori, Y., Schreiber, R. D., Hamilton, T. A. (1997) Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. *J Biol Chem* 272, 14899-907.

174. Ohmori, Y., Wyner, L., Narumi, S., Armstrong, D., Stoler, M., Hamilton, T. A. (1993) Tumor necrosis factor-alpha induces cell type and tissue-specific expression of chemoattractant cytokines in vivo. *Am J Pathol* 142, 861-70.
175. Campanella, G. S., Tager, A. M., El Khoury, J. K., Thomas, S. Y., Abraszinski, T. A., Manice, L. A., Colvin, R. A., Luster, A. D. (2008) Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. *Proc Natl Acad Sci U S A* 105, 4814-9.
176. Yoneyama, H., Narumi, S., Zhang, Y., Murai, M., Baggiolini, M., Lanzavecchia, A., Ichida, T., Asakura, H., Matsushima, K. (2002) Pivotal role of dendritic cell-derived CXCL10 in the retention of T helper cell 1 lymphocytes in secondary lymph nodes. *The Journal of experimental medicine* 195, 1257-66.
177. Ochiai, E., Sa, Q., Brogli, M., Kudo, T., Wang, X., Dubey, J. P., Suzuki, Y. (2015) CXCL9 is important for recruiting immune T cells into the brain and inducing an accumulation of the T cells to the areas of tachyzoite proliferation to prevent reactivation of chronic cerebral infection with *Toxoplasma gondii*. *Am J Pathol* 185, 314-24.
178. Kastenmuller, W., Brandes, M., Wang, Z., Herz, J., Egen, J. G., Germain, R. N. (2013) Peripheral prepositioning and local CXCL9 chemokine-mediated guidance orchestrate rapid memory CD8⁺ T cell responses in the lymph node. *Immunity* 38, 502-13.
179. Woodruff, M. C., Heesters, B. A., Herndon, C. N., Groom, J. R., Thomas, P. G., Luster, A. D., Turley, S. J., Carroll, M. C. (2014) Trans-nodal migration of resident dendritic cells into medullary interfollicular regions initiates immunity to influenza vaccine. *The Journal of experimental medicine* 211, 1611-21.
180. Oldham, W. M. and Hamm, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* 9, 60-71.
181. Wettschureck, N. and Offermanns, S. (2005) Mammalian G proteins and their cell type specific functions. *Physiol Rev* 85, 1159-204.
182. Marinissen, M. J. and Gutkind, J. S. (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol Sci* 22, 368-76.
183. Thelen, M. (2001) Dancing to the tune of chemokines. *Nat Immunol* 2, 129-34.
184. Thelen, M. and Stein, J. V. (2008) How chemokines invite leukocytes to dance. *Nat Immunol* 9, 953-9.

185. Smit, M. J., Verdijk, P., van der Raaij-Helmer, E. M., Navis, M., Hensbergen, P. J., Leurs, R., Tensen, C. P. (2003) CXCR3-mediated chemotaxis of human T cells is regulated by a Gi- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. *Blood* 102, 1959-65.
186. Miu, J., Mitchell, A. J., Muller, M., Carter, S. L., Manders, P. M., McQuillan, J. A., Saunders, B. M., Ball, H. J., Lu, B., Campbell, I. L., Hunt, N. H. (2008) Chemokine gene expression during fatal murine cerebral malaria and protection due to CXCR3 deficiency. *J Immunol* 180, 1217-30.
187. Klein, R. S., Lin, E., Zhang, B., Luster, A. D., Tollett, J., Samuel, M. A., Engle, M., Diamond, M. S. (2005) Neuronal CXCL10 directs CD8+ T-cell recruitment and control of West Nile virus encephalitis. *J Virol* 79, 11457-66.
188. Thapa, M. and Carr, D. J. (2009) CXCR3 deficiency increases susceptibility to genital herpes simplex virus type 2 infection: Uncoupling of CD8+ T-cell effector function but not migration. *J Virol* 83, 9486-501.
189. Thapa, M., Welner, R. S., Pelayo, R., Carr, D. J. (2008) CXCL9 and CXCL10 expression are critical for control of genital herpes simplex virus type 2 infection through mobilization of HSV-specific CTL and NK cells to the nervous system. *J Immunol* 180, 1098-106.
190. Mohan, K. and Issekutz, T. B. (2007) Blockade of chemokine receptor CXCR3 inhibits T cell recruitment to inflamed joints and decreases the severity of adjuvant arthritis. *J Immunol* 179, 8463-9.
191. Salomon, I., Netzer, N., Wildbaum, G., Schiff-Zuck, S., Maor, G., Karin, N. (2002) Targeting the function of IFN-gamma-inducible protein 10 suppresses ongoing adjuvant arthritis. *J Immunol* 169, 2685-93.
192. Frigerio, S., Junt, T., Lu, B., Gerard, C., Zumsteg, U., Hollander, G. A., Piali, L. (2002) Beta cells are responsible for CXCR3-mediated T-cell infiltration in insulinitis. *Nat Med* 8, 1414-20.
193. Menke, J., Zeller, G. C., Kikawada, E., Means, T. K., Huang, X. R., Lan, H. Y., Lu, B., Farber, J., Luster, A. D., Kelley, V. R. (2008) CXCL9, but not CXCL10, promotes CXCR3-dependent immune-mediated kidney disease. *J Am Soc Nephrol* 19, 1177-89.

194. Hsieh, M. F., Lai, S. L., Chen, J. P., Sung, J. M., Lin, Y. L., Wu-Hsieh, B. A., Gerard, C., Luster, A., Liao, F. (2006) Both CXCR3 and CXCL10/IFN-inducible protein 10 are required for resistance to primary infection by dengue virus. *J Immunol* 177, 1855-63.
195. Christen, U., McGavern, D. B., Luster, A. D., von Herrath, M. G., Oldstone, M. B. (2003) Among CXCR3 chemokines, IFN-gamma-inducible protein of 10 kDa (CXC chemokine ligand (CXCL) 10) but not monokine induced by IFN-gamma (CXCL9) imprints a pattern for the subsequent development of autoimmune disease. *J Immunol* 171, 6838-45.
196. Khan, I. A., MacLean, J. A., Lee, F. S., Casciotti, L., DeHaan, E., Schwartzman, J. D., Luster, A. D. (2000) IP-10 is critical for effector T cell trafficking and host survival in *Toxoplasma gondii* infection. *Immunity* 12, 483-94.
197. Groom, J. R., Richmond, J., Murooka, T. T., Sorensen, E. W., Sung, J. H., Bankert, K., von Andrian, U. H., Moon, J. J., Mempel, T. R., Luster, A. D. (2012) CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4⁺ T helper 1 cell differentiation. *Immunity* 37, 1091-103.
198. Sung, J. H., Zhang, H., Moseman, E. A., Alvarez, D., Iannacone, M., Henrickson, S. E., de la Torre, J. C., Groom, J. R., Luster, A. D., von Andrian, U. H. (2012) Chemokine guidance of central memory T cells is critical for antiviral recall responses in lymph nodes. *Cell* 150, 1249-63.
199. Yoneyama, H., Narumi, S., Zhang, Y., Murai, M., Baggiolini, M., Lanzavecchia, A., Ichida, T., Asakura, H., Matsushima, K. (2002) Pivotal Role of Dendritic Cell-derived CXCL10 in the Retention of T Helper Cell 1 Lymphocytes in Secondary Lymph Nodes. *The Journal of experimental medicine* 195, 1257-1266.
200. Maxwell, J. R., Ruby, C., Kerkvliet, N. I., Vella, A. T. (2002) Contrasting the roles of costimulation and the natural adjuvant lipopolysaccharide during the induction of T cell immunity. *J Immunol* 168, 4372-81.
201. Trumpfheller, C., Finke, J. S., Lopez, C. B., Moran, T. M., Molledo, B., Soares, H., Huang, Y., Schlesinger, S. J., Park, C. G., Nussenzweig, M. C., Granelli-Piperno, A., Steinman, R. M. (2006) Intensified and protective CD4⁺ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *The Journal of experimental medicine* 203, 607-17.

202. Soares, H., Waechter, H., Glaichenhaus, N., Mougneau, E., Yagita, H., Mizenina, O., Dudziak, D., Nussenzweig, M. C., Steinman, R. M. (2007) A subset of dendritic cells induces CD4⁺ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. *The Journal of experimental medicine* 204, 1095-106.
203. Boscardin, S. B., Hafalla, J. C., Masilamani, R. F., Kamphorst, A. O., Zebroski, H. A., Rai, U., Morrot, A., Zavala, F., Steinman, R. M., Nussenzweig, R. S., Nussenzweig, M. C. (2006) Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. *The Journal of experimental medicine* 203, 599-606.
204. Lee, S., Stokes, K. L., Currier, M. G., Sakamoto, K., Lukacs, N. W., Celis, E., Moore, M. L. (2012) Vaccine-elicited CD8⁺ T cells protect against respiratory syncytial virus strain A2-line19F-induced pathogenesis in BALB/c mice. *J Virol* 86, 13016-24.
205. Thompson, E. A., Liang, F., Lindgren, G., Sandgren, K. J., Quinn, K. M., Darrah, P. A., Koup, R. A., Seder, R. A., Kedl, R. M., Lore, K. (2015) Human Anti-CD40 Antibody and Poly IC:LC Adjuvant Combination Induces Potent T Cell Responses in the Lung of Nonhuman Primates. *J Immunol* 195, 1015-24.
206. Kurche, J. S., Burchill, M. A., Sanchez, P. J., Haluszczak, C., Kedl, R. M. (2010) Comparison of OX40 ligand and CD70 in the promotion of CD4⁺ T cell responses. *J Immunol* 185, 2106-15.
207. Mallett, S., Fossum, S., Barclay, A. N. (1990) Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes--a molecule related to nerve growth factor receptor. *Embo j* 9, 1063-8.
208. Godfrey, W. R., Fagnoni, F. F., Harara, M. A., Buck, D., Engleman, E. G. (1994) Identification of a human OX-40 ligand, a costimulator of CD4⁺ T cells with homology to tumor necrosis factor. *The Journal of experimental medicine* 180, 757-62.
209. Weinberg, A. D., Vella, A. T., Croft, M. (1998) OX-40: life beyond the effector T cell stage. *Semin Immunol* 10, 471-80.
210. Williams, C. A., Murray, S. E., Weinberg, A. D., Parker, D. C. (2007) OX40-mediated differentiation to effector function requires IL-2 receptor signaling but not CD28, CD40, IL-12Rbeta2, or T-bet. *J Immunol* 178, 7694-702.

211. al-Shamkhani, A., Birkeland, M. L., Puklavec, M., Brown, M. H., James, W., Barclay, A. N. (1996) OX40 is differentially expressed on activated rat and mouse T cells and is the sole receptor for the OX40 ligand. *Eur J Immunol* 26, 1695-9.
212. Rogers, P. R., Song, J., Gramaglia, I., Killeen, N., Croft, M. (2001) OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity* 15, 445-55.
213. Walker, L. S., Gulbranson-Judge, A., Flynn, S., Brocker, T., Raykundalia, C., Goodall, M., Forster, R., Lipp, M., Lane, P. (1999) Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. *The Journal of experimental medicine* 190, 1115-22.
214. Calderhead, D. M., Buhlmann, J. E., van den Eertwegh, A. J., Claassen, E., Noelle, R. J., Fell, H. P. (1993) Cloning of mouse Ox40: a T cell activation marker that may mediate T-B cell interactions. *J Immunol* 151, 5261-71.
215. Paterson, D. J., Jefferies, W. A., Green, J. R., Brandon, M. R., Corthesy, P., Puklavec, M., Williams, A. F. (1987) Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. *Mol Immunol* 24, 1281-90.
216. Takasawa, N., Ishii, N., Higashimura, N., Murata, K., Tanaka, Y., Nakamura, M., Sasaki, T., Sugamura, K. (2001) Expression of gp34 (OX40 ligand) and OX40 on human T cell clones. *Jpn J Cancer Res* 92, 377-82.
217. Baum, P. R., Gayle, R. B., 3rd, Ramsdell, F., Srinivasan, S., Sorensen, R. A., Watson, M. L., Seldin, M. F., Baker, E., Sutherland, G. R., Clifford, K. N., et al. (1994) Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. *Embo j* 13, 3992-4001.
218. Wang, H. C. and Klein, J. R. (2001) Multiple levels of activation of murine CD8(+) intraepithelial lymphocytes defined by OX40 (CD134) expression: effects on cell-mediated cytotoxicity, IFN-gamma, and IL-10 regulation. *J Immunol* 167, 6717-23.
219. Tanaka, Y., Inoi, T., Tozawa, H., Yamamoto, N., Hinuma, Y. (1985) A glycoprotein antigen detected with new monoclonal antibodies on the surface of human lymphocytes infected with human T-cell leukemia virus type-I (HTLV-I). *Int J Cancer* 36, 549-55.
220. Miura, S., Ohtani, K., Numata, N., Niki, M., Ohbo, K., Ina, Y., Gojobori, T., Tanaka, Y., Tozawa, H., Nakamura, M., et al. (1991) Molecular cloning and characterization of a

novel glycoprotein, gp34, that is specifically induced by the human T-cell leukemia virus type I transactivator p40tax. *Mol Cell Biol* 11, 1313-25.

- 221. Murata, K., Ishii, N., Takano, H., Miura, S., Ndhlovu, L. C., Nose, M., Noda, T., Sugamura, K. (2000) Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. *The Journal of experimental medicine* 191, 365-74.
- 222. Akiba, H., Oshima, H., Takeda, K., Atsuta, M., Nakano, H., Nakajima, A., Nohara, C., Yagita, H., Okumura, K. (1999) CD28-independent costimulation of T cells by OX40 ligand and CD70 on activated B cells. *J Immunol* 162, 7058-66.
- 223. Stuber, E., Neurath, M., Calderhead, D., Fell, H. P., Strober, W. (1995) Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. *Immunity* 2, 507-21.
- 224. Zingoni, A., Sornasse, T., Cocks, B. G., Tanaka, Y., Santoni, A., Lanier, L. L. (2004) Cross-talk between activated human NK cells and CD4⁺ T cells via OX40-OX40 ligand interactions. *J Immunol* 173, 3716-24.
- 225. Kashiwakura, J., Yokoi, H., Saito, H., Okayama, Y. (2004) T cell proliferation by direct cross-talk between OX40 ligand on human mast cells and OX40 on human T cells: comparison of gene expression profiles between human tonsillar and lung-cultured mast cells. *J Immunol* 173, 5247-57.
- 226. Nohara, C., Akiba, H., Nakajima, A., Inoue, A., Koh, C. S., Ohshima, H., Yagita, H., Mizuno, Y., Okumura, K. (2001) Amelioration of experimental autoimmune encephalomyelitis with anti-OX40 ligand monoclonal antibody: a critical role for OX40 ligand in migration, but not development, of pathogenic T cells. *J Immunol* 166, 2108-15.
- 227. Imura, A., Hori, T., Imada, K., Ishikawa, T., Tanaka, Y., Maeda, M., Imamura, S., Uchiyama, T. (1996) The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. *The Journal of experimental medicine* 183, 2185-95.
- 228. Song, J., Salek-Ardakani, S., Rogers, P. R., Cheng, M., Van Parijs, L., Croft, M. (2004) The costimulation-regulated duration of PKB activation controls T cell longevity. *Nat Immunol* 5, 150-8.

229. Song, J., So, T., Cheng, M., Tang, X., Croft, M. (2005) Sustained survivin expression from OX40 costimulatory signals drives T cell clonal expansion. *Immunity* 22, 621-31.
230. Gramaglia, I., Jember, A., Pippig, S. D., Weinberg, A. D., Killeen, N., Croft, M. (2000) The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J Immunol* 165, 3043-50.
231. Linton, P. J., Bautista, B., Biederman, E., Bradley, E. S., Harbertson, J., Kondrack, R. M., Padrick, R. C., Bradley, L. M. (2003) Costimulation via OX40L expressed by B cells is sufficient to determine the extent of primary CD4 cell expansion and Th2 cytokine secretion in vivo. *The Journal of experimental medicine* 197, 875-83.
232. Ohshima, Y., Yang, L. P., Uchiyama, T., Tanaka, Y., Baum, P., Sergerie, M., Hermann, P., Delespesse, G. (1998) OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. *Blood* 92, 3338-45.
233. Kim, M. Y., Gaspal, F. M., Wiggett, H. E., McConnell, F. M., Gulbranson-Judge, A., Raykundalia, C., Walker, L. S., Goodall, M. D., Lane, P. J. (2003) CD4(+)CD3(-) accessory cells costimulate primed CD4 T cells through OX40 and CD30 at sites where T cells collaborate with B cells. *Immunity* 18, 643-54.
234. Akiba, H., Miyahira, Y., Atsuta, M., Takeda, K., Nohara, C., Futagawa, T., Matsuda, H., Aoki, T., Yagita, H., Okumura, K. (2000) Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. *The Journal of experimental medicine* 191, 375-80.
235. Flynn, S., Toellner, K. M., Raykundalia, C., Goodall, M., Lane, P. (1998) CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blnr-1. *The Journal of experimental medicine* 188, 297-304.
236. Jember, A. G., Zuberi, R., Liu, F. T., Croft, M. (2001) Development of allergic inflammation in a murine model of asthma is dependent on the costimulatory receptor OX40. *The Journal of experimental medicine* 193, 387-92.
237. Yoshioka, T., Nakajima, A., Akiba, H., Ishiwata, T., Asano, G., Yoshino, S., Yagita, H., Okumura, K. (2000) Contribution of OX40/OX40 ligand interaction to the pathogenesis of rheumatoid arthritis. *Eur J Immunol* 30, 2815-23.

238. Weinberg, A. D., Bourdette, D. N., Sullivan, T. J., Lemon, M., Wallin, J. J., Maziarz, R., Davey, M., Palida, F., Godfrey, W., Engleman, E., Fulton, R. J., Offner, H., Vandenberg, A. A. (1996) Selective depletion of myelin-reactive T cells with the anti-OX-40 antibody ameliorates autoimmune encephalomyelitis. *Nat Med* 2, 183-9.
239. Huddleston, C. A., Weinberg, A. D., Parker, D. C. (2006) OX40 (CD134) engagement drives differentiation of CD4⁺ T cells to effector cells. *Eur J Immunol* 36, 1093-103.
240. Salek-Ardakani, S., Song, J., Halteman, B. S., Jember, A. G., Akiba, H., Yagita, H., Croft, M. (2003) OX40 (CD134) controls memory T helper 2 cells that drive lung inflammation. *The Journal of experimental medicine* 198, 315-24.
241. Weinberg, A. D., Wegmann, K. W., Funatake, C., Whitham, R. H. (1999) Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. *J Immunol* 162, 1818-26.
242. Humphreys, I. R., Edwards, L., Walzl, G., Rae, A. J., Dougan, G., Hill, S., Huscill, T. (2003) OX40 ligation on activated T cells enhances the control of *Cryptococcus neoformans* and reduces pulmonary eosinophilia. *J Immunol* 170, 6125-32.
243. Zubairi, S., Sanos, S. L., Hill, S., Kaye, P. M. (2004) Immunotherapy with OX40L-Fc or anti-CTLA-4 enhances local tissue responses and killing of *Leishmania donovani*. *Eur J Immunol* 34, 1433-40.
244. Weinberg, A. D., Thalhofer, C., Morris, N., Walker, J. M., Seiss, D., Wong, S., Axthelm, M. K., Picker, L. J., Urba, W. J. (2006) Anti-OX40 (CD134) administration to nonhuman primates: immunostimulatory effects and toxicokinetic study. *J Immunother* 29, 575-85.
245. Du, X., Zheng, G., Jin, H., Kang, Y., Wang, J., Xiao, C., Zhang, S., Zhao, L., Chen, A., Wang, B. (2007) The adjuvant effects of co-stimulatory molecules on cellular and memory responses to HBsAg DNA vaccination. *J Gene Med* 9, 136-46.
246. Munks, M. W., Mourich, D. V., Mittler, R. S., Weinberg, A. D., Hill, A. B. (2004) 4-1BB and OX40 stimulation enhance CD8 and CD4 T-cell responses to a DNA prime, poxvirus boost vaccine. *Immunology* 112, 559-66.
247. Salek-Ardakani, S., Moutaftsi, M., Crotty, S., Sette, A., Croft, M. (2008) OX40 drives protective vaccinia virus-specific CD8 T cells. *J Immunol* 181, 7969-76.

248. Morris, A., Vetto, J. T., Ramstad, T., Funatake, C. J., Choolun, E., Entwisle, C., Weinberg, A. D. (2001) Induction of anti-mammary cancer immunity by engaging the OX-40 receptor in vivo. *Breast Cancer Res Treat* 67, 71-80.
249. Kjaergaard, J., Tanaka, J., Kim, J. A., Rothchild, K., Weinberg, A., Shu, S. (2000) Therapeutic efficacy of OX-40 receptor antibody depends on tumor immunogenicity and anatomic site of tumor growth. *Cancer Res* 60, 5514-21.
250. Vetto, J. T., Lum, S., Morris, A., Sicotte, M., Davis, J., Lemon, M., Weinberg, A. (1997) Presence of the T-cell activation marker OX-40 on tumor infiltrating lymphocytes and draining lymph node cells from patients with melanoma and head and neck cancers. *Am J Surg* 174, 258-65.
251. Weinberg, A. D., Rivera, M. M., Prell, R., Morris, A., Ramstad, T., Vetto, J. T., Urba, W. J., Alvord, G., Bunce, C., Shields, J. (2000) Engagement of the OX-40 receptor in vivo enhances antitumor immunity. *J Immunol* 164, 2160-9.
252. Gough, M. J., Crittenden, M. R., Sarff, M., Pang, P., Seung, S. K., Vetto, J. T., Hu, H. M., Redmond, W. L., Holland, J., Weinberg, A. D. (2010) Adjuvant therapy with agonistic antibodies to CD134 (OX40) increases local control after surgical or radiation therapy of cancer in mice. *J Immunother* 33, 798-809.
253. Piconese, S., Valzasina, B., Colombo, M. P. (2008) OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection. *The Journal of experimental medicine* 205, 825-39.
254. Ali, S. A., Ahmad, M., Lynam, J., McLean, C. S., Entwisle, C., Loudon, P., Choolun, E., McArdle, S. E., Li, G., Mian, S., Rees, R. C. (2004) Anti-tumour therapeutic efficacy of OX40L in murine tumour model. *Vaccine* 22, 3585-94.
255. Valzasina, B., Guiducci, C., Dislich, H., Killeen, N., Weinberg, A. D., Colombo, M. P. (2005) Triggering of OX40 (CD134) on CD4(+)CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. *Blood* 105, 2845-51.
256. Vu, M. D., Xiao, X., Gao, W., Degauque, N., Chen, M., Kroemer, A., Killeen, N., Ishii, N., Li, X. C. (2007) OX40 costimulation turns off Foxp3+ Tregs. *Blood* 110, 2501-10.
257. Curti, B. D., Kovacsovics-Bankowski, M., Morris, N., Walker, E., Chisholm, L., Floyd, K., Walker, J., Gonzalez, I., Meeuwssen, T., Fox, B. A., Moudgil, T., Miller, W., Haley, D.,

- Coffey, T., Fisher, B., Delanty-Miller, L., Rymarchyk, N., Kelly, T., Crocenzi, T., Bernstein, E., Sanborn, R., Urba, W. J., Weinberg, A. D. (2013) OX40 is a potent immune-stimulating target in late-stage cancer patients. *Cancer Res* 73, 7189-98.
258. Morris, N. P., Peters, C., Montler, R., Hu, H. M., Curti, B. D., Urba, W. J., Weinberg, A. D. (2007) Development and characterization of recombinant human Fc:OX40L fusion protein linked via a coiled-coil trimerization domain. *Mol Immunol* 44, 3112-21.
 259. Grubin, C. E., Kovats, S., deRoos, P., Rudensky, A. Y. (1997) Deficient positive selection of CD4 T cells in mice displaying altered repertoires of MHC class II-bound self-peptides. *Immunity* 7, 197-208.
 260. Firpo, E. J., Kong, R. K., Zhou, Q., Rudensky, A. Y., Roberts, J. M., Franza, B. R. (2002) Antigen-specific dose-dependent system for the study of an inheritable and reversible phenotype in mouse CD4+ T cells. *Immunology* 107, 480-8.
 261. Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Itohara, S., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L., et al. (1992) Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* 360, 225-31.
 262. Sheehan, K. C., Lai, K. S., Dunn, G. P., Bruce, A. T., Diamond, M. S., Heutel, J. D., Dungo-Arthur, C., Carrero, J. A., White, J. M., Hertzog, P. J., Schreiber, R. D. (2006) Blocking monoclonal antibodies specific for mouse IFN-alpha/beta receptor subunit 1 (IFNAR-1) from mice immunized by in vivo hydrodynamic transfection. *J Interferon Cytokine Res* 26, 804-19.
 263. Unkeless, J. C. (1979) Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *The Journal of experimental medicine* 150, 580-96.
 264. Liu, W., Menoret, A., Vella, A. T. (2015) Responses to LPS boost effector CD8 T-cell accumulation outside of signals 1 and 2. *Cell Mol Immunol*.
 265. Koff, W. C., Burton, D. R., Johnson, P. R., Walker, B. D., King, C. R., Nabel, G. J., Ahmed, R., Bhan, M. K., Plotkin, S. A. (2013) Accelerating next-generation vaccine development for global disease prevention. *Science* 340, 1232910.
 266. Pasquale, A. D., Preiss, S., Silva, F. T., Garcon, N. (2015) Vaccine Adjuvants: from 1920 to 2015 and Beyond. *Vaccines (Basel)* 3, 320-43.

267. Janeway, C. A., Jr. (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1, 1-13.
268. Nossal, G. J. (1993) Tolerance and ways to break it. *Ann N Y Acad Sci* 690, 34-41.
269. Pulendran, B. and Ahmed, R. (2011) Immunological mechanisms of vaccination. *Nat Immunol* 12, 509-17.
270. Dubensky, T. W., Jr. and Reed, S. G. (2010) Adjuvants for cancer vaccines. *Semin Immunol* 22, 155-61.
271. Wortzman, M. E., Clouthier, D. L., McPherson, A. J., Lin, G. H., Watts, T. H. (2013) The contextual role of TNFR family members in CD8(+) T-cell control of viral infections. *Immunol Rev* 255, 125-48.
272. Garcon, N., Chomez, P., Van Mechelen, M. (2007) GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. *Expert Rev Vaccines* 6, 723-39.
273. Harper, D. M., Franco, E. L., Wheeler, C., Ferris, D. G., Jenkins, D., Schuind, A., Zahaf, T., Innis, B., Naud, P., De Carvalho, N. S., Roteli-Martins, C. M., Teixeira, J., Blatter, M. M., Korn, A. P., Quint, W., Dubin, G. (2004) Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *Lancet* 364, 1757-65.
274. Paavonen, J., Jenkins, D., Bosch, F. X., Naud, P., Salmeron, J., Wheeler, C. M., Chow, S. N., Apter, D. L., Kitchener, H. C., Castellsague, X., de Carvalho, N. S., Skinner, S. R., Harper, D. M., Hedrick, J. A., Jaisamrarn, U., Limson, G. A., Dionne, M., Quint, W., Spiessens, B., Peeters, P., Struyf, F., Wieting, S. L., Lehtinen, M. O., Dubin, G. (2007) Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial. *Lancet* 369, 2161-70.
275. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., Kimoto, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *The Journal of experimental medicine* 189, 1777-82.
276. Ahonen, C. L., Doxsee, C. L., McGurran, S. M., Riter, T. R., Wade, W. F., Barth, R. J., Vasilakos, J. P., Noelle, R. J., Kedl, R. M. (2004) Combined TLR and CD40 triggering induces potent CD8+ T cell expansion with variable dependence on type I IFN. *The Journal of experimental medicine* 199, 775-84.

277. Hoebe, K., Janssen, E. M., Kim, S. O., Alexopoulou, L., Flavell, R. A., Han, J., Beutler, B. (2003) Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. *Nat Immunol* 4, 1223-9.
278. De Smedt, T., Pajak, B., Muraille, E., Lespagnard, L., Heinen, E., De Baetselier, P., Urbain, J., Leo, O., Moser, M. (1996) Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *The Journal of experimental medicine* 184, 1413-24.
279. Shalek, A. K., Satija, R., Shuga, J., Trombetta, J. J., Gennert, D., Lu, D., Chen, P., Gertner, R. S., Gaublomme, J. T., Yosef, N., Schwartz, S., Fowler, B., Weaver, S., Wang, J., Wang, X., Ding, R., Raychowdhury, R., Friedman, N., Hacohen, N., Park, H., May, A. P., Regev, A. (2014) Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. *Nature* 510, 363-9.
280. McAleer, J. P. and Vella, A. T. (2010) Educating CD4 T cells with vaccine adjuvants: lessons from lipopolysaccharide. *Trends Immunol* 31, 429-35.
281. Cui, W., Joshi, N. S., Liu, Y., Meng, H., Kleinstein, S. H., Kaech, S. M. (2014) TLR4 ligands lipopolysaccharide and monophosphoryl lipid a differentially regulate effector and memory CD8+ T Cell differentiation. *J Immunol* 192, 4221-32.
282. Rawlings, D. J., Schwartz, M. A., Jackson, S. W., Meyer-Bahlburg, A. (2012) Integration of B cell responses through Toll-like receptors and antigen receptors. *Nat Rev Immunol* 12, 282-94.
283. Tokoyoda, K., Zehentmeier, S., Hegazy, A. N., Albrecht, I., Grun, J. R., Lohning, M., Radbruch, A. (2009) Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* 30, 721-30.
284. Mitchell, T. C., Hildeman, D., Kedl, R. M., Teague, T. K., Schaefer, B. C., White, J., Zhu, Y., Kappler, J., Marrack, P. (2001) Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat Immunol* 2, 397-402.
285. Myers, L., Lee, S. W., Rossi, R. J., Lefrancois, L., Kwon, B. S., Mittler, R. S., Croft, M., Vella, A. T. (2006) Combined CD137 (4-1BB) and adjuvant therapy generates a developing pool of peptide-specific CD8 memory T cells. *Int Immunol* 18, 325-33.

286. Srivastava, A. K., Dinc, G., Sharma, R. K., Yolcu, E. S., Zhao, H., Shirwan, H. (2014) SA-4-1BBL and monophosphoryl lipid A constitute an efficacious combination adjuvant for cancer vaccines. *Cancer Res* 74, 6441-51.
287. Croft, M., Benedict, C. A., Ware, C. F. (2013) Clinical targeting of the TNF and TNFR superfamilies. *Nat Rev Drug Discov* 12, 147-68.
288. Melero, I., Berman, D. M., Aznar, M. A., Korman, A. J., Perez Gracia, J. L., Haanen, J. (2015) Evolving synergistic combinations of targeted immunotherapies to combat cancer. *Nat Rev Cancer* 15, 457-72.
289. Rothenfusser, S., Hornung, V., Ayyoub, M., Britsch, S., Towarowski, A., Krug, A., Sarris, A., Lubenow, N., Speiser, D., Endres, S., Hartmann, G. (2004) CpG-A and CpG-B oligonucleotides differentially enhance human peptide-specific primary and memory CD8+ T-cell responses in vitro. *Blood* 103, 2162-9.
290. Tough, D. F., Sun, S., Sprent, J. (1997) T cell stimulation in vivo by lipopolysaccharide (LPS). *The Journal of experimental medicine* 185, 2089-94.
291. Maxwell, J. R., Rossi, R. J., McSorley, S. J., Vella, A. T. (2004) T cell clonal conditioning: a phase occurring early after antigen presentation but before clonal expansion is impacted by Toll-like receptor stimulation. *J Immunol* 172, 248-59.
292. Sin, W. X., Li, P., Yeong, J. P., Chin, K. C. (2012) Activation and regulation of interferon-beta in immune responses. *Immunol Res* 53, 25-40.
293. Berzofsky, J. A., Ahlers, J. D., Belyakov, I. M. (2001) Strategies for designing and optimizing new generation vaccines. *Nat Rev Immunol* 1, 209-19.
294. Butterfield, L. H. (2015) Cancer vaccines. *Bmj* 350, h988.
295. Hu, X., Chakravarty, S. D., Ivashkiv, L. B. (2008) Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. *Immunol Rev* 226, 41-56.
296. Eisen, T., Hedlund, G., Forsberg, G., Hawkins, R. (2014) Naptumomab estafenatox: targeted immunotherapy with a novel immunotoxin. *Curr Oncol Rep* 16, 370.
297. Elkord, E., Burt, D. J., Sundstedt, A., Nordle, O., Hedlund, G., Hawkins, R. E. (2015) Immunological response and overall survival in a subset of advanced renal cell carcinoma patients from a randomized phase 2/3 study of naptumomab estafenatox plus IFN-alpha versus IFN-alpha. *Oncotarget* 6, 4428-39.

298. Terman, D. S., Bohach, G., Vandenesch, F., Etienne, J., Lina, G., Sahn, S. A. (2006) Staphylococcal superantigens of the enterotoxin gene cluster (egc) for treatment of stage IIIb non-small cell lung cancer with pleural effusion. *Clin Chest Med* 27, 321-34.
299. Schenkel, J. M., Fraser, K. A., Beura, L. K., Pauken, K. E., Vezys, V., Masopust, D. (2014) T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science* 346, 98-101.
300. Curtsinger, J. M., Lins, D. C., Mescher, M. F. (2003) Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *The Journal of experimental medicine* 197, 1141-51.
301. Maxwell, J. R., Yadav, R., Rossi, R. J., Ruby, C. E., Weinberg, A. D., Aguila, H. L., Vella, A. T. (2006) IL-18 bridges innate and adaptive immunity through IFN-gamma and the CD134 pathway. *J Immunol* 177, 234-45.
302. Ruby, C. E., Montler, R., Zheng, R., Shu, S., Weinberg, A. D. (2008) IL-12 is required for anti-OX40-mediated CD4 T cell survival. *J Immunol* 180, 2140-8.
303. Croft, M. (2014) The TNF family in T cell differentiation and function--unanswered questions and future directions. *Semin Immunol* 26, 183-90.
304. Mikucki, M. E., Fisher, D. T., Matsuzaki, J., Skitzki, J. J., Gaulin, N. B., Muhitch, J. B., Ku, A. W., Frelinger, J. G., Odunsi, K., Gajewski, T. F., Luster, A. D., Evans, S. S. (2015) Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. *Nat Commun* 6, 7458.
305. Tamaru, M., Nishioji, K., Kobayashi, Y., Watanabe, Y., Itoh, Y., Okanoue, T., Murai, M., Matsushima, K., Narumi, S. (2000) Liver-infiltrating T lymphocytes are attracted selectively by IFN-inducible protein-10. *Cytokine* 12, 299-308.
306. Swiecki, M. and Colonna, M. (2010) Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. *Immunol Rev* 234, 142-62.
307. Hu, W., Jain, A., Gao, Y., Dozmorov, I. M., Mandraju, R., Wakeland, E. K., Pasare, C. (2015) Differential outcome of TRIF-mediated signaling in TLR4 and TLR3 induced DC maturation. *Proc Natl Acad Sci U S A* 112, 13994-9.
308. Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y. M., Gale, M., Jr., Akira, S., Yonehara, S., Kato, A., Fujita, T. (2005) Shared

- and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 175, 2851-8.
309. Gough, M. J., Ruby, C. E., Redmond, W. L., Dhungel, B., Brown, A., Weinberg, A. D. (2008) OX40 agonist therapy enhances CD8 infiltration and decreases immune suppression in the tumor. *Cancer Res* 68, 5206-15.
 310. Datta, S. K., Redecke, V., Prilliman, K. R., Takabayashi, K., Corr, M., Tallant, T., DiDonato, J., Dziarski, R., Akira, S., Schoenberger, S. P., Raz, E. (2003) A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J Immunol* 170, 4102-10.
 311. Ngoi, S. M., Tovey, M. G., Vella, A. T. (2008) Targeting poly(I:C) to the TLR3-independent pathway boosts effector CD8 T cell differentiation through IFN-alpha/beta. *J Immunol* 181, 7670-80.
 312. Djeu, J. Y., Heinbaugh, J. A., Holden, H. T., Herberman, R. B. (1979) Augmentation of mouse natural killer cell activity by interferon and interferon inducers. *J Immunol* 122, 175-81.
 313. Zabaleta, A., Arribillaga, L., Llopiz, D., Dotor, J., Lasarte, J. J., Prieto, J., Borrás-Cuesta, F., Esteban, J. I., Quer, J., Vayreda, F., Sarobe, P. (2007) Induction of potent and long-lasting CD4 and CD8 T-cell responses against hepatitis C virus by immunization with viral antigens plus poly(I:C) and anti-CD40. *Antiviral Res* 74, 25-35.
 314. Barrios, K. and Celis, E. (2012) TriVax-HPV: an improved peptide-based therapeutic vaccination strategy against human papillomavirus-induced cancers. *Cancer Immunol Immunother* 61, 1307-17.
 315. Kumai, T., Lee, S., Cho, H.-I., Sultan, H., Kobayashi, H., Harabuchi, Y., Celis, E. (2017) Optimization of Peptide Vaccines to Induce Robust Antitumor CD4 T-cell Responses. *Cancer Immunology Research* 5, 72.
 316. Llopiz, D., Dotor, J., Zabaleta, A., Lasarte, J. J., Prieto, J., Borrás-Cuesta, F., Sarobe, P. (2008) Combined immunization with adjuvant molecules poly(I:C) and anti-CD40 plus a tumor antigen has potent prophylactic and therapeutic antitumor effects. *Cancer Immunol Immunother* 57, 19-29.

317. Mullen, A. C., High, F. A., Hutchins, A. S., Lee, H. W., Villarino, A. V., Livingston, D. M., Kung, A. L., Cereb, N., Yao, T. P., Yang, S. Y., Reiner, S. L. (2001) Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292, 1907-10.
318. Mullen, A. C., Hutchins, A. S., High, F. A., Lee, H. W., Sykes, K. J., Chodosh, L. A., Reiner, S. L. (2002) Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. *Nat Immunol* 3, 652-8.
319. Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., Beary, H., Hayes, D., Frankel, S. S., Birx, D. L., Lewis, M. G. (2000) Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* 6, 207-10.
320. Moorthy, V. S. and Ballou, W. R. (2009) Immunological mechanisms underlying protection mediated by RTS,S: a review of the available data. *Malar J* 8, 312.
321. Dorrell, L., Yang, H., Ondondo, B., Dong, T., di Gleria, K., Suttill, A., Conlon, C., Brown, D., Williams, P., Bowness, P., Goonetilleke, N., Rostron, T., Rowland-Jones, S., Hanke, T., McMichael, A. (2006) Expansion and diversification of virus-specific T cells following immunization of human immunodeficiency virus type 1 (HIV-1)-infected individuals with a recombinant modified vaccinia virus Ankara/HIV-1 Gag vaccine. *J Virol* 80, 4705-16.
322. Thaiss, C. A. and Kaufmann, S. H. (2010) Toward novel vaccines against tuberculosis: current hopes and obstacles. *Yale J Biol Med* 83, 209-15.
323. Bejon, P., Peshu, N., Gilbert, S. C., Lowe, B. S., Molyneux, C. S., Forsdyke, J., Lang, T., Hill, A. V., Marsh, K. (2006) Safety profile of the viral vectors of attenuated fowlpox strain FP9 and modified vaccinia virus Ankara recombinant for either of 2 preerythrocytic malaria antigens, ME-TRAP or the circumsporozoite protein, in children and adults in Kenya. *Clin Infect Dis* 42, 1102-10.
324. Zhang, S., Li, W., Xia, Z., Mao, Y. (2011) CD4 T cell dependent tumor immunity stimulated by dendritic cell based vaccine. *Biochem Biophys Res Commun* 413, 294-8.
325. Kreiter, S., Vormehr, M., van de Roemer, N., Diken, M., Lower, M., Diekmann, J., Boegel, S., Schrors, B., Vascotto, F., Castle, J. C., Tadmor, A. D., Schoenberger, S. P., Huber, C., Tureci, O., Sahin, U. (2015) Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* 520, 692-6.

326. Kumai, T., Matsuda, Y., Ohkuri, T., Oikawa, K., Ishibashi, K., Aoki, N., Kimura, S., Harabuchi, Y., Celis, E., Kobayashi, H. (2015) c-Met is a novel tumor associated antigen for T-cell based immunotherapy against NK/T cell lymphoma. *Oncoimmunology* 4, e976077.
327. Kumai, T., Matsuda, Y., Oikawa, K., Aoki, N., Kimura, S., Harabuchi, Y., Celis, E., Kobayashi, H. (2013) EGFR inhibitors augment antitumour helper T-cell responses of HER family-specific immunotherapy. *Br J Cancer* 109, 2155-66.
328. McAleer, J. P. and Vella, A. T. (2008) Understanding how lipopolysaccharide impacts CD4 T-cell immunity. *Crit Rev Immunol* 28, 281-99.
329. Wang, H., Li, M., Hung, C. Y., Sinha, M., Lee, L. M., Wiesner, D. L., LeBert, V., Lerksuthirat, T., Galles, K., Suresh, M., DeFranco, A. L., Lowell, C. A., Klein, B. S., Wuthrich, M. (2016) MyD88 Shapes Vaccine Immunity by Extrinsically Regulating Survival of CD4⁺ T Cells during the Contraction Phase. *PLoS Pathog* 12, e1005787.
330. Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., Colonna, M. (1999) Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* 5, 919-23.
331. Schulz, O., Diebold, S. S., Chen, M., Naslund, T. I., Nolte, M. A., Alexopoulou, L., Azuma, Y. T., Flavell, R. A., Liljestrom, P., Reis e Sousa, C. (2005) Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433, 887-92.
332. Dhodapkar, K. M., Banerjee, D., Connolly, J., Kukreja, A., Matayeva, E., Veri, M. C., Ravetch, J. V., Steinman, R. M., Dhodapkar, M. V. (2007) Selective blockade of the inhibitory Fcγ receptor (FcγRIIB) in human dendritic cells and monocytes induces a type I interferon response program. *The Journal of experimental medicine* 204, 1359-69.