

Targeting lymph node niches enhances type 1 immune responses to immunization

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Jeffrey Lian

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Targeting lymph node niches enhances type 1 immune responses to immunization**ABSTRACT**

The generation of a robust CD4⁺ T helper cell type 1 (Th1) response is an essential component of protective type 1 immunity following infection or vaccination, providing critical support for cytotoxic T cell responses and the production of protective antibodies. This process requires the co-localization and interaction of innate and adaptive immune cells within the reactive lymph node (LN), including the redistribution of CD4⁺ T cells from the deep paracortex to microenvironments in the peripheral regions of the LN. The mechanisms governing the spatial and temporal coordination of immune cell localization within the LN, and the ability to target aspects of this response through immunization strategies, are incompletely understood.

In this work, we examined whether immunization formulation associated with enhanced vaccine efficacy could promote antigen distribution and recruitment of immune cells into LN niches associated with optimal type 1 responses. In contrast to immunization with antigen and toll-like receptor agonist in saline alone, immunization with an identical mixture emulsified in paraffin oil (Incomplete Freund's Adjuvant) promoted a rapid targeting of antigen to interfollicular regions (IFR) of the LN near B cell areas, which led to increased differentiation of polyfunctional interferon- γ and tumor necrosis factor- α double-producing Th1 cells. Inflammatory monocytes infiltrated the immunized LN, localized toward antigen-rich regions, and highly upregulated CXCL10 in a type I interferon-dependent manner. In turn, CD4⁺ T cells mobilized out of the T cell zone and into the IFR/B cell regions in a manner not dependent on injection site depot, where they were strategically positioned to encounter IL-12⁺ inflammatory DCs in an

environment rich with CXCR3 ligand-producing cells for Th1 differentiation. Depletion of monocyte-derived cells in MM-DTR mice resulted in a reduction in CD4⁺ T cell mobilization out of the T cell zone and a loss of enhanced Th1 differentiation in response to emulsified immunization.

These data suggest that oil emulsion preferentially targets antigen to LN regions where responding immune cells create an inflammatory niche advantageous for supporting Th1 differentiation. Formulations that can replicate this effect by delivering antigen and adjuvant to distinct LN niches such as IFR may improve vaccine efficacy.

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DEDICATION

To Elizabeth,

For though she may never read a letter herein
she is written everywhere within.

**CHAPTER 1: Introduction to the organization of the lymph node and cues directing T cell
activation *in vivo***

A brief overview of the immune system

The mammalian immune system is capable of responding to a diverse array of pathogens and malignancies with specificity and long-lasting memory. A successful response involves both branches of the immune system, the innate and adaptive. The generation of protective immunity requires the coordination of a multitude of cell types across both systems as well as multiple organs in a kinetically and spatially organized manner. In a type 1 immune response, cooperation between innate and adaptive immune cells provides protection against intracellular bacteria, viruses and cancer.

Cells of the innate immune system include phagocytes such as neutrophils, monocytes, macrophages and dendritic cells (DCs), and provide a first line of defense in response to a pathogen. The constitutive expression of nonclonal, germline-encoded pattern recognition receptors (PRRs) allows innate immune cells to discriminate between self and non-self through the recognition of evolutionarily conserved components of bacteria and viruses (Akira et al., 2006). Activation through PRRs induces effector responses that can act locally at the site of pathogen encounter and recruit other immune cells to help limit damage and dissemination. The machinery for this response system is highly conserved among species but is not without some limitations. PRRs recognize a broad but limited set of components without antigen specificity. In addition, a germline-encoded repertoire means that any evasion away from the existing set of receptors or the evolution of defense mechanisms by a pathogen represents a permanent deficiency for the host. Furthermore, the innate immune system in general operates independent of immunologic memory, such that the quality of the response is relatively fixed upon subsequent encounters with the same pathogens. Nevertheless, the innate immune system can provide sufficient protection to some simple and common pathogens and is critical for initiating the adaptive immune response.

In order to deal with constantly mutating pathogens of higher complexity, the adaptive immune system first evolved in jawed vertebrates and consists primarily of T and B lymphocytes expressing receptors determined by rearrangeable immunoglobulin genes and able to recognize specific antigens (Pancer and Cooper, 2006). These features allow adaptive immune cells to generate a repertoire specific for an almost unlimited set of antigens. Although this response takes more time to develop to protective levels, the resulting clonal expansion of effector cells and generation of high-affinity antibodies allows the host to mount a strong and highly specific response originating from a low frequency of precursors. Unlike the innate immune system, a hallmark of adaptive immunity is the ability to generate a pool of memory cells, such that upon successive encounters with the same pathogen, a faster and stronger quality response is produced.

Despite the advantages provided by antigen-specificity and memory formation, the generation of adaptive immune responses still depends on instructions provided by the innate immune system. For example, the T cell receptor recognizes cognate peptide presented in the context of MHC and additionally requires costimulatory signals to mount an activated response. The discovery of the classical DC (Steinman and Cohn, 1973) and its ability to potently promote T cell activation (Steinman and Witmer, 1978) paved the way for its role as the bridge between innate and adaptive immunity and has served as the foundation for understanding the cooperation between these two arms of the immune system to provide optimal host protection.

Lymph nodes: marketplaces of the immune system

The naïve T cell repertoire is incredibly diverse consisting of tens of millions of unique receptors, but a cell with a given receptor only exists at very low frequency (Arstila et al., 1999). In order to generate an effective adaptive immune response *in vivo*, the immune system must then solve the dilemma of promoting the encounter of a naïve lymphocyte of limited precursor

frequency with cognate antigen to ensure timely and effective immune surveillance. Lymph nodes (LNs) are located throughout the body and are sites where antigen and a multitude of cell types, both innate and adaptive, congregate for the exchange of information and the initiation of adaptive immune responses. Circulating naïve lymphocytes enter LNs through specialized high endothelial venules (HEVs) (Marchesi V. T. et al., 1964) and survey the organ in search of their cognate antigen. Meanwhile, antigens originating in peripheral tissues arrive to the draining LN (dLN) either directly through passive drainage or carried by migratory cells, usually DCs, via afferent lymphatics (Merad et al., 2013; Platt and Randolph, 2013). Upon activation, a naïve T cell can differentiate and undergo clonal expansion, and B cells can acquire antigen and receive help from activated T cells to undergo somatic hypermutation and produce high-affinity antibodies. However, given the limited frequency of a naïve T cell specific for a given antigen, stochastic encounter between a naïve T cell and its cognate antigen displayed on the appropriate antigen-presenting cell even once within the LN represents a very low probability event. The ability to rapidly and efficiently coordinate not only the arrival of the necessary cells to the reactive LN but also to direct their positioning within the LN is therefore critical for a protective immune response. This process is achieved through the combination of a highly organized microanatomy within the LN containing a diversity of cell types that is orchestrated by a network of chemotactic cytokines, or chemokines, that control immune cell migration patterns and positioning.

Organization of the lymph node

The LN can be roughly divided into the T cell zone in the deep LN parenchyma, B cell zones organized into follicles situated around the LN periphery, and interfollicular regions (IFR) connecting these areas. Together they are surrounded by the subcapsular sinus (SCS) on the cortical side of the lymph node proximal to afferent lymphatics, which connect with the medullary

region in the lower hemisphere leading to the efferent lymphatics. Fluid from afferent lymph drains into the SCS of the LN before percolating through the cortical and medullary sinus and collecting in efferent lymph vessels that exit the LN. Antigen draining the LN does not freely permeate the parenchyma but is captured by macrophages lining the floor of the LN sinus (Clark, 1962; Nossal et al., 1965). SCS macrophages are thus uniquely positioned to function as a physical barrier at the intersection of lymph and tissue, and also to act as important participants in immune surveillance and initiating the early LN immune response.

CD169⁺ SCS are capable of acquiring antigen from the draining lymph and transferring antigen across the SCS floor to activate B cells (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007). CD169⁺ medullary sinus macrophages (MSMs) can be distinguished from SCS macrophages by their expression of F4/80, SIGNR1 (CD209b) and MARCO, and despite their name, cells of this phenotype can be found not only in medullary regions of the LN but also in IFR (Gray and Cyster, 2012). Medullary sinus macrophages are phenotypically similar to marginal zone macrophages in the spleen, and expression of the C-type lectin SIGNR1 facilitates their phagocytic capability (Kang et al., 2004). This subset of macrophages can capture lymph borne virus in response to vaccination and play an important role in host antiviral defense (Gonzalez et al., 2010).

The anatomical organization of the LN is supported by chemokines, which are constitutively produced but can also be rapidly induced during inflammation. Chemokines are 7-12 kDa proteins that can be mainly divided into 4 groups based on their cysteine structure: CC, CXC, CX3C, and XC. Chemokines can be bound by a 7-transmembrane G protein-coupled signaling receptor sensitive to PTX, or by an atypical G protein-independent receptor that may serve through scavenging functions (Zlotnik and Yoshie, 2012). Classical chemokine receptor signaling induces calcium flux, actin cytoskeleton rearrangement, integrin activation, and cellular migration toward increasing concentrations of ligand (Rot and von Andrian, 2004). The

regulation between chemokine receptor expression and chemokine production is thus able to provide a combinatorial template for dictating specific immune cell localization.

Naïve T cells circulate throughout the body, using lymph vessels and the thoracic duct to travel between blood, tissues and secondary lymphoid organs (Gowans James Learmonth et al., 1964). Entry into LNs through HEVs involves a multi-step cascade that involves initial tethering of L-selectin to peripheral node addressin and activated LFA-1-mediated sticking (Warnock et al., 1998), the latter of which requires engagement of CCR7 with its ligand CCL21 (Gunn et al., 1998a; Stein et al., 2000). Within the LN, a stromal cell network provides structure to the LN and compartmentalizes the organ into distinct microenvironments (Bajénoff et al., 2006a). CCR7 expressed by naïve T cells within the LN allows for congregation into the central T cell zone in the paracortex (Förster et al., 1996), which is organized along stromal fibroblastic reticular cells (FRCs) producing the CCR7 ligands CCL19 and 21, as well as the cytokine IL-7 to promote T cell homeostasis and survival (Link et al., 2007; Luther et al., 2000). Conversely, naïve B cells enter LNs via CCR7 but upregulate CXCR5 after entry (Förster et al., 1996) and cluster around stromal follicular dendritic cells (FDCs) producing CXCL13 and BAFF situated along the outer parenchyma underneath the LN sinus (Gunn et al., 1998b; Hase et al., 2004). The segregation of the LN into distinct microenvironments in the steady-state allows each cell type to maintain exposure to unique survival signals. Connecting the SCS with the LN parenchyma and occupying the space between the T and B cell zones is the IFR, which consists of a distinct stromal cell network and contains cortical ridges where migratory DCs accumulate (Katakai et al., 2004a) and will be discussed in greater detail later. At the interface between the IFR and the SCS, atypical chemokine receptor ACKR4 promotes CCR7-mediated DC migration across the SCS floor, potentially through scavenging of CCL19 and 21 to create a local chemokine gradient (Ulvmar et al., 2014). Together, these microenvironments allow a single organ to support homeostatic niches of multiple cell types. However, this spatial

segregation also dictates the need for the ability to specifically direct cell mobilization upon the onset of inflammation to promote the interaction between different cell types.

Arrival of antigen to the draining LN

Tissue-derived antigen can arrive in the dLN either freely through afferent lymphatics or carried by cells, predominantly migratory DCs. Lymphatic vessels are situated throughout peripheral tissues collecting interstitial fluid and flow unidirectionally, passing through the LNs and eventually returning to blood circulation. They serve as a primary means of redistributing fluid and proteins from interstitial space and provide a major route of transport for immune cells (Swartz, 2001). *In vivo* migration of DCs from the periphery to the dLN during both homeostasis and inflammation requires CCR7 (Förster et al., 1999; Ohl et al., 2004). Immature DCs are relatively sessile and specialized for antigen sampling of their surroundings and uptake through endocytosis. Engagement of PRRs, including TLRs and C-type lectins, triggers maturation of DCs resulting in the upregulation of pathways and machinery for antigen presentation and a transformation to a mobile state including increased expression of CCR7 (Sallusto et al., 1998a; Yanagihara et al., 1998). Activated DCs can then utilize CCR7 ligand gradients and enter lymphatics for transit to dLNs (Robbiani et al., 2000; Saeki et al., 1999).

Alternatively, freely draining antigen can arrive in the proximal dLN prior to the appearance of migratory DCs (Ingulli et al., 2002; Itano et al., 2003; Pior et al., 1999). Antigen arriving in this manner first enters into the SCS and provides the opportunity for engagement with the aforementioned LN macrophages lining the LN capsule for downstream activation or antigen transfer. In addition, soluble substances of lower molecular weight including antigens and chemokines can directly access the LN parenchyma through FRC conduits for potential interaction with DCs located along these routes or presentation on HEVs (Gretz et al., 2000; Palframan et al., 2001; Sixt et al., 2005). The ability for antigen to reach dLNs directly thus

provides opportunities for the initiation of LN inflammation in a timely manner while awaiting arrival of DCs from the periphery.

DCs can be classified into two major subgroups that express CD11c and MHCII: classical DCs (cDCs) and lymphocyte-like plasmacytoid DCs (pDCs) (Cella et al., 1999; Siegal et al., 1999). cDCs derive from the common dendritic cell progenitor (Naik et al., 2007; Onai et al., 2007), and can be further subdivided into *batf3* and *irf8*-dependent cDC1s and *ifn4*-dependent CD11b⁺ cDC2s (Grajales-Reyes et al., 2015; Schlitzer et al., 2015; Williams et al., 2013). Both cDC1s and cDC2s contain migratory and LN-resident subsets. In mouse lymphoid tissue, cDC1s express CD8 α ⁺ and XCR1⁺ and are now understood to be the principal DC subset responsible cross-priming CD8⁺ T cells *in vivo* (Dorner et al., 2009; den Haan et al., 2000; Hildner et al., 2008; Pooley et al., 2001). cDC2s appear more primarily associated with CD4⁺ T cell responses, although are more difficult to distinguish due to heterogeneity among cells expressing CD11b (Dudziak et al., 2007; Gao et al., 2013; Gerner et al., 2015; Kumamoto et al., 2013; Suzuki et al., 2004).

Th1 cell differentiation

In part through the production of their hallmark cytokine interferon-gamma (IFN γ), CD4⁺ T helper type 1 (Th1) cells contribute to immunity against intracellular bacteria, viruses, and tumors. Complete deficiency of IFN γ R in humans is rare and life-threatening, with survivors demonstrating increased susceptibility to infection especially stemming from mycobacteria (Newport et al., 1996; Pierre-Audigier et al., 1997). Th1 cells are required to provide help signals to activate macrophages for the destruction of intracellular pathogens (Suzuki et al., 1988), and are required for the generation of cytotoxic T cells that are capable of providing long-lived protection (von Herrath et al., 1996; Keene and Forman, 1982). CD40L stimulation provided by

Th1 cells augments co-stimulatory capabilities of APCs for CTL priming, while production of IL-2 during CD8⁺ T cell priming is critical for their memory formation (Bennett et al., 1997, 1998; Schoenberger et al., 1998; Williams et al., 2006). The induction of polyfunctional Th1 cells, able to produce IFN γ , TNF γ and IL-2, can be a correlate of vaccine response (Darrah et al., 2007).

The identification of Th1 cells as a unique subset of differentiated naïve CD4⁺ T cells derived from the ability of this clonotype to produce large amounts of IFN γ upon stimulation, distinct from Th2 cell production of IL-4 (Killar et al., 1987; Mosmann et al., 1986). Differentiation of Th1 cells *in vitro* could be driven through their exposure to IL-12 and neutralization of IL-4, while the use of heat-killed *Listeria monocytogenes* to stimulate IL-12 production in those cultures underscores the relationship between Th1 cells and immunity against intracellular pathogens (Hsieh et al., 1993). The identification of T-bet as a master transcriptional regulator of IFN γ expression and Th1 cell commitment established these cells as a *bona fide* T helper cell lineage and fundamentally linked Th1 cells with production of its hallmark cytokine IFN γ (Szabo et al., 2000). While TCR signals augment T-bet expression and are required for T cell activation, cytokines are crucial for successful lineage commitment by providing signals to promote and stabilize the transcriptional Th1 differentiation program through IL-12/STAT4 signaling and also supported by IFN γ (Grogan et al., 2001; Lighvani et al., 2001; Mullen et al., 2001).

In this manner, the immune system regulates the generation of inflammatory effector T cells by requiring the recognition of cognate antigen presented on MHC in addition to the exposure to skewing cytokines. *In vitro*, when the factors are combined simultaneously in close proximity, this effect can be achieved using a variety of conditions and antigen-presenting cells. However, the *in vivo* process is much more complex, and requires the coordination of immune cells across both space and time.

T cell activation *in vivo* and the importance of the LN interfollicular region

T cells require multiple interactions with DCs for activation and lineage commitment, while different types of DCs have shown distinct kinetics and localization behavior within the LN (Bajénoff et al., 2003; Braun et al., 2011; Itano et al., 2003; Mempel et al., 2004). Given our rapidly improving knowledge of the diverse stromal microenvironments contained within the LN and the ability for chemokines to induce activated T cell migration toward DCs, it is becoming clear that preferential localization within the LN may exist for several immune cell types and may dynamically change during inflammation. The development of advanced imaging techniques including confocal microscopy and 2-photon microscopy has allowed for the study of T cell activation *in situ* and *in vivo*. A method for quantitative image analysis of multiplex staining (histo-cytometry) has allowed for the identification of DC subsets *in situ* and revealed a spatial organization of DCs in the LN, with resident cDC1s primarily in the paracortex, resident CD11b⁺ DCs in the medullary region and near lymphatic sinuses, and migratory CD11b⁺ DCs localizing to the IFR (Gerner et al., 2012). LN-resident DCs appear capable of intranodal migration in response to inflammation (Gonzalez et al., 2010; Iannacone et al., 2010; Woodruff et al., 2014). The mechanisms underlying these localization behaviors in the steady-state and inflamed conditions remain unclear.

In vivo studies of CD8⁺ T cell activation in response to infection, as opposed to a common experimental approach of transferring isolated populations of activated DCs, revealed that CD8⁺ T cells preferentially interacted with infected DCs, and migrated into the IFR to encounter them for activation, as opposed to receiving signals in the deep parenchyma (Hickman et al., 2008; Norbury et al., 2002). CCR5 expression on CD8⁺ T cells was important for this localization behavior, although it is not singularly required depending on the model (Hickman et al., 2011; Kastenmüller et al., 2013). Regardless, these studies established that after initial priming, terminal effector differentiation occurs in peripheral regions of the LN.

Kastemüller *et al.* additionally examined CD8⁺ memory T cell positioning, and together with Sung *et al.* determined that IFN γ produced during the memory recall response prepositioned CXCR3-expressing CD8⁺ memory T cells in the cortical medullary ridge and IFR for rapid interaction with infected APCs in peripheral regions of the LN (Kastemüller *et al.*, 2013; Sung *et al.*, 2012).

Th1 cell activation dynamics *in vivo* have been relatively less well established. The chemokines CXCL9 and CXCL10 are strongly induced by interferons and are selectively bound by their receptor CXCR3 which is highly expressed on activated T cells (Farber, 1990; Loetscher *et al.*, 1996; Luster *et al.*, 1985). The link between IFN γ -inducible CXCR3 ligands and type 1 immunity suggests a strong relationship between the CXCR3 chemokine system and Th1 cells. Indeed, CXCR3 is preferentially expressed on Th1 versus Th2 cells and corresponds with their migration behavior accordingly (Bonecchi *et al.*, 1998; Sallusto *et al.*, 1998b). T-bet directly transactivates CXCR3, and the loss of T-bet results in a loss of CXCR3 expression and impaired migration to inflamed tissue (Lord *et al.*, 2005). Work done in our lab generated a transgenic dual-reporter mouse on the BL/6 background allowing for the visualization of CXCL9- and CXCL10-producing cells *in vivo* (Groom *et al.*, 2012) (functional CXCL11 is not expressed in this mouse background (Sierro *et al.*, 2007)). Groom *et al.* demonstrated that, in response to immunization with soluble TLR ligands, CXCR3 expression on CD4⁺ T cells was required for intranodal migration toward CXCL9- and CXCL10-producing cells in LN IFR and medullary regions for differentiation into polyfunctional TNF γ /IFN γ double-producing polyfunctional Th1 cells (**Figure 1.1**). CXCL10 was also important for formation of stable contacts with transferred DCs, agreeing with previous reports that chemokines can play a role not only in global cell positioning but local DC-T interactions (Friedman *et al.*, 2006; Molon *et al.*, 2005).

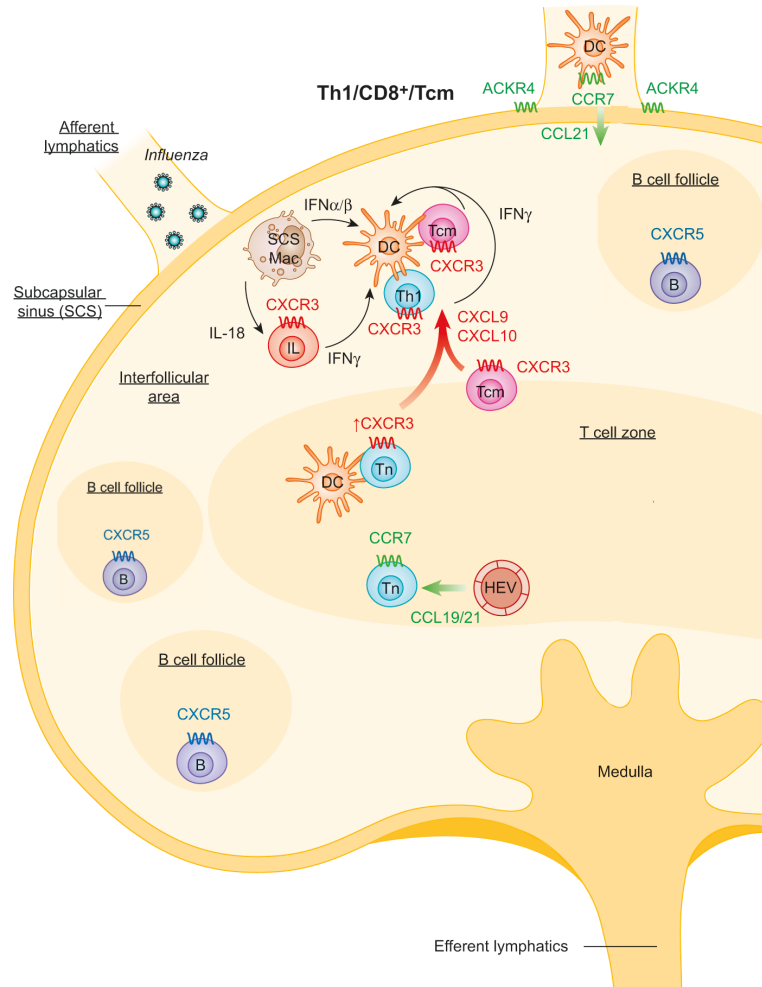


Figure 1.1 Chemokines direct intranodal T cell positioning for activation *in vivo*. Antigen can arrive in the dLN via passive drainage or carried by migratory CCR7⁺ DCs via afferent lymphatics. ACKR4 facilitates DC entry across the SCS floor. CCR7⁺ naïve T cells accumulate in the LN paracortex, while CXCR5⁺ B cells cluster in B cell follicles along the periphery. CXCR3 directs CD4⁺ cells toward CXCL9/10 gradients in LN IFR for Th1 differentiation, and prepositions memory CD8⁺ T cells for rapid mobilization to LN periphery. Adapted from (Lian and Luster, 2015).

Two recent studies have elegantly shown the interplay between CD4⁺ and CD8⁺ T cell priming in response to models of viral infection. Using different models of viral infection, these groups showed that initial priming of CD4⁺ and CD8⁺ T cells occur in distinct time and place. CD4⁺ T cells were primed earlier than CD8⁺ T cells, and licensed XCR1⁺ cDC1s to promote cross-priming of CTLs. XCR1⁺ DCs located in the IFR served as a bridge where CD4⁺ and CD8⁺ T cells interacted on the same DC to promote memory CTL formation (Eickhoff et al., 2015; Hor et al., 2015).

These studies of *in vivo* T cell priming dynamics in the LN have sharpened our understanding of cues regulating intranodal T cell positioning and highlighted the importance of peripheral regions of the LN such as the IFR as critical microenvironments where Th1 responses are initiated. With this in mind, we wanted to examine how the LN microenvironment may be altered by vaccine formulation.

Vaccine adjuvants

The concept of conferring immunity to a nonimmune individual was well established throughout Asia and Africa by the 10th century. During this period, in an effort to combat smallpox, fresh biological material was transferred from an infected individual to a nonimmune recipient, thus inoculating the recipient with a small dose of live virus (Gross and Sepkowitz, 1998). In China, the practice of intranasal inoculation as a way to immunize individuals against smallpox appeared to be common, although these types of practices were risky and could lead to active infection in the recipient. Centuries later, Edward Jenner's landmark observations of milkmaids acquiring immunity to smallpox through exposure to cowpox led to the development of a smallpox vaccine in the late 18th century and became one of the major underpinnings of modern preventative vaccinology. The ability to generate protective immunity through the safe administration of vaccines is one of the miracles of modern medicine, and has led to the near

elimination of reported cases in the United States of several afflictions previously common throughout much of the 20th century, including measles, pertussis, diphtheria, and hepatitis A (CDC, 2018).

Early vaccines generally consisted of live attenuated pathogens, which contained endogenous adjuvants, and were thus capable of stimulating a robust immune response. However, for most diseases, the ability to create vaccines based on inactivated pathogens or recombinant components offers a much safer and customizable alternative. The downside is the loss in immunogenicity provided by the natural form of the pathogen originally containing a multitude of innate immune system and PRR stimuli. The discovery of aluminum salts (alum) as an effective vaccine adjuvant in the 1920's by Alexander T. Glenny and colleagues (Glenny et al., 1926) led to its foundational use in vaccines treating diphtheria and tetanus, and the ability to restore potent adjuvant activity through vaccine formulation has led to the inclusion of alum in many current inactivated and subunit vaccines.

The establishment of antigen depot in the tissue was historically proposed as a primary mechanism for the adjuvant effects of classical adjuvants such as alum and oil emulsions. Alum has been shown to form a depot with some antigens but not others (Gupta et al., 1996; de Veer et al., 2010). Additional studies with alum used radiolabeled antigen to track antigen persistence, or excised the injection site to find that a tissue-site depot was not needed for its adjuvant effects, which has also been confirmed using the oil emulsion MF59 (Dupuis et al., 1999; Hutchison et al., 2011; Noe et al., 2010). These studies highlight the need to further examine immunization drainage in the proximal LN and the effects on immune activation.

Jules Freund initially observed that paraffin oil could be used to enhance and prolong antibody formation, which led to the development of Complete and Incomplete Freund's Adjuvant (Freund, 1947, 1951; Freund et al., 1937). Whereas Complete Freund's Adjuvant contains heat-killed *Mycobacterium tuberculosis* with endogenous PRR ligands and therefore

can on its own stimulate type 1-skewed cellular immunity, Incomplete Freund's Adjuvant lacks the mycobacterial components and consists solely of non-metabolizable oil (85% paraffin oil and 15% mannide monooleate). Granuloma and abscess formation at the injection site with CFA precluded its use in humans. Attempts to use IFA in clinical applications in influenza vaccines in the 1950s did result in enhanced antibody production (Salk and Laurent, 1952), but adverse reactions at the injection site likely due to impure formulation resulted in the use of IFA in humans to fall out of favor. Nevertheless, Freund's Adjuvants have remained useful tools and long been used to generate boosted immune responses in experimental models, and more recent improvements to formulation purity of IFA preparations have led to an increase in its safety profile and the revisiting of its clinical applicability in some cases such as HIV and cancer (Apostólico et al., 2016).

Following the implementation of alum in vaccines, no new adjuvants were approved until several decades later when the oil-in-water adjuvant MF59 was approved in the influenza vaccine for use in the elderly in the 1990s, with only another handful of currently licensed adjuvant systems developed since (Del Giudice et al., 2018). Several diseases remain without a successful vaccine or require improvements to existing formulations, including HIV, influenza, and cancer. Therefore, a large need remains for continuing to understand how the immune system can be targeted to promote robust cellular immune responses to vaccination, and what events are associated with effective vaccine adjuvants and formulations.

Dissertation Objectives

Given recent advances in our understanding of *in vivo* CD4⁺ T cell priming and dLN microenvironments, we wanted to examine whether immunization formulation associated with enhanced immune responses is able to promote immune cell recruitment to LN niches where optimal Th1 differentiation takes place. This study aims to address whether antigen distribution

in the LN is affected by oil emulsion, and to better understand how chemokines help determine immune cell positioning in the LN. By elucidating the mechanisms underlying LN activation in response to effective adjuvants, we hope to identify immune pathways that can be utilized to improve rational vaccine design.

CHAPTER 2: Targeting lymph node niches enhances type 1 immune responses to immunization

Parts of this chapter are adapted from work currently in preparation for submission as:

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Author contributions:

Jeffrey Lian designed and performed most experiments and analyzed all data. Aleksandra Ozga assisted with CUBIC-clearing of LNs, performed light sheet microscopy, and provided valuable input. Caroline L. Sokol, and Andrew D. Luster helped design the study and provided valuable input.

INTRODUCTION

The generation of an effective adaptive immune response requires the convergence of multiple cell types in the same anatomical location. A network of secondary lymphoid organs are strategically positioned throughout the body and provide hubs where naïve lymphocytes from circulation can accumulate to survey antigens and mount an adaptive immune response. After pathogen encounter at a barrier surface, antigens derived from the pathogen arrive in the draining lymph node (dLN) via afferent lymphatics primarily through direct drainage or carried by migratory dendritic cells (DCs) that have seen the pathogen in the context of inflammatory signals. LNs contain a highly regulated and specialized microanatomy that facilitates the activation of naïve T cells through encounter with cognate antigen presented on antigen-presenting cells and exposure to inflammatory signals for differentiation and clonal expansion (Junt et al., 2008).

CD4⁺ T cells can differentiate into one of several lineages of helper T cell subsets depending on the context of activation, such as T-helper type 1 (Th1), Th2, Th17, T regulatory and T follicular helper cells, and can modulate the activity and function of other cells (Zhu et al.,

2010). Polyfunctional Th1 cells can secrete high levels of interferon-gamma (IFN γ) and tumor necrosis factor-alpha (TNF α) and are critical orchestrators of effective immunity against intracellular pathogens and tumor cells. Although inflammatory cues required for Th1 differentiation are well defined, less is known about the timing and environmental context required *in vivo* for optimal T cell differentiation. CD4⁺ T cell priming and lineage commitment involves multiple interactions between T cells and DCs in the LN (Celli et al., 2005; Eickhoff et al., 2015; Itano et al., 2003; Mempel et al., 2004). Prior to activation and clonal expansion, a naïve CD4⁺ T cell specific for a given antigen exists only at extremely low frequency (Jenkins and Moon, 2012). If left to chance, productive DC-T interactions would represent very low probability events and be insufficient to provide timely protection for the host. Therefore, the rapid and efficient recruitment of necessary cells into the dLN as well as their positioning within the dLN is critical for generating a robust adaptive immune response.

Chemokines are produced at steady state and can be rapidly induced upon inflammation, thereby serving as essential cues to organize the distribution of cells at homeostasis and also to direct immune cell re-localization in response to environmental changes (Griffith et al., 2014). Within the LN, chemokine microenvironments support organization into distinct immune compartments. In the steady state, expression of CCL19 and 21 in the deep paracortex establishes an inner T cell zone containing CCR7-expressing naïve T cells (Förster et al., 1999; Gunn et al., 1999), whereas CXCR5 expression on naïve B cells guides their organization around CXCL13-expressing follicles (Förster et al., 1996). Upregulation of chemokines during inflammation directs intranodal immune cell localization and promotes DC-T interactions (Tang and Cyster, 1999). The interfollicular region (IFR) separating the B and T cell zones connects the subcapsular sinus (SCS) with the LN cortex, where CCL19 and 21 gradients are created through scavenging activity of ACKR4 (Ulvmar et al., 2014). DCs

preferentially accumulate in this region, which is anatomically positioned to serve as a crossroads bridging adaptive and innate immunity (Katakai et al., 2004a).

The IFR has been shown to play an important role in type 1 inflammation. Previous work from our lab demonstrated that expression of the CXCR3 ligands CXCL9 and CXCL10 are highly upregulated in LN IFR and are required for optimal Th1 differentiation (Groom et al., 2012). In response to type 1 stimuli, CXCR3 is rapidly upregulated *in vivo* on CD4⁺ T cells in the dLN prior to proliferation and is critical for directing CD4⁺ T cells towards LN IFR (Groom et al., 2012), and in response to vaccination CXCR3 upregulation is observed within the first 12h (Woodruff et al., 2014). The IFR has also been shown to play an important role as the site where CD4⁺ T cells co-localize with cross-presenting DCs and deliver help to CD8⁺ cytotoxic lymphocytes (Eickhoff et al., 2015; Hor et al., 2015; Qi et al., 2014), further underscoring the importance of this region in generating a robust immune response to type 1 pathogens.

A better understanding of the events leading to CD4⁺ T cell localization to LN IFR, optimal Th1 differentiation and strategies to promote these pathways, are necessary for the creation of new and more effective vaccines. Inactivated and recombinant subunit vaccines can be poorly immunogenic, while live attenuated vaccines are not suitable for some pathogens or always safe to use in certain segments of the population such as young children and the elderly. Vaccines formulated with oil emulsions have been shown to promote a stronger immune response over the same dose but without the addition oil, including the generation of robust antibody titers and cellular immunity (Coffman et al., 2010; Di Pasquale et al., 2015). This suggests that antigenic stimulation even in the presence of pattern recognition receptor agonist may not be sufficient for producing the best vaccine responses without delivery in the context of optimal formulation. Responses to oil emulsions have historically been partially attributed to the establishment of an antigen depot at the injection site, but studies using alum provide a notable example of modes of action independent of injection site depot (Hutchison et al., 2011; Noe et

al., 2010). Injected antigen depending on size can directly access afferent lymphatics and rapidly enter the dLN through passive drainage (Rozenendaal et al., 2009; Sixt et al., 2005), and the pattern of antigen dispersal in the LN can have profound effects on T cell activation (Gerner et al., 2017). We sought to better understand how oil emulsion might affect antigen targeting in the dLN, and whether these patterns could lead to differences in directing the intranodal positioning of immune cells to LN microenvironments capable of supporting robust Th1 differentiation.

In this study, we found that immunization with the model antigen OVA and the toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) emulsified in Incomplete Freund's Adjuvant (IFA) enhanced Th1 responses compared to an identical dose of OVA and LPS but without IFA. Immunization with or without IFA rapidly drained into the dLN but to different locations depending on the presence of IFA. Immunization with IFA preferentially targeted antigen and adjuvant to the IFR, while immunization without IFA preferentially targeted antigen and adjuvant to the medullary regions. The unique pattern of antigen distribution following immunization with the combination of OVA/LPS/IFA correlated with enhanced recruitment of inflammatory monocytes into the IFR that produced CXCL10 in a type I interferon-dependent manner, which amplified CXCR3 ligand-gradients to direct CD4⁺ T cell localization to the IFR. CD4⁺ T cell localization to these niches was important for encountering DCSIGN⁺ IL-12-producing DCs in the context of CXCR3 ligands and driving polyfunctional Th1 differentiation. These findings demonstrate that the formulation of an immunogen can have a significant effect on directing immune cell localization within the LN and can be utilized for the development of more effective vaccines.

RESULTS

Oil emulsion enhances the type 1 response to immunization

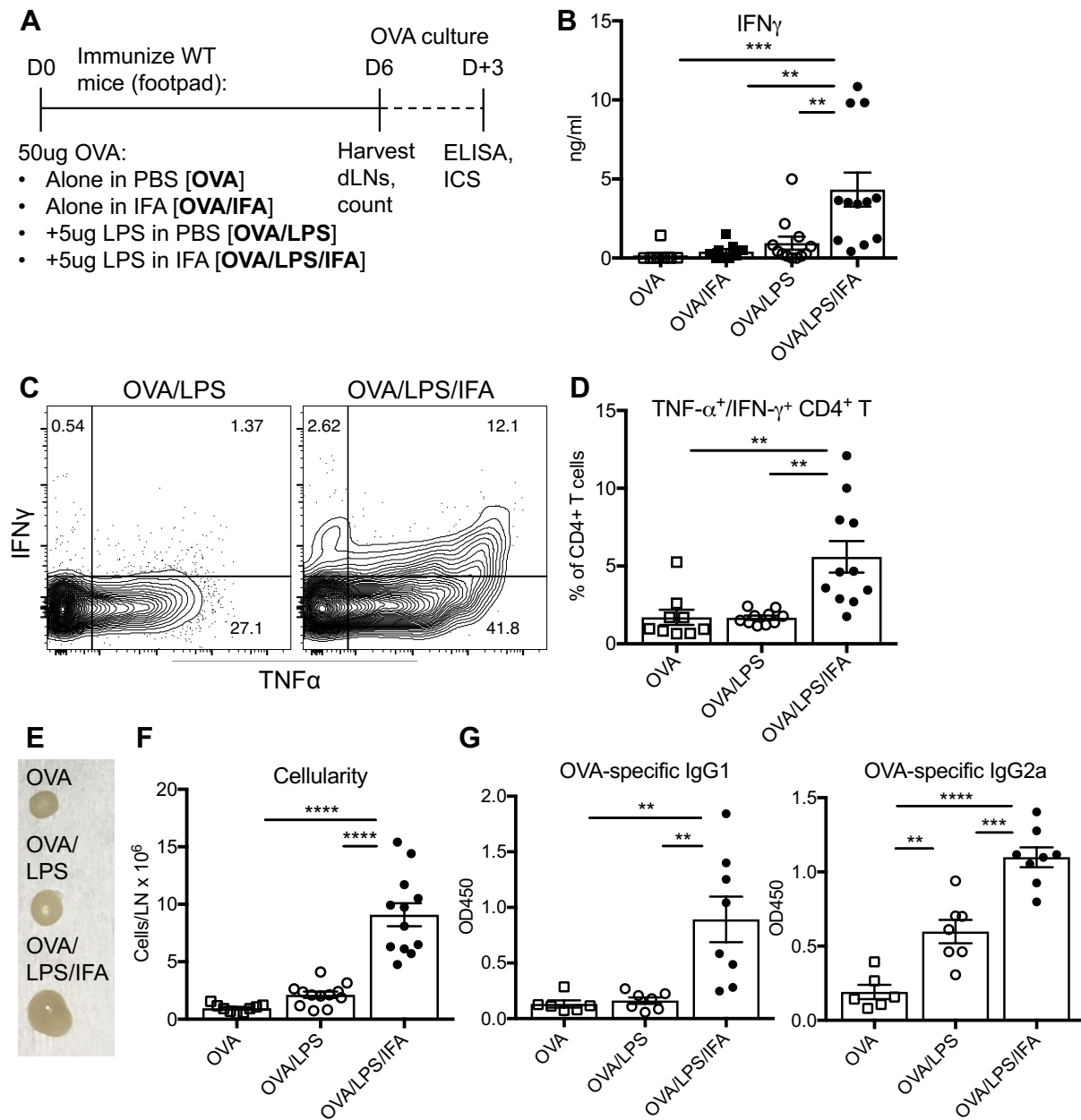
To understand the vaccine components required to induce a robust type 1 immune response, we immunized mice in the footpad with OVA protein as a model antigen, with or without LPS as a type-1 skewing TLR agonist, either in PBS alone or emulsified 1:1 with Incomplete Freund's Adjuvant (IFA) and harvested dLNs 6 days later (**Figure 2.1A**). Although immunization with OVA and LPS in PBS (OVA/LPS) contained a high dose of TLR agonist, this was not sufficient to generate a robust type 1 response compared to the identical dose of OVA and LPS emulsified in IFA (OVA/LPS/IFA). When dLN cells from immunized mice were cultured with OVA, only the cells from mice immunized with the combination of OVA/LPS/IFA produced a significant amount of IFN γ whereas cells from mice immunized either with OVA alone, OVA/IFA (without LPS) or OVA/LPS did not produce a high level of IFN γ (**Figure 2.1B**). Since OVA/IFA without TLR agonist (LPS) was unable to induce the production of the hallmark type 1 cytokine IFN γ in the dLN, it was not included for further analysis and we proceeded to try and better understand why immunization with antigen in combination with a potent TLR agonist in saline required emulsion in IFA to induce robust IFN γ production. To determine if the increased level of IFN γ production from OVA/LPS/IFA-immunized dLNs cultured with OVA was associated with Th1 cell responses, intracellular cytokine staining of these same cells were analyzed. An increased frequency of polyfunctional Th1 cells co-producing TNF γ and IFN γ was seen in OVA-cultured dLN cells after OVA/LPS/IFA immunization compared to OVA or OVA/LPS (**Figures 2.1C & 1D**). 6 days post-immunization, compared to immunization with OVA alone, *ex vivo* LN size and cellularity were expanded in response to immunization with OVA/LPS/IFA but not OVA/LPS (**Figure 2.1E & 2.1F**). In addition, OVA/LPS/IFA immunization generated the highest levels of OVA-specific IgG1 and IgG2a antibodies in the serum 21 days post-immunization

compared to OVA alone or OVA/LPS (**Figure 2.1G**). OVA/LPS immunization was able to induce the generation of moderate levels of OVA-specific IgG2a antibodies in the serum compared to OVA alone, suggesting that TLR agonist without emulsification does provide type 1 skewing signals and is capable of stimulating an adaptive immune response to some degree.

These data suggest that while the presence of a high dose of LPS in an immunization in saline can generate some production of type 1 skewed antibodies, emulsification in IFA leads to a more robust type 1 response, including higher levels of antigen-specific antibody production and enhanced Th1 differentiation.

Figure 2.1. Immunization with IFA leads to a more robust Th1 response. (A-F) WT mice were immunized in the footpad with OVA with or without LPS in PBS or IFA, and dLNs harvested 6 days later. **(A)** Schematic of experimental design. **(B)** Total dLN cells were cultured with 100µg/mL of OVA protein for 3 days and IFN γ in the supernatant was measured by ELISA. **(C)** dLNs cultured with OVA as in (B) were stained for intracellular cytokines and CD4⁺ T cells analyzed. Representative flow cytometry plot. **(D)** Frequency of polyfunctional Th1 cells among CD4⁺ T cells from dLNs cultured with OVA. **(E)** Representative photograph of excised LNs on day 6. **(F)** Total cellularity of LNs as determined by flow cytometry. **(G)** Relative OVA-specific IgG antibody levels from serum harvested 21 days post-immunization were measured by ELISA (OD450). Data are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test; ** $p \leq 0.01$, *** ≤ 0.001 , **** ≤ 0.0001 . Results in (A-F) are pooled from 3 independent experiments (n=9-11 mice per group). Results in (G) are pooled from 2 independent experiments (n=6-8 mice per group).

Figure 2.1 (Continued)



Oil emulsion targets antigen to interfollicular regions of the lymph node

Since immunization with a high dose of LPS did not result in robust type-1 responses unless it was emulsified in IFA, we hypothesized that changes to the biophysical nature of the immunization formulation after emulsification may be leading to an altered pattern of antigen distribution in the dLN and thereby affect downstream immune pathways, potentially leading to increased Th1 differentiation. To test this, we labeled OVA with the fluorescent small molecule dye Alexa Fluor 488 (AF488-OVA) and immunized mice with either AF488-OVA/LPS or AF488-OVA/LPS/IFA and performed light sheet microscopy on CUBIC-cleared LNs (Abe et al., 2016). This technique allowed us to examine the distribution of antigen in whole intact LNs in three dimensions. These images and movies revealed that OVA/LPS/IFA was targeted to different regions of the dLN compared to OVA/LPS. 24 hours after OVA/LPS/IFA immunization, labeled OVA appeared targeted to cortical regions to a greater degree compared to OVA/LPS immunization, and demonstrated a ringed pattern suggestive of antigen deposition to IFRs surrounding B cell areas (**Figure 2.2A, Supplementary Movies 2.1A & 2.1B**). To better elucidate the LN architecture and antigen distribution pattern over time, we injected CD21/35 and MECA79 antibodies to label LN follicles and HEVs, respectively, and harvested LNs at various timepoints post-immunization. We confirmed that OVA/LPS/IFA was indeed targeting antigen to IFRs around LN follicles and that this difference compared to OVA/LPS was established as quickly as 6 hours following immunization (**Figure 2.2B, Supplementary Movies 2.2A-2.2F**). The kinetics suggest that this effect was due to passive drainage of the antigen from the injection site to the dLN preceding tissue-derived DC migration and potentially leading to early activation of LN niches. Flow cytometry analysis of dLNs harvested from mice immunized with AF488-OVA/LPS or AF488-OVA/LPS/IFA at various timepoints showed that the total number of cells taking up OVA was not different between the two immunizations, suggesting that the effect of IFA was not simply a result of an increase in the overall number of

cells in the dLN that had acquired antigen (**Figure 2.3A & 2.3B**). Taken together, these data suggest that oil emulsion is capable of promoting a more robust type-1 immune response by altering antigen distribution in the dLN, preferentially targeting niches important for Th1 differentiation.

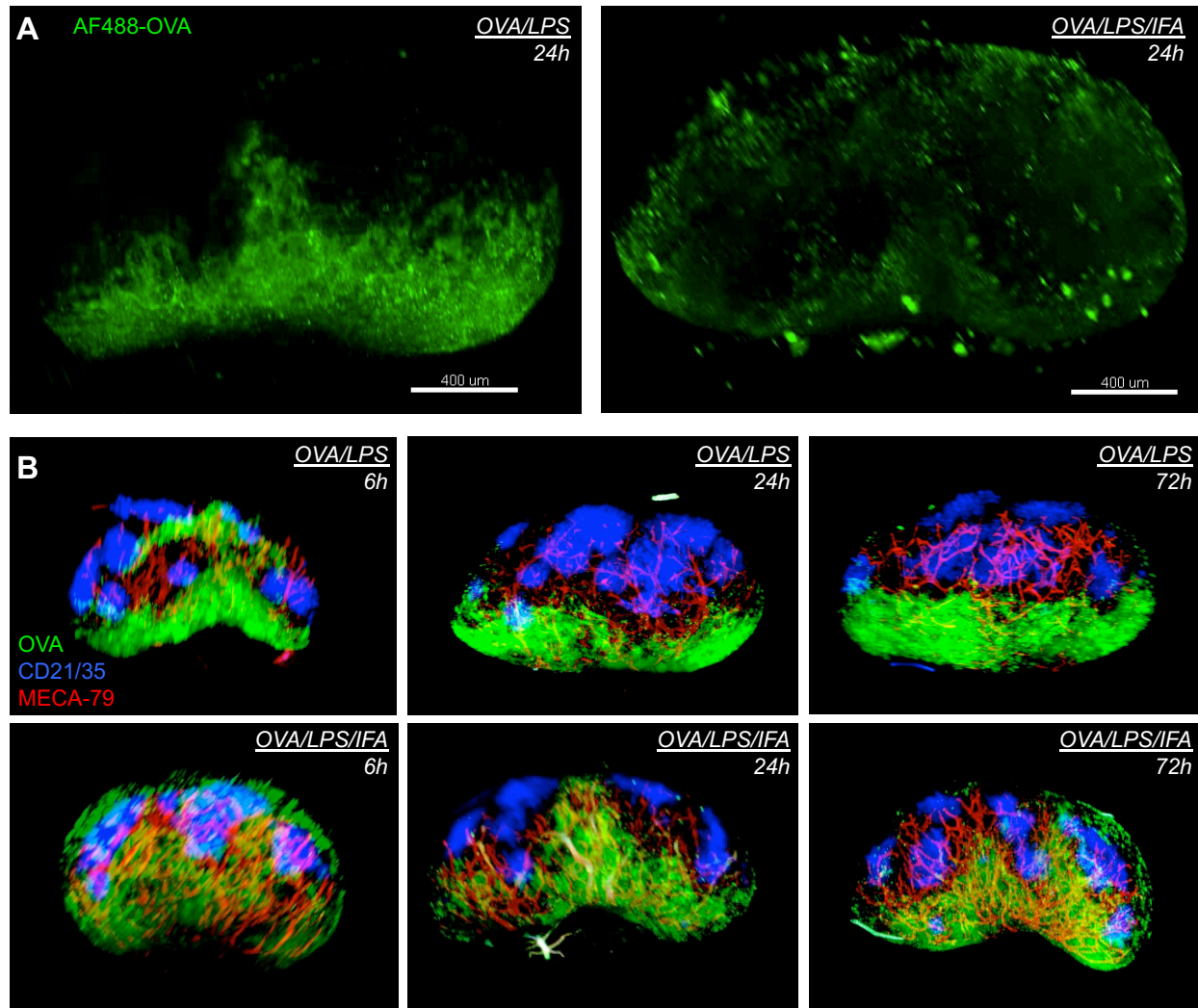


Figure 2.2 IFA-containing immunization targets antigen and adjuvant to IFR/B cell areas.

(A) WT mice were immunized in the footpad with AF488-OVA and LPS in PBS (OVA/LPS, left) or AF488-OVA and LPS emulsified in IFA (OVA/LPS/IFA, right). LNs were harvested after 24h, cleared in CUBIC and analyzed intact by light sheet microscopy. **(B)** WT mice were immunized as in (A) and LNs harvested at the indicated timepoints. CD21/35 (blue) was injected in the footpad 24h prior to harvest to label follicles and MECA-79 (red) was injected i.v. 2min. prior to harvest to label HEV. Representative images are shown from 2 independent experiments. Images in (A) are still captures from **Supplementary Movies 2.1A & 2.1B**, and images in (B) from **Supplementary Movies 2.2A – 2.2F**.

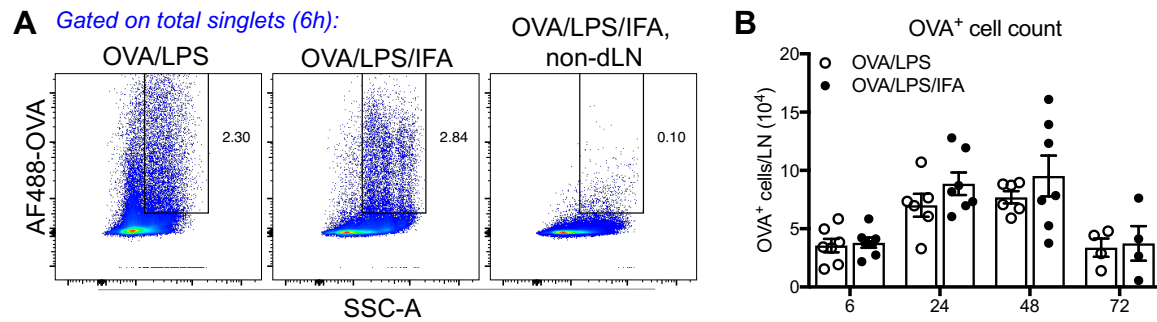


Figure 2.3 OVA/LPS/IFA does not lead to an increase in the overall number of antigen-bearing cells. Mice were immunized with AF488-OVA/LPS or AF488-OVA/LPS/IFA and dLNs harvested at the indicated timepoints. **(A)** Representative flow cytometry plot to identify OVA⁺ cells. **(B)** OVA⁺ cell numbers at the indicated timepoints post-immunization. Data are presented as mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA with Tukey's multiple comparisons test. Results are pooled from 2 independent experiments (n=4-7 mice per group).

OVA/LPS/IFA promotes inflammatory monocyte infiltration into the dLN from the blood in response to antigen targeting

To identify which myeloid or DC populations were acquiring antigen in the dLN and could be responsible for augmenting the immune response after OVA/LPS/IFA, we performed additional flow cytometry analysis of dLNs from mice immunized with AF488-OVA/LPS or AF488-OVA/LPS/IFA at various timepoints and identified myeloid and DC subsets according to the gating strategy in **Figure 2.4**.

We found that very early on (6-24h post-immunization), the total number of OVA⁺ inflammatory monocytes in the dLN after OVA/LPS/IFA immunization was increased compared to OVA/LPS with a trend towards an increase at later timepoints (48-72h), whereas the number of CD8⁺ cDC1s remained similar between the two immunizations throughout the timecourse. We also decided to look for a CD11b⁺ subset of DCs that expressed MHCII and the C-type lectin CD209a/DCSIGN (DCSIGN DCs), since upregulation of DCSIGN is associated with TLR4 stimulation and Th1 cell responses (Bergman et al., 2004; Geijtenbeek et al., 2003; Steeghs et al., 2006). We noticed an increase in the number of OVA⁺ DCSIGN DCs at 48h and a trend towards an increase at 72h, suggesting these DCs may play a role later on in the immune response (**Figure 2.5A**). Total cell numbers for these populations followed a similar trend, with an increase in inflammatory monocytes at early timepoints after OVA/LPS/IFA immunization compared to OVA/LPS, similar numbers of CD8⁺ DCs, and increased numbers of DCSIGN DCs at later timepoints (**Figure 2.5B**). Over half of the inflammatory monocytes were positive for OVA at 6h and 24h post-immunization, suggesting that OVA/LPS/IFA was resulting in preferential targeting of antigen to this compartment early in the immune response.

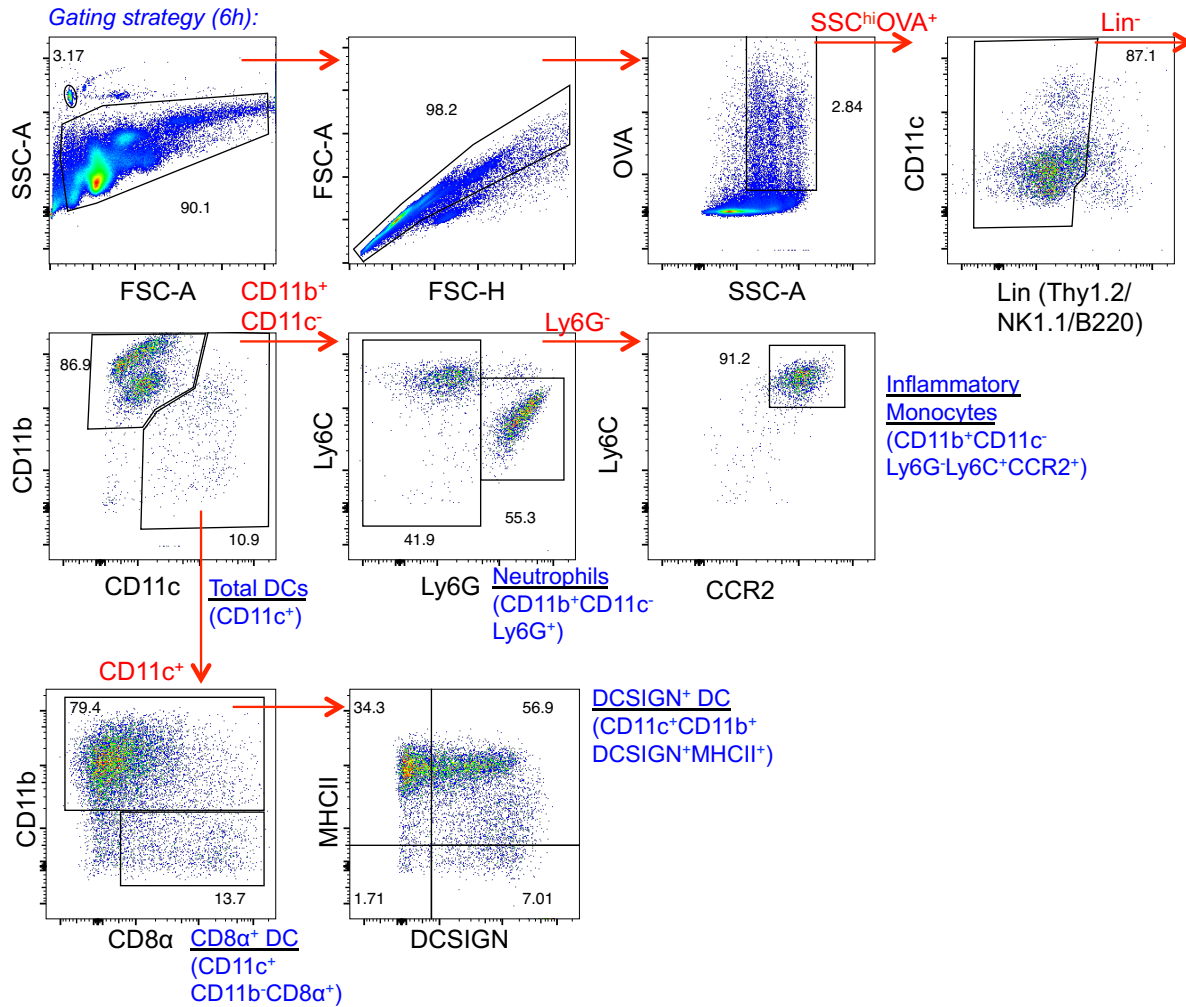


Figure 2.4. Gating strategy to identify innate immune cell subsets in the LN.

Representative flow cytometry plots showing gates and definitions for indicated cell subsets.

OVA⁺ gate only applies for Figure 2.5.

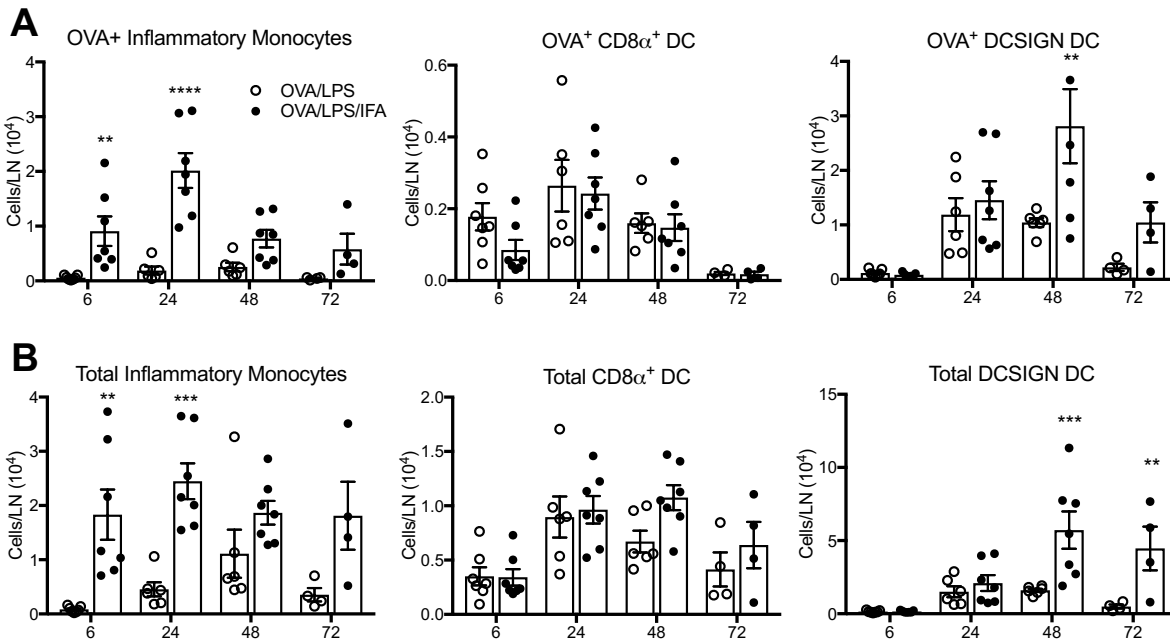


Figure 2.5. Antigen-bearing inflammatory monocytes are increased in the dLN shortly after IFA-containing immunization. WT mice were immunized in the footpad with AF488-OVA/LPS or AF488-OVA/LPS/IFA and dLNs harvested at the indicated timepoints. Cell subsets were analyzed by flow cytometry and identified according to the gating strategy in Figure 2.4. **(A)** OVA⁺ cell numbers of the indicated subsets. **(B)** Total cell numbers of the subsets from (A). Data are presented as mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA with Tukey's multiple comparisons test; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Results are pooled from 2 independent experiments (n=4-7 mice per group).

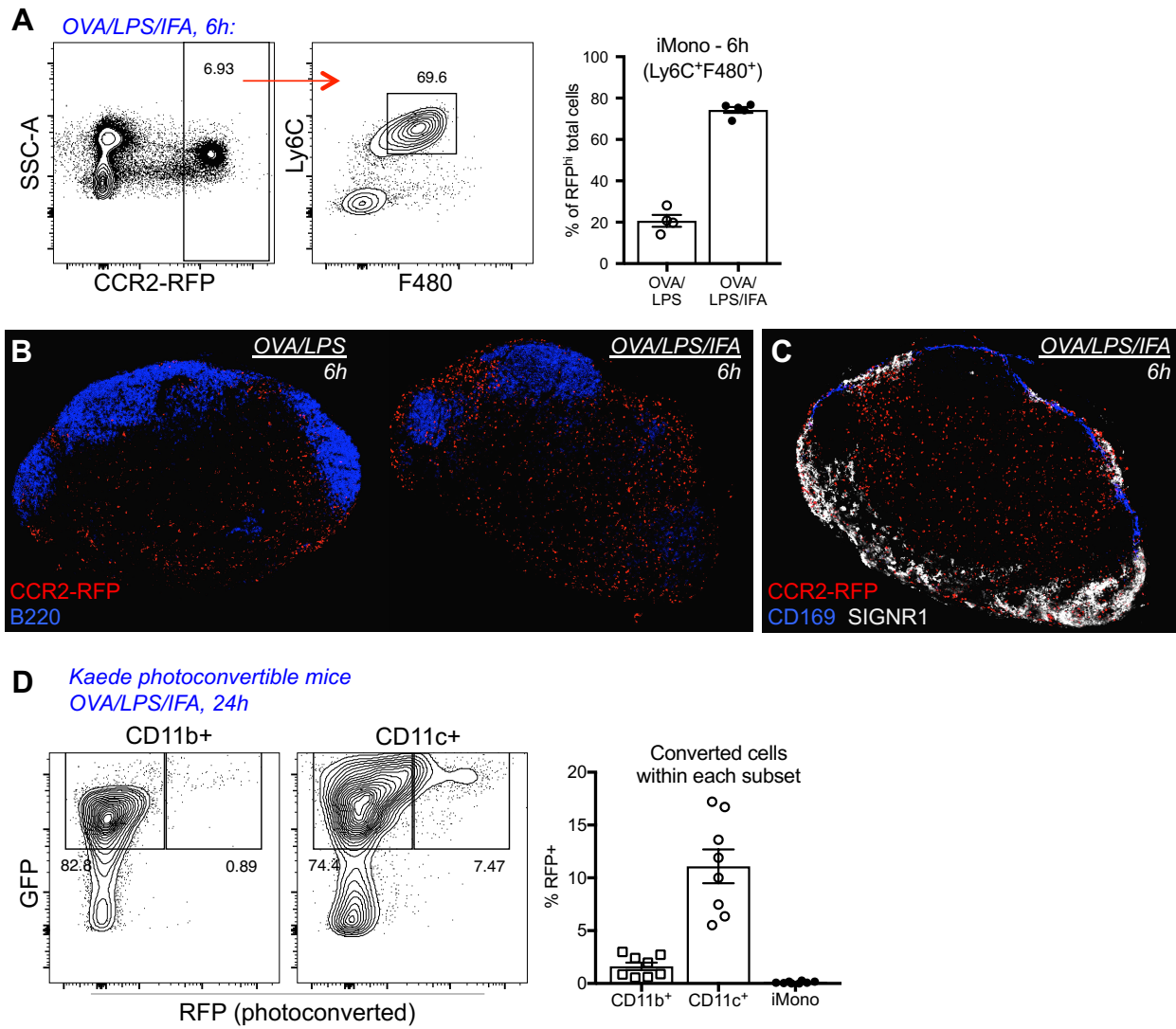
Given the rapid increase in both total and OVA⁺ inflammatory monocyte numbers in the dLN after OVA/LPS/IFA immunization, we wanted to determine if the early infiltrating inflammatory monocytes were localizing towards antigen in the LN, in regions similar to the unique antigen distribution pattern seen after OVA/LPS/IFA. Although tools to exclusively identify inflammatory monocytes *in situ* are lacking, we took advantage of the fact that inflammatory monocytes highly express CCR2 and immunized reporter mice in which RFP expression is driven by the CCR2 promoter (Saederup et al., 2010). Approximately 75% of RFP⁺ cells in the dLN of reporter mice were of the inflammatory monocyte phenotype after OVA/LPS/IFA, although this proportion was lower in OVA/LPS-immunized dLNs owing to the fewer inflammatory monocytes present in the dLN after immunization (**Figure 2.6A**). Analysis of frozen sections by confocal microscopy showed a distribution of RFP⁺ cells within the dLN at 6h post-immunization similar to the pattern of antigen targeting after OVA/LPS or OVA/LPS/IFA immunization (**Figure 2.6B**). Serial sections of the same LNs were stained with CD169 and SIGNR1 to identify LN macrophages and revealed the accumulation of RFP⁺ cells near SIGNR1⁺ macrophages appearing to correspond with medullary sinus and medullary sinus-related macrophages that reside in medullary regions and IFR (Gray and Cyster, 2012) (**Figure 2.6C**).

Since we observed inflammatory monocyte accumulation in the dLN as early as 6h after immunization, we suspected that their arrival might precede skin-derived cell migration through afferent lymphatics and may be infiltrating the LN directly from circulation. To determine if inflammatory monocytes originated from the injection site, we immunized Kaede transgenic photoconvertible mice in which cells express a photoconvertible green fluorescence protein that becomes red upon exposure to violet light (Tomura et al., 2008). Since we were unable to successfully photoconvert cells in the footpad perhaps due to an increased resistance to the penetration of violet light, we photoconverted patches of shaved skin at the tail base prior to

immunization with OVA/LPS/IFA and harvested inguinal LNs 24h post-immunization, when some tissue-derived DC migration to the LN has begun to take place. Photoconverted red cells in the dLN were predominantly DCs with a negligible proportion of photoconverted CD11b⁺ cells, and virtually none of these being inflammatory monocytes (**Figure 2.6D**). Taken together, these data demonstrate that OVA/LPS/IFA promotes rapid inflammatory monocyte infiltration into the reactive LN from the blood, where they localize to antigen-containing LN niches targeted by the immunization.

Figure 2.6. Inflammatory monocytes infiltrate the dLN from circulation after IFA-containing immunization and localize toward antigen-targeted IFR/B cell areas. (A) dLNs from CCR2-RFP reporter mice were harvested 6h post-immunization and analyzed for inflammatory monocytes within RFP⁺ cells by flow cytometry. Representative plot (left). Data are pooled from 2 independent experiments (n=4-5 mice per group). **(B)** Frozen sections from dLNs in (A) were stained with B220 (blue) to identify B cells and analyzed by confocal microscopy. **(C)** Serial sections from (B) were stained with CD169 (blue) and SIGNR1 (white) to identify LN macrophages and analyzed by confocal microscopy. (B-C) Images are representative of two independent experiments (n=4-5). **(D)** Shaved skin patches at the tail base of Kaede transgenic mice were photoconverted and then immunized with OVA/LPS/IFA. dLNs (inguinal) were harvested 24h later and frequency of photoconverted (red) cells within the indicated subsets was determined by flow cytometry. Results are pooled from 3 independent experiments (n=8) and presented as mean $\pm$ SEM.

Figure 2.6 (Continued)



Infiltrating inflammatory monocytes are a prominent source of early, type I interferon-dependent CXCL10

Previous studies have established the importance of various LN niches involved in CD4⁺ T cell activation (Eickhoff et al., 2015; Gerner et al., 2015; Groom et al., 2012; Hor et al., 2015; Woodruff et al., 2014), and have identified CXCR3 as a critical receptor that directs CD4⁺ T cells to IFR and medullary regions of the LN for optimal Th1 differentiation (Groom et al., 2012). Having found that inflammatory monocytes rapidly infiltrate the reactive LN in large numbers and localize to areas of antigen targeting, we hypothesized that they may play a role as a highly mobile cell capable of amplifying chemokine gradients in order to direct cell localization in the LN. To identify cellular sources of CXCR3 ligands, we utilized REX3-Tg (REX3) mice in which CXCL9-producing cells express RFP and CXCL10-producing cells express BFP (Groom et al., 2012). 24h after OVA/LPS/IFA immunization, flow cytometry analysis revealed that inflammatory monocytes were highly expressing CXCL10-BFP in the dLN (**Figure 2.7A**). The expression pattern among inflammatory monocytes contrasted with that of CD8⁺ DC1s, which highly expressed CXCL9-RFP but did not highly express CXCL10-BFP. Although neutrophils and CD8⁺ DCs were technically CXCL10-BFP⁺ by traditional quadrant gating, the MFI of these cells was negligible compared to inflammatory monocytes, suggesting they are only minor contributors (**Figure 2.7B**). Among total BFP^{hi} cells, approximately 80% were comprised of inflammatory monocytes regardless of immunization type (**Figure 2.7C**). Analysis of dLN sections 24h following immunization showed localization of BFP^{hi} CXCL10-producing cells enriched in the IFR in response to OVA/LPS/IFA compared to OVA/LPS (**Figure 2.7D**), which corresponded with the distribution pattern of CCR2-RFP⁺ cells from Figure 2.6.

To determine whether type I or type II interferons were regulating CXCL10 production during the early response to OVA/LPS/IFA immunization in the LN, we crossed REX3 mice with *ifnar*^{-/-} (REX3/IFNAR^{-/-}) and *ifngr1*^{-/-} (REX3/IFN γ R^{-/-}) mice, respectively. Compared to dLNs from

REX3 mice 24h post-immunization (Figure 2.7D, right panel), we observed a decrease in CXCL10-BFP expression in the dLNs of REX3/IFNAR^{-/-} but not REX3/IFN γ R^{-/-} mice, while CXCL9-RFP appeared to be preserved (**Figure 2.8A**). Accordingly, there was a reduction in the overall proportion of CXCL10-BFP⁺ cells in the dLNs of REX3/IFNAR^{-/-} but not REX3/IFN γ R^{-/-} mice, including a loss of CXCL10-BFP expression within inflammatory monocytes (**Figure 2.8B & 2.8C**). These results suggest that as a consequence of its antigen-targeting pattern, OVA/LPS/IFA immunization recruits inflammatory monocytes in the IFR produce CXCL10 in a type I interferon-dependent manner and establishes an inflammatory niche potentially beneficial for Th1 differentiation.

Figure 2.7. OVA/LPS/IFA immunization leads to early production of CXCL10 in the dLN IFR, and is highly expressed by inflammatory monocytes. (A) REX3 reporter mice were immunized with OVA/LPS/IFA and LNs analyzed by flow cytometry 24h later. Representative CXCL9-RFP and CXCL10-BFP expression within indicated cell subsets. **(B)** Median fluorescence intensity of CXCL10-BFP of indicated cell subsets. Data are presented as mean $\pm$ SEM. Statistical significance was determined by unpaired t-test; **** $p \leq 0.0001$. Results are pooled from 3 independent experiments (n=11). **(C)** REX3 mice were immunized with OVA/LPS or OVA/LPS/IFA and dLNs analyzed by flow cytometry 24h later. Total BFP^{hi} cells were analyzed for Ly6C/CCR2⁺ inflammatory monocytes. Results are pooled from 2 independent experiments (n=6). **(D)** dLNs from REX3 mice 24h post-immunization with OVA/LPS or OVA/LPS/IFA were stained with B220 and analyzed by confocal microscopy (B220 = gray; CXCL9-RFP = red; CXCL10-BFP = blue). Images are representative of 2 independent experiments (n=6).

Figure 2.7 (Continued)

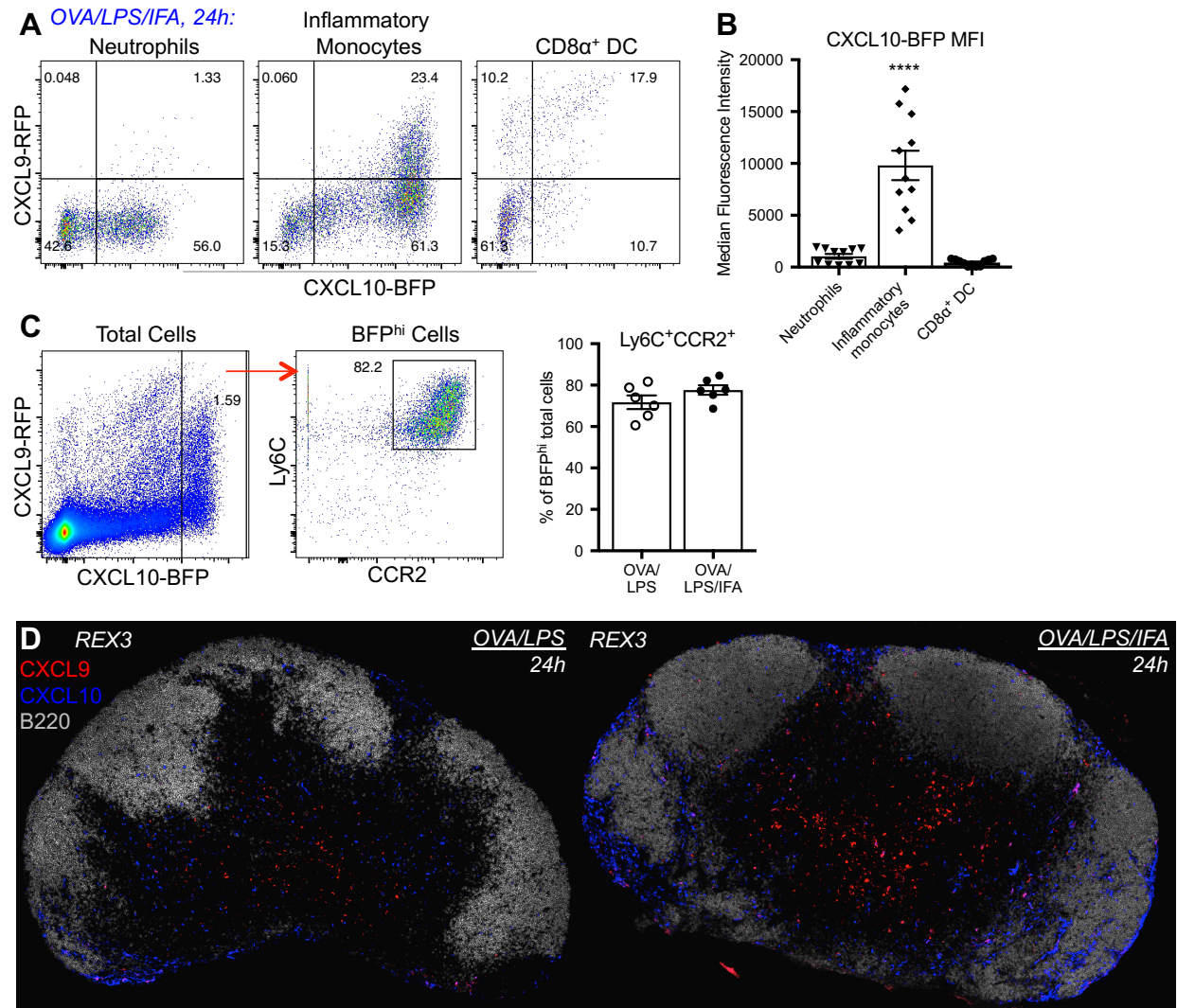
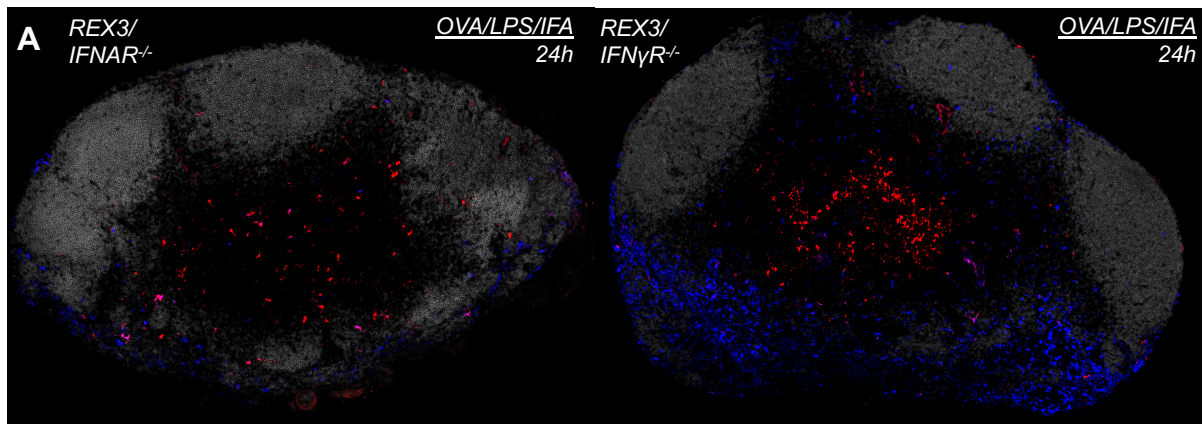
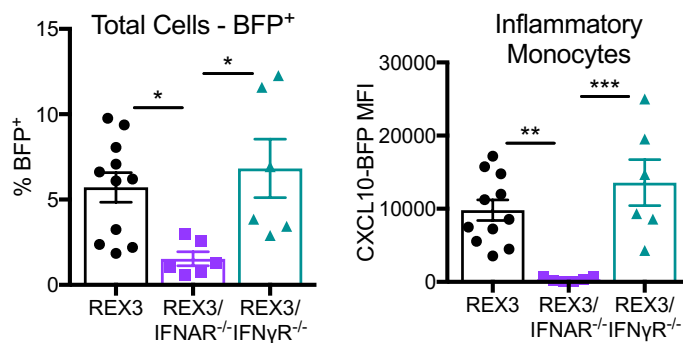


Figure 2.8. Early CXCL10 production in the dLN after IFA-containing immunization is dependent on type I interferon signaling. (A) dLNs from REX3/IFNAR^{-/-} or REX3/IFN β R^{-/-} mice were harvested 24h after OVA/LPS/IFA and analyzed by confocal microscopy (B220 = gray; CXCL9-RFP = red; CXCL10-BFP = blue). Images are representative of two independent experiments (n=5). **(B)** dLNs from REX3, REX3/IFNAR^{-/-} and REX3/IFN β R^{-/-} reporter mice were immunized with OVA/LPS/IFA and LNs analyzed 24h later by flow cytometry. Proportion of CXCL10-BFP⁺ expressers among total cells (left) and CXCL10-BFP⁺ median fluorescence intensity of inflammatory monocytes (right). **(C)** Representative flow cytometry plot of CXCL9-RFP and CXCL10-BFP expression within inflammatory monocytes from (B). Data are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test; * $p \leq 0.05$, ** ≤ 0.01 , *** ≤ 0.001 . Results are pooled from 2-3 independent experiments (n=6-11 per group).

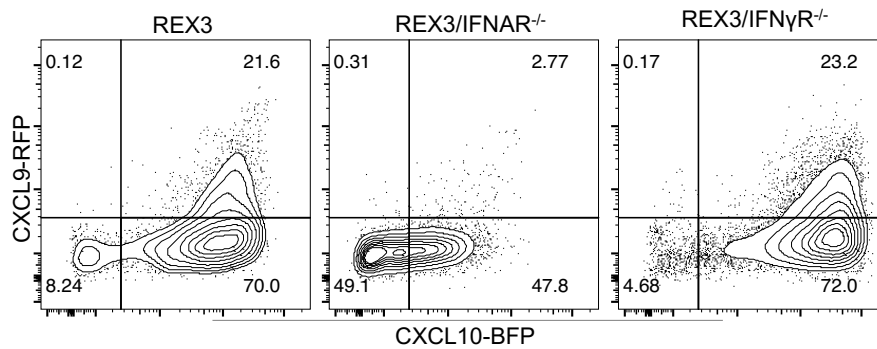
Figure 2.8 (Continued)



B *OVA/LPS/IFA, 24h:*



C *Inflammatory monocytes:*



Emulsified immunization promotes antigen-specific CD4⁺ T cell localization into the IFR

Since we observed a robust accumulation of CXCL10-producing cells in the IFR in response to OVA/LPS/IFA immunization, we hypothesized that this response might be directing CD4⁺ T cells out of the deep LN parenchyma and toward niches activated by antigen/adjuvant containing inflammatory signals beneficial for Th1 differentiation. In order to visualize antigen-specific CD4⁺ T cell localization in an immunized mouse, we isolated naïve CD4⁺CD62L⁺ T cells from Actin-GFP/Thy1.1⁺ OTII mice and adoptively transferred 2.5×10^5 cells per mouse into WT mice. Recipients were immunized one day later in the presence of FTY720, and dLNs harvested after 72 hours. Frozen sections of dLNs were stained with B220 and analyzed by confocal microscopy, and the localization of transferred OTII cells within the LN was determined relative to B cell staining (**Figure 2.9**). Since we observed relatively few GFP⁺ cells localized to the most central area of B cell follicles, we chose to combine IFR/B cell regions for quantitation to simplify the classification of the many GFP⁺ cells positioned on the border of B220 staining.

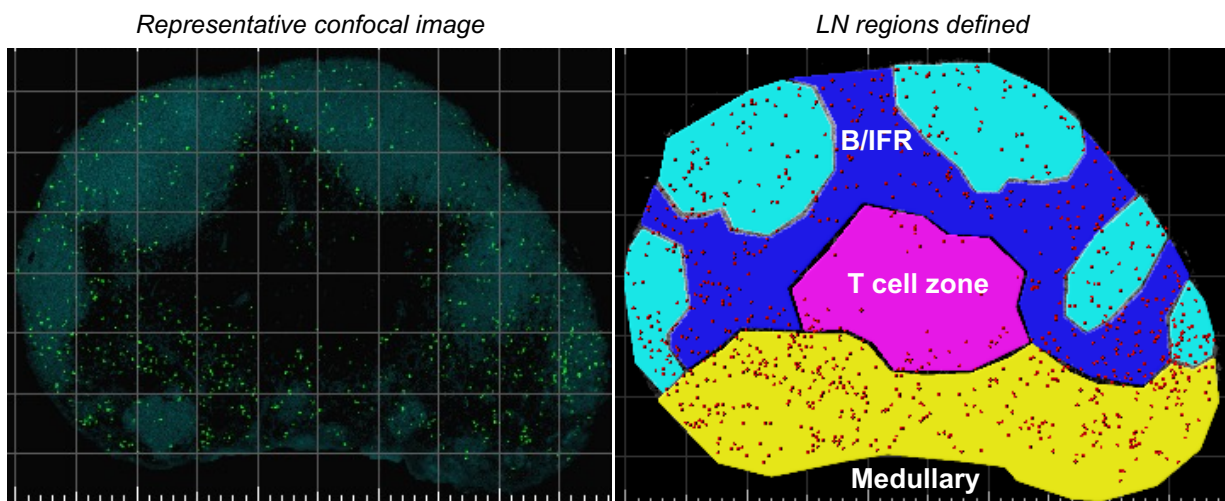


Figure 2.9. Defined LN niches. (Left panel) Representative confocal image of transferred GFP⁺ cells (green) stained with B220 (turquoise), left panel. (Right panel) LN niches as defined in Imaris based on B cell staining. T cell zone = pink; Medullary Region = yellow; B/IFR = turquoise/blue; transferred CD4⁺ cells (red).

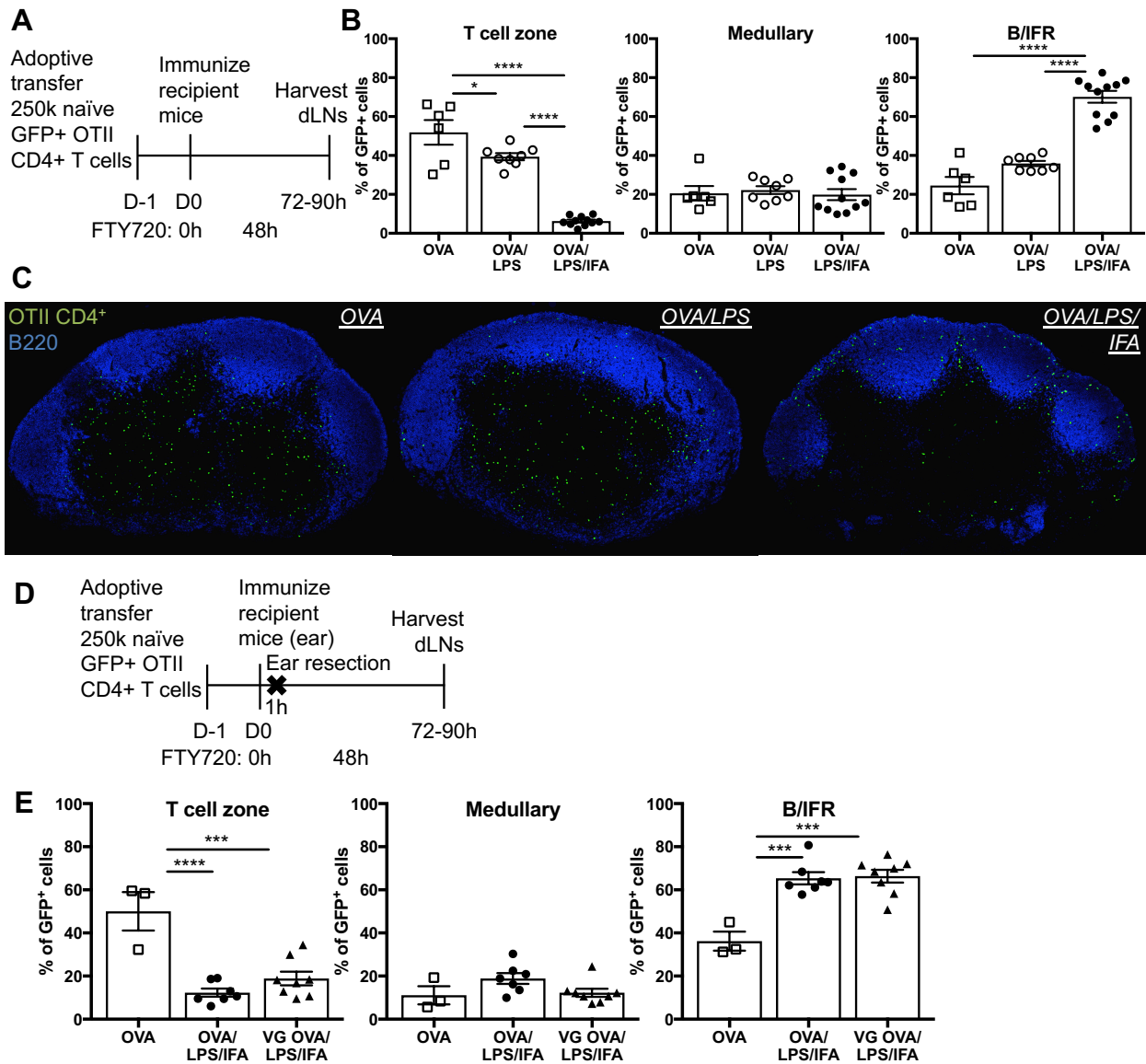
By 72 hours after OVA/LPS/IFA immunization in our adoptive transfer model (**Figure 2.10A**), the transferred GFP⁺ OTII cells had almost completely mobilized out of the deep parenchymal T cell zone, with the vast majority localizing to the peripheral IFR/B cell areas of the LN (**Figure 2.10B – filled circles, Figure 2.10C – right panel**). In contrast, dLNs from mice immunized with OVA/LPS contained a greater proportion of GFP⁺ cells remaining in the T cell zone (**Figure 2.10B – open circles, Figure 2.10C – middle panel**), while the proportion of GFP⁺ cells in the IFR/B areas was similar to dLNs from mice immunized with OVA alone (**Figure 2.10B – open squares, Figure 2.10C – left panel**). CD4⁺ T cell localization to the medullary region was not significantly different between the immunization groups. These data demonstrate that immunization with antigen in the presence of a type 1-skewing ligand alone (OVA/LPS) may not be as effective in directing optimal CD4⁺ T cell positioning in the dLN, whereas the identical mixture emulsified in oil (OVA/LPS/IFA) induced a higher degree of CD4⁺ T cell mobilization out of the central T cell zone and directed them into the IFR/B cell areas.

In order to test the whether an injection site depot effect following immunization with IFA was responsible for the localization pattern of antigen-specific CD4⁺ T cells we observed, we performed the same adoptive transfer of naïve GFP⁺ OTII CD4⁺ T cells and instead immunized the ear, which allowed us to resect the injection site shortly after immunization (**Figure 2.10D**). Ear immunization with OVA/LPS/IFA resulted in transferred CD4⁺ T cell localization patterns in the dLN similar to the results after footpad immunization, with a greater proportion of GFP⁺ cells mobilizing out of the T cell zone and localizing to IFR/B areas (**Figure 2.10E – filled circles**) compared to immunization OVA alone in PBS (**Figure 2.10E – open squares**). Ear resection 1 hour after immunization with OVA/LPS/IFA did not alter the pattern of GFP⁺ cell localization (**Figure 2.10E – filled triangles**), suggesting that effects resulting from direct drainage of emulsified antigen and adjuvant to the dLN are sufficient to mediate T cell mobilization to IFR/B areas after OVA/LPS/IFA, and are not strictly dependent on antigen depot at the injection site.

Figure 2.10. IFA-containing immunization promotes antigen-specific CD4⁺ T cell

localization to IFR/B cell areas of the dLN. Naïve CD62L⁺CD4⁺ T cells were magnetically isolated from Actin-GFP⁺ OTII mice. 2.5×10^5 cells were adoptively transferred into WT recipients 1d prior to immunization. FTY720 was administered i.p. at 0h and 48h and LNs harvested 72-90h later. **(A)** Schematic of experimental design. **(B)** dLNs were stained with B220 (blue) and analyzed by confocal microscopy. Transferred CD4⁺ T cell (green) localization to LN niches is expressed as % of GFP⁺ cells in each LN region. **(C)** Representative confocal images from (B). Data are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test; $*p \leq 0.05$, $**** \leq 0.0001$. Results are pooled from 3 independent experiments (n=4-7 mice per group). (D-E) GFP⁺ naïve OTII cells were adoptively transferred as in (A) and recipient mice were immunized in the ear in the presence of FTY720. Injection site was excised 1h after immunization ("VG OVA/LPS/IFA"). **(D)** Schematic of experimental design. **(E)** Transferred CD4⁺ T cell localization at 72-90h. Data are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test; $***p \leq 0.001$, $**** \leq 0.0001$. Results are pooled from 3 independent experiments (n=3-6 mice per group).

Figure 2.10 (Continued)



Enhanced antigen-specific Th1 differentiation induced by OVA/LPS/IFA immunization is dependent on the monocyte-macrophage lineage

In order to determine the degree of Th1 differentiation among antigen-specific CD4⁺ T cells, we performed *ex vivo* intracellular cytokine staining in our adoptive transfer model. At 90h following OVA/LPS/IFA immunization, there was an increase in the total number of transferred OTII CD4⁺ T cells in the draining LN as well as the proportion of these cells that had become polyfunctional Th1 cells that were able to simultaneously produce IFN γ and TNF γ , similar to what we previously observed in dLNs cultured with OVA (**Figures 2.11A-2.11C**). At this late timepoint, the enhanced differentiation of polyfunctional Th1 cells in response to OVA/LPS/IFA immunization was associated with an increase in the number of inflammatory monocytes and DCSIGN DCs in the dLN compared to OVA or OVA/LPS, while the number of CD8 γ ⁺ cDC1s was not significantly different between OVA/LPS/IFA and OVA/LPS (**Figure 2.11D**).

Approximately two-thirds of the DCs after OVA/LPS/IFA immunization were DCSIGN/MHCII⁺ (**Figure 2.11E**), suggesting that this DC subset was potentially playing a role in the enhanced generation of Th1 cells after OVA/LPS/IFA.

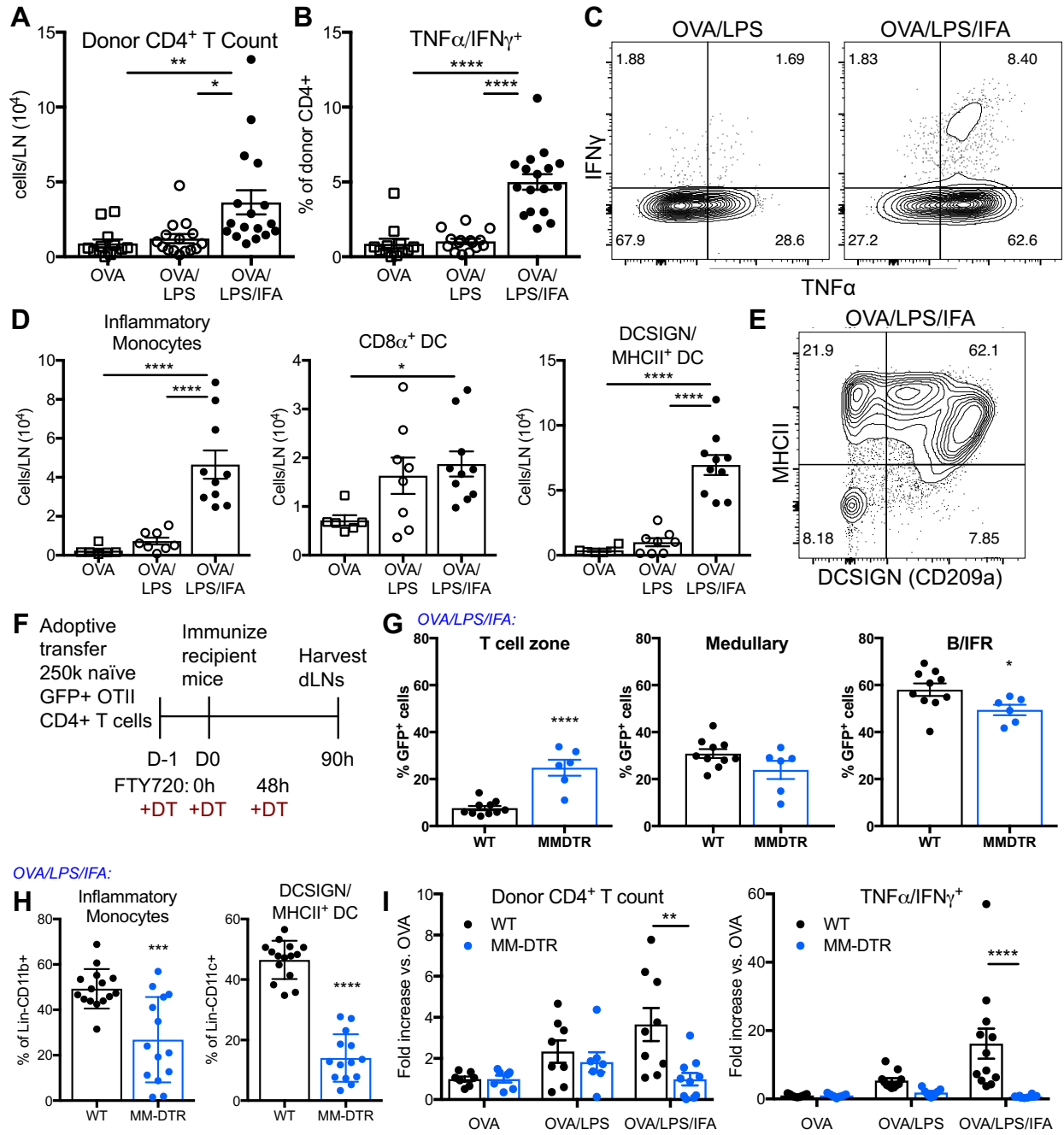
In order to determine if monocyte-derived cells were mediating the increase in antigen-specific CD4⁺ T cell localization to IFR and enhanced Th1 differentiation seen after OVA/LPS/IFA immunization, we performed the same adoptive transfer of GFP⁺ naïve OTII cells into WT or MM-DTR mice in which administration of diphtheria toxin (DT) specifically depletes cells of the monocyte-macrophage lineage based on dual expression of *lysm* and *csf1r* (Schreiber et al., 2013). Recipient mice were immunized with OVA/LPS/IFA in the presence of FTY720 as before, and DT was administered to all mice 1 day prior to, immediately after, and 2 days following immunization (**Figure 2.11F**). In dLNs from DT-treated MM-DTR mice, more transferred CD4⁺ T cells remained in the T cell zone with fewer cells mobilizing to IFR/B areas of the LN compared

to DT-treated WT mice (**Figure 2.11G**). The decreased peripheralization of transferred CD4⁺ T cells corresponded with a decrease in the frequency of inflammatory monocytes and DCSIGN DCs recovered from dLNs of MM-DTR compared to WT mice, although some inflammatory monocytes were able to rapidly repopulate (**Figure 2.11H**). In MM-DTR but not WT mice, DT treatment abrogated the increase in both the total number of transferred CD4⁺ T cells and enhanced polyfunctional Th1 differentiation of these cells promoted by OVA/LPS/IFA immunization (**Figure 2.11I**). Taken together, these data suggest that depletion of the monocyte/macrophage lineage impairs the optimal localization of CD4⁺ T cells in the dLN along with the enhanced Th1 differentiation induced by OVA/LPS/IFA immunization.

Figure 2.11. IFA-containing immunization promotes antigen-specific Th1 differentiation

that is dependent on monocyte-derived cells. (A-E) Naïve CD62L⁺CD4⁺ T cells were magnetically isolated from Actin-GFP⁺ OTII mice. 2.5×10^5 cells were adoptively transferred into WT recipients 1d prior to immunization. FTY720 was administered i.p. at 0h and 48h. and dLNs harvested at 90h. **(A)** Cell count of GFP⁺ OTII CD4⁺ T cells. **(B)** dLN cells were restimulated for 4h with OVA peptide (323-339) and α CD28 in the presence of Brefeldin A and stained for intracellular Th1 cytokines. Results are pooled from 4 independent experiments (n=12-17 per group). **(C)** Representative flow cytometry plots from (B). **(D)** Cell counts of indicated cell subsets. **(E)** Representative flow cytometry plot gated on CD11c⁺ DCs. Results are pooled from 3 independent experiments (n=6-10 mice per group). (A-E) Data are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test; * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. (F-I) GFP⁺ naïve OTII cells were adoptively transferred into WT or MMDTR mice and immunized in the presence of FTY720 as in (A-E), and diphtheria toxin (DT) administered at the indicated timepoints. **(F)** Schematic of experimental design. **(G)** Transferred OTII CD4⁺ T cell localization to LN niches. Data are presented as mean $\pm$ SEM. Statistical significance was determined by unpaired t-test; * $p \leq 0.05$, **** $p \leq 0.0001$. Results are pooled from 4 independent experiments (n=6-10 mice per group). **(H)** Proportion of inflammatory monocytes among Lin⁻CD11b⁺ cells (left) and DCSIGN⁺ DC among Lin⁻CD11c⁺ cells (right). Data are presented as mean $\pm$ SEM. Statistical significance was determined by unpaired t-test; *** $p \leq 0.001$, **** $p \leq 0.0001$. Results are pooled from 5 independent experiments (n=13-14 mice per group). **(I)** Cell count and Th1 differentiation of transferred CD4⁺ T cells. Data are presented as mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA with Sidak's multiple comparisons test; ** $p \leq 0.01$, **** $p \leq 0.0001$. Results are pooled from 4 independent experiments (n=7-12 mice per group).

Figure 2.11 (Continued)



OVA/LPS/IFA promotes IL-12p40 production by DCSIGN DCs in the dLN and is produced alongside CXCR3 ligands in the IFR

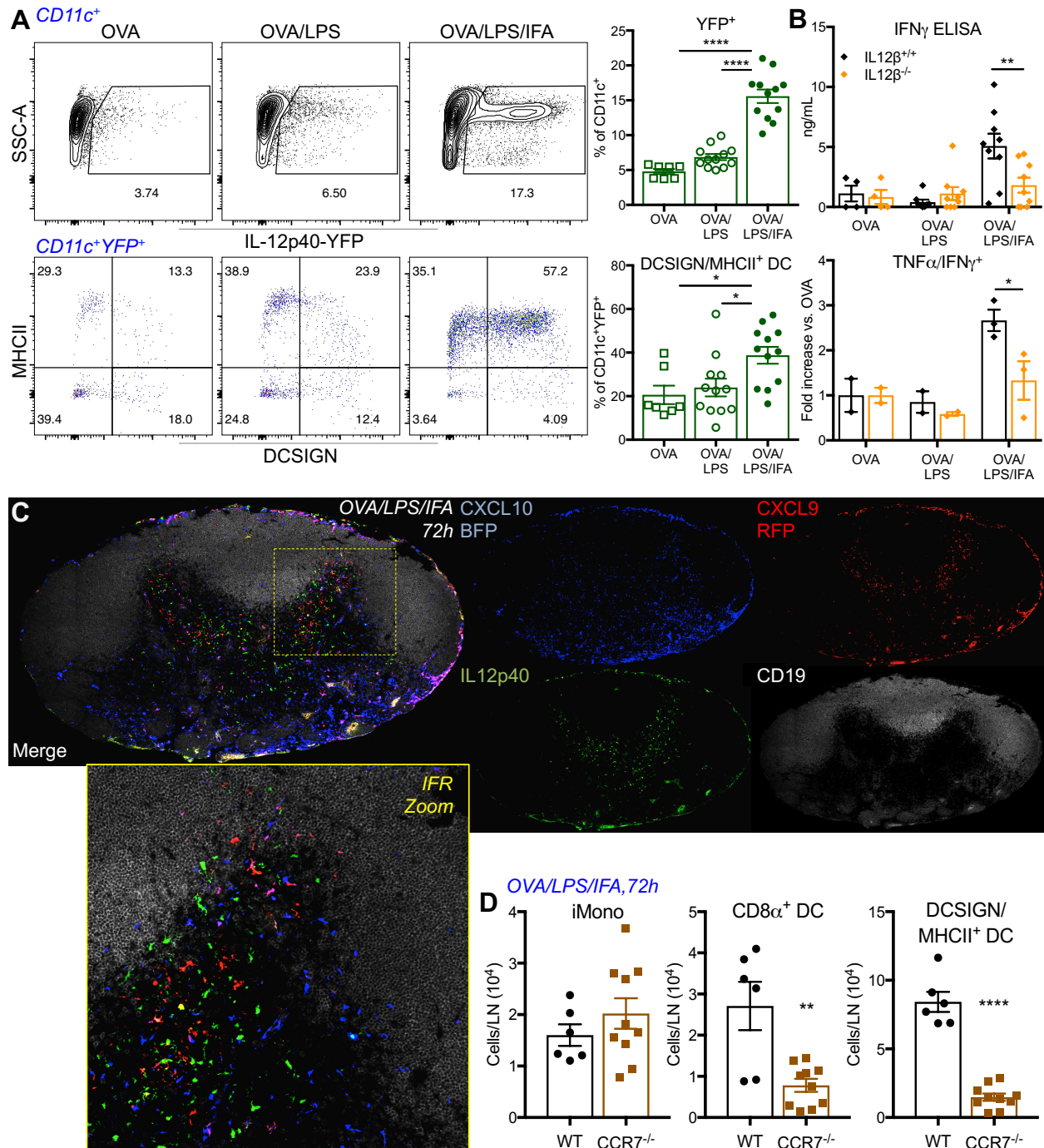
Having observed an increase in the accumulation of DCSIGN DCs in the dLN at later time points following OVA/LPS/IFA immunization, we investigated the role for these cells in promoting Th1 differentiation. IL-12 is a pro-inflammatory cytokine that can promote and sustain Th1 differentiation (Ho and Glimcher, 2002; Murphy and Reiner, 2002). In order to determine the key IL-12-producing immune cell population after OVA/LPS/IFA immunization, we performed flow cytometry on immunized IL-12p40-YFP (IL12-YFP) mice (Reinhardt et al., 2006). 72h after OVA/LPS/IFA immunization, we found an increase in the frequency of YFP⁺ DCs in the dLN compared to OVA/LPS or OVA immunization (**Figure 2.12A, upper panels**). Within YFP⁺ DCs, the proportion that were DCSIGN DCs was also increased after OVA/LPS/IFA immunization compared to OVA/LPS or OVA immunization, becoming roughly half of all YFP⁺ DCs after OVA/LPS/IFA compared to only ~20% after the other immunizations (**Figure 7B, lower panels**). YFP expression among CD11b⁺CD11c⁻ cells including neutrophils and monocytes was not detected (data not shown). To determine if IL-12 was critical for enhanced Th1 responses after OVA/LPS/IFA, we immunized mice that were deficient for *il12b* (IL12^{-/-}). After 6 days, cells from dLNs of IL12^{-/-} mice immunized with OVA/LPS/IFA and cultured with OVA were unable to produce increased levels of IFN γ over OVA/LPS or OVA immunization compared to WT. Enhanced differentiation of polyfunctional Th1 cells was also impaired in cells from IL12^{-/-} dLNs compared to WT (**Figure 2.12B**).

In order to determine whether IL-12p40⁺ cells were positioned in the same areas as CXCR3 ligand producing cells, we crossed IL12-YFP with REX3 mice. Confocal microscopy revealed substantial YFP⁺ cell localization in IFR at 72h after OVA/LPS/IFA immunization, along with cells producing CXCR3 ligands (**Figure 2.12D**). Although we observed BFP⁺ CXCL10-

producing cells in other areas of the dLN such as medullary regions, there were few IL12p40-YFP⁺ cells in these areas, suggesting that LN IFR are unique niches where CD4⁺ T cells can respond to CXCR3 ligands and also encounter type 1 skewing cytokine signals from IL12p40⁺ cells that support robust Th1 differentiation. Mice deficient for *ccr7* (CCR7^{-/-}) immunized with OVA/LPS/IFA had reduced numbers of CD8⁺ DCs and DCSIGN DCs in the dLN compared to WT, while inflammatory monocytes numbers were similar (**Figure 2.12E**), suggesting that DCSIGN DCs and inflammatory monocytes may rely on different mechanisms for their migration into the reactive LN and that the timing of IL-12 signals provided by DCSIGN DCs in the later stages after immunization may be tied to the kinetics of CCR7-dependent migration. Together, these data demonstrate that in response to OVA/LPS/IFA immunization, early-infiltrating, CXCL10^{hi}, CCR7-independent inflammatory monocytes cooperate with later-arriving, CCR7-dependent, IL-12⁺ DCSIGN DCs to create a niche in the dLN IFR that promotes optimal Th1 differentiation.

Figure 2.12. DCSIGN⁺ DCs produce IL12p40 in a type-I-interferon dependent manner after IFA-containing immunization and help promote Th1 responses. (A) Representative flow cytometry plots from IL-12p40-YFP reporter mice 72h after the indicated immunizations. (B) Proportion of YFP⁺ cells among total DCs (upper), and proportion of DCSIGN/MHCII⁺ DCs among YFP⁺ DCs (lower). Data are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test; * $p \leq 0.05$, **** ≤ 0.0001 . Results are pooled from 4 independent experiments (n=7-10 mice per group). (C) WT or IL12 β ^{-/-} mice were immunized and analyzed for IFN γ production and Th1 differentiation as in Figure 1. Data are presented as mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA with Sidak's multiple comparisons test; ** $p \leq 0.01$, **** ≤ 0.0001 . Results are pooled from 2 independent experiments. (D) Confocal image from a REX3/IL12p40-YFP reporter mouse 72h after OVA/LPS/IFA (B220 = gray; CXCL9-RFP = red; CXCL10-BFP = blue; IL12p40-YFP = green). Representative image from 4 independent experiments (n=8). (E) WT or CCR7^{-/-} mice were immunized and dLNs harvested 72h later. Cell counts of indicated cell subsets. Data are presented as mean $\pm$ SEM. Statistical significance was determined by unpaired t-test; ** $p \leq 0.01$, **** ≤ 0.0001 . Results are pooled from 3 independent experiments (n=6-10 mice).

Figure 2.12 (Continued)



DISCUSSION

In this study, we demonstrated that immunization with an oil formulation enhanced Th1 responses in the LN. Oil emulsion demonstrated a superior ability compared to saline in targeting antigen to IFR of the dLN. This correlated with a rapid and enhanced influx of inflammatory monocytes into the dLN, where they accumulated in IFRs corresponding to the unique pattern of antigen deposition promoted by oil emulsion. Inflammatory monocytes in the dLN highly upregulated CXCL10 in a type I interferon-dependent manner early in the response to emulsified immunization, and contributed to gradients directing the mobilization of antigen-specific CD4⁺ T cells out of the deep LN parenchyma and positioning within the IFR. Experiments in Kaede photoconvertible mice, together with experiments excising the injection site and DT-mediated depletion of monocyte-derived cells, suggest that these early events occur prior to the arrival of migratory DCs and are amplified by CCR7-independent inflammatory monocytes infiltrating into the LN from circulation. The enhanced differentiation of polyfunctional Th1 cells after OVA/LPS/IFA immunization was dependent on inflammatory signals received within the IFR, including IL-12 provided by later-arriving CCR7-dependent DCSIGN DCs, suggesting a cooperation between rapid inflammatory monocyte influx and slower arrival of IL-12-producing DCs for generating optimal Th1 responses.

Previous work has established CXCR3 and its ligands CXCL9 and CXCL10 (CXCL11 is functionally absent in C57BL/6 mice) as a critical chemokine system that directs intranodal positioning of CD4⁺ T cells to IFR for optimal Th1 differentiation (Groom et al., 2012). Using the REX3 reporter mouse developed in that study, we show here that inflammatory monocytes are capable of highly expressing CXCL10 and serve as dynamic responders to amplify this chemokine system in the LN in response to immunization with OVA/LPS/IFA. We observed that cells highly expressing CXCL9 were mainly restricted to DC1. Accordingly, CXCL9 has been shown to act in prepositioning memory CD8⁺ T cells for activation during the recall response

(Kastenmüller et al., 2013; Sung et al., 2012). These findings suggest that DC production of CXCL9 might play an important role in CD8⁺ T cell activation, whereas CD4⁺ T cells could potentially be more dependent on CXCL10 from monocyte-derived cells and DC2, although this may depend on the nature and context of the inflammatory stimulus. Whether there exists any bias toward CXCL9 or CXCL10 by CXCR3⁺ CD8⁺ versus CD4⁺ T cells, and whether these requirements differ during various phases of acute inflammation and memory recall requires further study.

IFRs are an important LN microenvironment where innate and adaptive immune cells converge. LN macrophages located at IFR have been shown to release type I interferon in response to influenza vaccination (Chatziandreou et al., 2017). We found that emulsified immunization promoted the accumulation of CCR2⁺ cells toward SIGNR1⁺ LN macrophages located at IFRs, and that CCR2⁺ inflammatory monocytes produced CXCL10 dependent on the ability to sense type I interferon. Together, these results suggest that SIGNR1⁺ LN macrophages may be potential upstream mediators of inflammatory monocyte activation and may be targeted by OVA/LPS/IFA. Discerning whether LN macrophages play a role in directly influencing intranodal recruitment and/or activation of inflammatory monocytes requires further study, and would be greatly aided by the development of better tools to specifically interrogate these populations. It is possible that oil emulsions may specifically target other LN-resident immune cells (Bajénoff et al., 2006a; Gaya et al., 2018; Gerner et al., 2015; Kastenmüller et al., 2012) or stromal cells (Bajénoff et al., 2006b; Katakai et al., 2004b; Rodda et al., 2018) positioned in the IFR, and such a property could potentially be utilized to more efficiently deliver vaccine to cells and boost early activation of LN IFR. In addition, DC1 in IFR of the LN serve as cross-presenting DCs and platforms for the delivery of CD4⁺ help to CTLs (Eickhoff et al., 2015; Hor et al., 2015). Our finding that OVA/LPS/IFA immunization promotes CXCR3 ligand and IL-12p40

production in IFRs may suggest that targeted delivery of antigen to these regions through the use of oil emulsions could potentially promote cDC1 cross-presentation and CTL responses.

DCSIGN DCs but not inflammatory monocytes in the dLN were dependent on CCR7, which suggests that optimal Th1 responses require cooperation between these two cell types. Migratory DCs require CCR7 for trafficking from the skin to the dLN (Ohl et al., 2004), but the process for these cells to upregulate CCR7, enter draining lymphatics and arrive at the LN can take at least 24 hours after activation (Itano et al., 2003). Accordingly, we did not see an increased accumulation of DCSIGN DCs in dLNs of OVA/LPS/IFA-immunized mice until 48h after immunization, whereas increased accumulation of inflammatory monocytes was detected as early as 6h post-immunization. This early activation period represents a window of opportunity for rapidly mobilized inflammatory monocytes to respond to potent but tightly regulated inflammatory mediators induced by antigen/adjuvant directly draining into the LN, including type I interferons, and amplify the early immune response. Inflammatory monocyte activation in the IFR may therefore be beneficial to promote the timely formation of LN niches and chemokine gradients contributing to the pre-positioning of CD4⁺ T cells to these regions, which serve as corridors for DCs arriving from the periphery (Schumann et al., 2010; Ulvmar et al., 2014). In response to OVA/LPS/IFA immunization, we found DCSIGN DCs but not CD11b⁺ inflammatory monocytes to be a major source of IL-12, a critical cytokine cue for driving Th1 differentiation. These migratory DCs may contain important tissue-derived information that the inflammatory monocytes infiltrating from blood circulation cannot alone provide. Therefore our data suggest that, *in vivo*, an optimal and robust Th1 response depends on timely and spatially coordinated action of both CXCL10-producing inflammatory monocytes and IL-12-producing DCSIGN DCs.

IFA was originally developed in the 1950s. Although its use in clinical vaccines demonstrated improved immune responses in patients, severe adverse reactions at the injection

site ultimately limited further development (Chang et al., 1998). However, recent improvements in formulation design have led to the successful use of other oil emulsions, most notably AS03 and MF59, which have been licensed for use in both seasonal and pandemic influenza vaccines in young children and the elderly (Wilkins et al., 2017). Emerging approaches to vaccine design include the use of nanoparticles and bioengineered scaffolds as platforms for delivery, and aim to solve outstanding issues including kinetics and bioavailability without adverse reactions (Moyer et al., 2016). As more advanced approaches allow the design of vaccines that can target more specific cell subsets or surface receptors, it will be important to better understand the consequences of antigen targeting to different LN microenvironments and identify the proper balance for the desired outcome. Strategies targeting specific cell subsets should not neglect robust support for both LN resident and migratory immune cells in order to ensure the generation and amplification of LN niches such as IFR that are important for the best immune response. The development of formulations that can efficiently deliver vaccine components to these regions in a controlled manner could provide a substantial benefit to vaccine efficacy and the treatment of infectious diseases and cancer.

MATERIALS AND METHODS

Mice

C57BL/6 (WT) were obtained from Charles River Laboratories. CCR2-RFP, Actin-eGFP, IL12p40-eYFP, *Il12b*^{-/-}, *Ifngr1*^{-/-}, LysMcre, *Csf1r*-DTR^{fl}-Tg, and Thy1.1 mice were obtained from Jackson Laboratories. OTII (male-linked) were obtained from Taconic. *Ifnar*^{-/-} mice (Muller et al., 1994), were a gift from Michel Aguet (University of Zurich, Zurich, Switzerland). *Ccr7*^{-/-} mice were a gift from Thorsten R. Mempel (Harvard Medical School, Boston, Massachusetts, USA). REX3-Tg mice were generated by our lab as previously described (Groom et al., 2012). Kaede transgenic mice were obtained from Dr. Osami Kanagawa (RIKEN Institute) (Tomura et al., 2008), rederived at Taconic and then bred at Massachusetts General Hospital. All mice were maintained on the C57BL/6 background. REX3-Tg and IL12p40-eYFP mice were bred and maintained in SPF plus Helicobacter and Pasteurella Pneumotropica free conditions at the Massachusetts General Hospital Center for Comparative Medicine; all others were bred and maintained under specific pathogen free (SPF) conditions. All procedures were approved and carried out according to the standards and guidelines set forth by the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

Immunizations, adoptive transfer

Mice were anesthetized and immunized in the footpad with 50µg ovalbumin (OVA; grade V, Sigma) with or without 5µg LPS-EK (Invivogen) in 50µl of PBS or emulsified 1:1 in Incomplete Freund's Adjuvant (Sigma). For OTII adoptive transfer experiments, naïve CD4⁺ T cells were isolated from Actin-GFP/OT2/Thy1.1⁺ mice using magnetic bead separation (CD4⁺CD62L⁺ T cell isolation kit; Stemcell). 2.5 x 10⁵ cells per mouse were injected retro-orbitally 1 day prior to immunization. 1mg/kg FTY720 (Cayman Chemicals) was injected intraperitoneally every other

day starting immediately after immunization in order to block T cell egress. For ear immunizations, mice were immunized in the ear pinnae with identical concentrations as above but in 20µl total volume and the injection site excised 1 hour after immunization. To deplete monocyte/macrophages in MMDTR experiments, 4ng/g of DT (Sigma-Aldrich) was injected intraperitoneally into mice 1 day prior to immunization, immediately following, and on day 2. In some experiments, OVA protein was labeled with Alexa Fluor 488 Protein Labeling Kits (Invitrogen) according to manufacturer's instructions prior to injection.

LN restimulation

3×10^5 total cells from dLNs were cultured at 37°C in 5% CO₂ for 3 days in 96-well round bottom plates containing 100µg/mL OVA protein in 200µL of complete RPMI (10% heat-inactivated fetal bovine serum [FBS], 2mmol/L Glutamax, 100U/mL penicillin, 100µg/mL streptomycin, 0.01M HEPES), and supernatants collected and stored at -80°C. To assess polyfunctional T cell responses from cells in OVA cultures, cells were washed and incubated with 20ng/mL PMA + 1µg/mL ionomycin in the presence of Brefeldin A in complete RPMI at 37°C for 4 hours. For *ex vivo* intracellular cytokine staining in OTII adoptive transfer experiments, dLNs were harvested 4 days after immunization and restimulated with 20µg/mL OVA peptide (323-339, Invivogen) + 2µg/mL soluble $\square$ CD28 with Brefeldin A in complete RPMI for 4 hr. For OVA-specific ELISAs, serum was collected from immunized mice after 21 days and frozen at -80°C.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants from restimulated LNs were collected and mouse IFN $\square$ measured by ELISA (Biolegend) according to manufacturer's protocols. Briefly, samples were incubated at room temperature in triplicate with antibody-coated plates for 1 hour, detection antibody for 1 hour,

followed by streptavidin-horseradish peroxidase for 30 minutes. TMB substrate solution was added and the reaction stopped with 2M sulfuric acid. Samples were read at 450nm with Softmax Pro Software and concentrations determined from a standard curve. For OVA-specific ELISA, mice were immunized on D0 and given an identical booster on D14, and serum was harvested on D21. High-binding assay plates were coated with 10 μ g/mL OVA in PBS at 4°C overnight, then blocked with 5% dried milk at room temperature for 2 hours. Serial dilutions of serum in duplicate were incubated at room temperature with OVA-coated plates for 2 hours, followed by Goat anti-mouse IgG1- or IgG2a-human adsorbed-HRP (Southern Biotech) for 1 hour. 1-step Ultra TMB (ThermoFisher) was added and the reaction stopped with 2M sulfuric acid. Samples were read at 450nm with Softmax Pro Software and reported as OD450 at the appropriate dilution (IgG1, 1:1,000,000; IgG2a, 1:1,000).

Flow cytometry

LNs were digested with 0.26 units/mL Liberase DH (Sigma) and 0.1mg/mL DNase I (Sigma) in RPMI at 37°C for 20 min, minced, and pipetted over a 35 μ M filter. Ice-cold RPMI containing 10% FBS and 10mM EDTA was added to stop digestion. Cells were washed twice in ice cold PBS, blocked with anti-CD16/32 (TruStain FcX; Biolegend) at 4°C for 10 min in FACS buffer and stained with indicated antibodies at 4°C for 30 min. For intracellular cytokine staining, cell surface staining was performed, and then cells were fixed and permeabilized with intracellular fixation and permeabilization buffer (eBioscience) and stained for intracellular cytokines. Cells were washed and resuspended in FACS buffer and acquired on a Fortessa X-20 flow cytometer (BD Biosciences). Viability was determined using Fixable Viability Dye eFluor 780 (eBioscience) and cell counts determined using CountBright counting beads (Invitrogen). Data was analyzed using FlowJo software (FlowJo, LLC).

Light Sheet Microscopy

Mice were immunized with AF488-OVA and LNs were fixed in 4% PFA, dehydrated in 30% sucrose, and cleared in CUBIC as previously described (Abe et al., 2016). Cleared LNs were embedded in agarose and light sheet microscopy was performed using a Lightsheet.Z1 Microscope (Carl Zeiss). In some experiments, α CD21/35 was labeled with AF647 and MECA79 labeled with AF594 (Invitrogen microscale protein labeling kits). To label LN follicles and HEVs, AF647- α CD21/35 was injected into the footpad 18 hr prior and AF594-MECA79 i.v. 2 min. prior to harvest.

Confocal microscopy

LNs were fixed in PLP at 4°C for 4 hr, dehydrated in 30% sucrose overnight, embedded in OCT (Tissue-Tek) and frozen on 2-methylbutane over liquid nitrogen. 12-16 μ M sections were cut from approximately the center of the LN using a Cryostat at -20°C (Microm HM 505 E, GMI). Sections were rehydrated in PBS at room temperature for 20 minutes, blocked with 5% normal goat serum in 0.1% Tween-20 in PBS for 30 minutes, and stained with indicated antibodies for 1 hour at room temperature in the dark. If necessary, samples were incubated for an additional hour with secondary antibodies. Coverslips were mounted with ProLong Diamond Antifade Mountant (Invitrogen) and cured overnight at room temperature in the dark. Images were captured with an LSM 780 Confocal Microscope and tile scans compiled with Zen Blue software (Carl Zeiss). LN regions were defined based on B cell staining using IMARIS software (Bitplane) and center point spots within each region were calculated.

Kaede photoconversion

Mice were anesthetized and skin patches on both sides of the tail base were shaved and remaining hair removed by application of Nair for 30 seconds followed by washing with PBS. Shaved skin patches were exposed to violet light (420 nm) using a Bluewave LED visible light curing unit (Dymax) equipped with a 420 nm bandpass filter (Andover Corp) with the light source at maximum power, 7.5 cm away from the skin for 5 minutes. Mice were immunized subcutaneously in skin patches and inguinal lymph nodes harvested for analysis.

Statistical analysis

Statistics were calculated using Prism software (GraphPad) using unpaired t test (between two columns), one-way ANOVA with Tukey's multiple comparisons test (3 or more columns), or two-way ANOVA with Sidak's multiple comparisons tests (grouped comparisons). * $P \leq 0.05$, ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 . Data are shown as mean $\pm$ SEM.

CHAPTER 3: Conclusions and Future Directions

Summary

Our work has highlighted the importance of antigen targeting to dLN IFR on directing downstream immune activation in support of type 1 immune responses, and suggests that oil emulsion may promote immunization targeting to and activation of this LN niche. We found that immunizing mice with antigen and TLR agonist emulsified in IFA resulted in robust type 1 immune responses, whereas an identical dose of antigen and TLR agonist in saline was not able to induce a significant type 1 cellular immune response. OVA/LPS/IFA immunization resulted in a distinctly altered distribution pattern of antigen compared to OVA/LPS immunization with a greater degree of labeled OVA targeted to IFR surrounding B cell areas, which correlated with a rapid influx of inflammatory monocytes to these same areas that upregulated CXCL10 in a type I interferon-dependent manner. A consequence of immunization delivery to these areas was the early activation in the LN IFR that resulted in inflammatory niches capable of attracting CD4⁺ T cells and supporting enhanced Th1 differentiation through exposure to IL-12 in the context of CXCR3 ligands (**Figure 3.1**). The implications of the present study on providing a better understanding of the mechanisms leading to activation of specific LN niches through immunization, future directions, and the potential to influence the design of vaccine formulations to promote protective adaptive immunity, will be discussed here in greater detail.

Figure 3.1. OVA/LPS/IFA preferentially targets antigen to LN IFRs resulting inflammatory monocyte-mediated formation of niches beneficial for promoting enhanced type 1 responses. (Left) During the early response to immunization (within the first 24 hours), emulsified immunization promotes antigen distribution to cortical areas of the LN including the IFR. Inflammatory monocytes infiltrating from circulation localize toward antigen-rich regions and upregulate CXCL10 in response to type-I-interferon, possibly produced by SIGNR1⁺ macrophages in the IFR, and amplify CXCR3 ligand gradients to direct CD4⁺ T cells out of the LN paracortex and toward IFRs. (Right) Later in the immune response, CD4⁺ T cells are advantageously positioned within an inflammatory niche in IFRs to encounter IL-12 signals from migratory DCSIGN DCs in the context of CXCR3 ligands, supporting differentiation into polyfunctional Th1 cells and leading to robust type 1 responses.

<u>Model – Emulsified Immunization</u> <u>(OVA/LPS/IFA)</u>	
<u>Early response:</u>	<u>Later events:</u>
- Antigen targeted to IFR and type 1 IFN release	- CD4 ⁺ T cells have localized to IFR
- Inflammatory monocyte infiltration, localization toward antigen-rich areas and upregulation of CXCL10	- DCSIGN accumulation and production of IL-12
- CD4 ⁺ T cells begin to mobilize to IFR	- Inflammatory niche established in IFR rich in CXCR3 ligand- and IL-12-producing cells
	- Enhanced polyfunctional Th1 differentiation and type 1 immunity



Altered antigen distribution in the dLN caused by oil emulsion

Although altered antigen distribution to the dLN has been proposed as a mechanism of action for oil adjuvants, the methods have not been available until more recently to formally examine this hypothesis. Our experimental model imaging CUBIC-cleared LNs with light sheet microscopy shows antigen localization in structurally intact LNs and demonstrates the ability of an immunization delivered in an oil emulsion to alter antigen/adjuvant distribution in the dLN. Our experiments with injection site excision suggest that the events leading to CD4⁺ T cell mobilization to LN IFR in the LN can be triggered by freely draining antigen/adjuvant arriving at the dLN.

Although the fully metabolizable squalene oil that forms the basis for MF59 is different from non-metabolizable paraffin oil in IFA, its success clinically has led to an interest in its effect on the immune system, and some observations intersect with our study presented here. While partially dependent on MyD88, MF59 is not itself a TLR agonist (Seubert et al., 2011). Intramuscular injection of labeled OVA and MF59 resulted in an increase in monocytes in the dLN within hours, while neutrophils were dispensable for MF59 effects on increased antibody production (Calabro et al., 2011). Intramuscular injection of PE and MF59 resulted in detection in both cortical SCS regions and medullary regions within hours, in contrast to PE alone, which was not effectively retained in the LN (Cantisani et al., 2015). A more recent study from the same group again injected differentially labeled PE and MF59 intramuscularly and showed localization of MF59 more toward the medullary region of the lymph node, although PE could be detected throughout the LN including cortical regions (Cioncada et al., 2017). They observed monocyte-derived DCs, as defined by surface expression of CD64 (FcγR1), accumulating in the medullary region of the dLN, and the ability of these DCs to stimulate CD4⁺ T cell proliferation *in vitro*.

The correlation between MF59 targeting to LN macrophages and monocyte appearance in the LN agrees with our data, suggesting that the ability to target and potentially activate LN macrophages as well as recruit monocytes to the dLN is a shared property among oil emulsions and may be a result of the altered antigen targeting of these substances. An important difference in these studies was their lack of TLR agonist, whereas our immunization included LPS as a potent TLR agonist. Whether TLR agonists themselves contribute to antigen distribution patterns in the LN is an open question. The creation of oil emulsions is known to result in the formation of droplets, which under the right conditions might ensure delivery of antigen and agonists together (Fox et al., 2013). We did not specifically label LPS or IFA in our study, which precludes us from formally determining whether localization of those components in the dLN differed from that of OVA, although we might speculate that droplets formed by emulsion in IFA are capable of delivering our immunization components together. Differential fluorescent labeling of individual immunization components could try and address which compartments and cells are targeted by each, and could reveal interesting differences in intranodal cell dynamics and determine whether antigen/adjuvant are required intact together in specific LN niches for optimal immune stimulation.

LN macrophages as upstream initiators of the LN response to oil adjuvants

LN macrophages are capable of limiting the systemic spread of lymph-borne pathogens, in part through activation of type I interferon responses (Iannacone et al., 2010; Junt et al., 2007). Iannacone *et al.* observed that SCS macrophages themselves were a source of type I interferon in addition to recruiting type I interferon-producing cells. Subsequent studies determined that type I interferon production early in the anti-viral response could drive CXCL10 production in the primary immune response (Sung et al., 2012), and vaccination with UV-inactivated influenza led to the production of IFN- α and IL-1 β by LN macrophages in the IFR

within 12 hours (Chatziandreou et al., 2017). Together with our data here, it is likely that LN macrophages play a role in the early response to OVA/LPS/IFA. We observed SIGNR1⁺ macrophages in cortical regions of the LN in IFR, which could represent interfollicular macrophages, but given the heterogeneity of cells in this region, a more detailed analysis is required to more accurately determine their identity. Our observation that CXCL10-producing cells were enriched in IFR and were dependent on the ability to sense type I but not type II interferons for chemokine production suggests that LN macrophages could be the upstream mediators of CXCL10 production by inflammatory monocytes. Detection of IFN- γ *in situ* remains challenging, although IFN- γ detection is achievable and is of interest in our model. Preliminary data suggests that IFN- γ mRNA levels in the dLN are potentially increased early in response to OVA/LPS/IFA compared to OVA/LPS (data not shown), which could be a result of preferential antigen targeting and activation of specific LN macrophage subsets, possibly SIGNR1⁺ macrophages. However, the available tools make this difficult to formally prove. Commercially available depletion models, including antibody-mediated depletion, clodronate liposome delivery, and *in vivo* genetic targeting models, are not able to specifically deplete only monocytes or only macrophages with a high degree of confidence. A mouse expressing floxed *ifnar1* is now available, which would be useful in tandem with a specific cre-expressing counterpart for monocytes or macrophages should one emerge.

Injection of MF59 or the TLR4 adjuvant GLA emulsified in squalene oil results in antigen uptake by SIGNR1⁺ macrophages, so additional study of these cells and their participation in responses to adjuvant are of high interest (Cantisani et al., 2015; Desbien et al., 2016). These studies focused on the medullary region of the LN, where an abundance of SIGNR1⁺ macrophages can be observed. However, given our observation of OVA/LPS/IFA preferentially targeted to cortical LN regions that also contain SIGNR1⁺ macrophages, an outstanding

question is whether SIGNR1⁺ macrophages in medullary regions are identical to SIGNR1⁺ macrophages seen in IFR. In addition, it would be interesting to determine if preferential uptake of oil adjuvant was mediated by cell surface receptors expressed on this subset, which have been shown to demonstrate increased expression of TLR4 compared to SCS macrophages (Phan et al., 2007).

A recent study showed that SIGNR1⁺ MSMs in this location regulate CCR8⁺ DC entry to the LN in response to allergic sensitization through production of CCL8 (Sokol et al., 2018). The identification of a novel function for these cells raises the question of whether SIGNR1⁺ macrophages in IFR have distinct functions, even if they are phenotypically similar to those in the medullary region, and whether differences in function might be regulated by unique signals derived from their microenvironment.

Inflammatory monocytes as amplifiers of the innate LN response

Monocytes are mononuclear phagocytes that are connected with macrophages and DCs as part of the mononuclear phagocyte system. By radiolabeling cells of the bone marrow with thymidine-³H and following them in mice *in vivo*, it was determined that monocytes circulating in the blood came from precursors in the bone marrow with the main purpose to replenish the tissue macrophage compartment (Furth and Cohn, 1968). However, it is now understood that most tissue-resident macrophages have a distinct ontogeny originating from the yolk sac and fetal liver and are capable of self-renewal (Ginhoux and Jung, 2014). In addition, antigen-carrying monocytes are present in LNs in steady-state retaining a transcriptional profile distinct from macrophages or DCs, and have been shown to have an impact on CD4⁺ T cell responses during inflammation (Hohl et al., 2009; Jakubzick et al., 2013; Samstein et al., 2013; Serbina et al., 2003). Our findings here agree with a role for inflammatory monocytes with their own effector function, in this case the production of chemokines to amplify gradients for T cell

localization. We found that CXCL10 production by inflammatory monocytes was impaired in the absence of type I interferon but not type II interferon receptor signaling. This agrees with the biology of CXCL10 regulation, which can be strongly induced by either type I or type II interferons through cooperation of IRSE and NF- κ B promoter elements (Ohmori and Hamilton, 1993). We have observed in a different setting that CXCL10 production by monocyte-derived cells was dependent on type II interferon but not type I interferon signaling during a model of experimental cerebral malaria, although those observations were at near-terminal stages of disease in cells with a more matured DC phenotype and represents a much different inflammatory context than the one in this study (Sorensen et al., 2018). Nevertheless, it will be important to further dissect the factors regulating different interferon signaling requirements for CXCR3 ligand production in inflammatory monocytes *in vivo* and how these might differ in various disease settings.

We observed a robust accumulation of inflammatory monocytes in the LN induced by immunization with OVA/LPS/IFA. While we were not able to directly determine the path of inflammatory monocyte migration to the inflamed LN in our model, our finding that these cells accumulated in the OVA/LPS/IFA-immunized LN of *ccr7*^{-/-} mice and were not photoconverted from UV-exposed immunized skin in Kaede transgenic mice, both indirectly support their infiltration from blood circulation. Nakano et al. observed the accumulation of CD11b⁺Gr1⁺ inflammatory monocytes able to enter inflamed LNs after immunization with Complete Freund's Adjuvant in mice either lacking CCR7 or unable to produce CCR7 ligands, which was impaired in the absence of CCR2 but not its ligand CCL2 (Nakano et al., 2009). We found an upregulation of CCL2 mRNA in the dLN within the first 2 hours after OVA/LPS/IFA immunization compared to OVA/LPS, but not other CCR2 ligands such as CCL7 or CCL12 (**Figure 3.2**). However, this assay does not capture the contribution of soluble chemokines draining from inflamed tissue through afferent lymph which can be presented on HEV (Palframan et al., 2001). It is possible

that CCL2 is acting to promote inflammatory monocyte entry from circulation to the inflamed LN. Another possibility is that CCL2 plays a role in directing positioning of CCR2⁺ cells within the LN, including inflammatory monocytes, with increased local production in IFR promoted by OVA/LPS/IFA immunization. Preliminary data from sorted LN macrophage subsets suggests that OVA/LPS/IFA may induce CCL2 mRNA expression preferentially within CD169⁺SIGNR1⁺ macrophages, but additional experiments are needed to confirm this finding (data not shown). A recent study found that depletion of macrophages using clodronate liposomes prior to vaccination with UV-inactivated influenza virus (PR8) resulted in a modest but significant decrease in overall CCL2 protein production in the LN (Chatziandreou et al., 2017). The production of CCL2 by LN macrophages could be a potential mechanism for directing localization of CCR2⁺ cells, but further study is required and a role for CCR2⁺ on intranodal positioning has yet to be fully demonstrated.

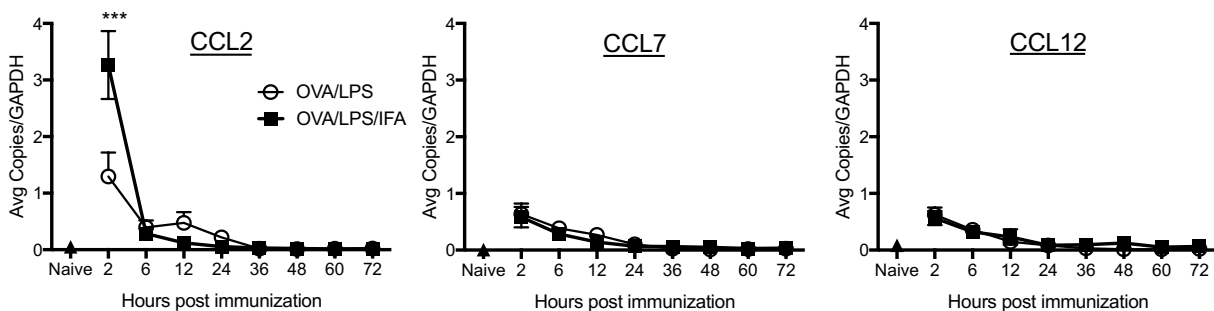


Figure 3.2. OVA/LPS/IFA rapidly induces CCL2 upregulation in the dLN in response to immunization. dLNs from immunized mice were harvested at the indicated timepoints and mRNA levels of CCR2 ligands measured by qPCR. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test; *** $p \leq 0.001$. Results are pooled from 2 independent experiments (n=4 mice per group).

Our finding that inflammatory monocytes rapidly can enter inflamed LNs directly from circulation and produce chemokines positions them as potentially critical cells to bridge the gap between initial LN activation mediated by resident cells and the later wave mediated by migratory cells. While LN resident cells can serve a number of important functions in initiating inflammation, their potential for a large expansion in cell numbers early in the response are relatively more limited than that which can be achieved with a circulating sentinel with a rapid rate of replenishment. In addition, while a degree of repositioning amongst some LN-resident cells is now appreciated, macrophages and stromal cells are likely more fixed in position compared to highly mobile inflammatory monocytes, making them well suited to serve as amplifiers of directional cues toward specific LN compartments.

The ability for monocytes to differentiate into DCs is another mechanism by which they can contribute to inflammation in the LN. In highly inflamed settings such as the one in this study, it is difficult to distinguish them from conventional DCs. The transcription factor *zbtb46* has been shown to define and deplete the cDCs (Meredith et al., 2012; Satpathy et al., 2012). However, it's possible under different conditions that monocyte-derived DCs may upregulate this transcription factor as well as the cDC2-associated *irf4* as part of an inflammatory DC program (Briseño et al., 2016). Preliminary data in our model suggests that DC depletion using *zbtb46*-DTR (zDC-DTR) mice did not result in a decrease in IFN γ production in the dLN, suggesting that compensatory mechanisms provided by monocyte-derived or other APCs and/or IL-12 sources may be able to preserve type 1 responses to some degree in response to OVA/LPS/IFA immunization, but additional experiments to examine the depletion efficiency of different myeloid and DC subsets are necessary to better interpret this result (**Figure 3.3**).

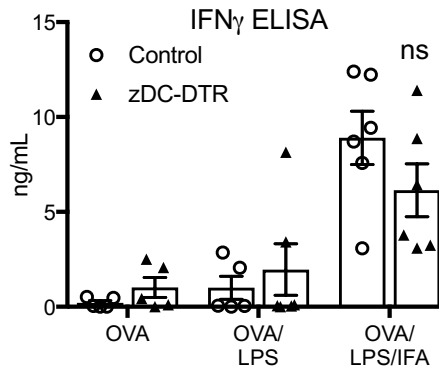


Figure 3.3. Depletion of cDCs may not result in impaired IFN γ production after

OVA/LPS/IFA immunization. WT hosts were lethally irradiated and reconstituted with WT or zDC-DTR bone marrow. dLNs were harvested 6 days after immunization and cultured with OVA protein for 3 days, and IFN γ from supernatants measured by ELISA. Data are presented as mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA with Sidak's multiple comparisons test. Results are pooled from 2 independent experiments (n=5-6 mice per group).

CD64 (FC γ R1) and DCSIGN (CD209a) have been used as cell surface receptors to define monocyte-derived DCs (Cheong et al., 2010; Langlet et al., 2012). However, in our hands there is a substantial heterogeneity in cells expressing these markers. The decrease in DCSIGN DCs observed in our experiments with MM-DTR mice does not necessarily prove that these DCs are of monocyte origin, but could be a mixed population and/or depend on cells depleted in the MM-DTR setting for their accumulation and/or survival in the dLN in response to inflammation. We noticed subtle differences in MFI expression in MHCII and DCSIGN expression among these cells, and preliminary data suggests that differences in the capability to produce IL-12 may correspond with this heterogeneity (data not shown). We are not yet confident that these surface markers can necessarily be uniquely used to define a monocyte or

cDC ontogeny of these cells, particularly between highly inflamed settings when expression of these markers can be vary.

In our model, DCSIGN DCs depended on CCR7 for accumulation in the LN and arrived at later timepoints following immunization (48-90h), suggesting that they migrate from the injected tissue. These DCSIGN DCs made up roughly half of all IL-12p40⁺ DCs at 72h post-OVA/LPS/IFA immunization. This response was likely due, at least in part, to our use of LPS and resulting TLR4 activation on this subset of DCs. Transcriptional analyses comparing sorted IL-12⁺ DC SIGNs, DCSIGN DCs not producing IL-12, as well other cDC2s capable of making IL-12 could potentially reveal important pathways that are capable of being activated by oil adjuvants and thus attractive for vaccine targeting. In addition, we did not analyze cells in the injected tissue, which could reveal interesting subsets of cells activated by OVA/LPS/IFA. Nevertheless, it is likely that both migratory and LN monocyte-derived DCs along with cDC2s together form a general pool of inflammatory DC2s that can promote CD4⁺ T cell responses including Th1 differentiation and provide some redundancy and/or synergy, especially in highly inflamed settings or during cell-tropic pathogen infections. Better tools to discriminate these cells *in vivo* are needed to more completely understand the functional differences between them.

DC targeting considerations and intranodal DC positioning

In addition to the difficulty in discerning between monocyte-derived DCs and cDC2s during inflammation, the heterogeneity of resident DC subsets in the LN has made it difficult to formally discern these populations *in situ*. Histo-cytometry has allowed for multiplex analysis of tissue sections and revealed that the residence of different DC subsets in the LN is non-uniform and highly compartmentalized, with cDC2s preferentially located in more peripheral areas of the LN near cortical sinuses, as opposed to cDC1s in the deeper paracortex (Gerner et al., 2012). This agrees with studies in the spleen, where early descriptions of asymmetric DC residence

(Steinman et al., 1997) has been more recently confirmed through identification of resident XCR1⁺ cDC1s in the T cell zone of the inner white pulp and 33D1⁺ cDC2s residing in the peripheral bridging channels (Calabro et al., 2016). This outlines the potential for highly variable outcomes in cellular immunity depending on antigen targeting in the LN. Indeed, injection of soluble fluorescently labeled proteins were able to rapidly drain into LNs and enter conduits, and regionalized uptake of antigen by cDC2s in more peripheral regions of the LN was able to induce the upregulation of early activation surface markers on CD4⁺ T cells (Gerner et al., 2017). This agrees with findings that LN-resident cDC2s positioned near lymphoid sinuses are capable of promoting CD4⁺ T cell activation in response to immunization or infection (Gerner et al., 2015; Gonzalez et al., 2010; Woodruff et al., 2014).

The use of histo-cytometry in the 2015 Gerner *et al.* study revealed a previously unappreciated LN-resident CD11b⁺ DC subset residing within lymphoid sinus endothelium and enriched in IFRs and cortico-medullary junctions able to capture lymph-borne particles. These lymphoid-sinus DCs (LS-DCs) could induce a modest level of CD4⁺ T cell activation in response to injected antigen, although the role of inflammatory monocytes in this study was not examined. Chemokine production by LS-DCs is not yet known. We have identified inflammatory monocytes as significant producers of CXCL10 based on our REX3-Tg reporter mice, and it is possible that LS-DCs also produce CXCR3 ligands. Based on our data alongside previous work demonstrating a role for CXCL10 in driving prolonged CD4⁺ T-DC contacts for optimal activation (Groom et al., 2012), LS-DCs may be CXCR3 ligand producers in response to interferon or PRR signaling which could attract CD4⁺ T cells or other CXCR3⁺ cells to their microenvironment, and inflammatory monocytes may help amplify these gradients to promote encounter with CD4⁺ T cell encounter with LS-DCs. Alternatively, LS-DCs could produce other chemoattractants responsible for directing intranodal inflammatory monocyte migration toward areas activated by lymph-borne antigens possibly including OVA/LPS/IFA. However, CD4⁺ T cell responses in this

model were diminished in the absence of migratory DCs, suggesting that LS-DCs may not be sufficient on their own to induce a robust and optimal type 1 response, which is reinforced by our finding that CCR7-dependent DCSIGN DCs are a significant source of IL-12 after OVA/LPS/IFA.

Therefore, targeting any one individual innate immune cell subset may not represent the best approach absent the ability to specifically target certain LN niches and including additional agonists to ensure an appropriately skewed response. For example, antigen coupled to the DC-selective receptors DEC-205 (Hawiger et al., 2001) or CLEC9A (Joffre et al., 2010) are able to efficiently target antigen DCs for presentation in the dLN, but CD4⁺ T cells were only transiently activated and unable to differentiate into IFN γ -producing cells, leading to anergic or tolerogenic T cell responses. In both cases, inclusion of a potent adjuvant was required for immune response skewing. Antigen conjugated with DEC-205 in combination with α CD40 led to effective antitumor responses (Bonifaz et al., 2004), whereas CLEC9A-conjugated antigen could promote T cell responses in the presence of poly(I:C) (Joffre et al., 2010). The DCs targeted with these strategies are primarily DC1s more tightly linked with cross-priming CD8⁺ T cells, but nevertheless underscore the importance of ensuring that targeting antigen to specific cell subsets is not achieved through sacrificing the optimal context of stimulation. A vaccine approach that requires additional examination is the combination of specific DC-targeting of antigen alongside the delivery of adjuvants/formulation that promote inflammatory LN niches through a more global activation of non-DC innate immune cells in those microenvironments.

Previous work from our lab demonstrated the requirement for CXCR3 expression on CD4⁺ T cells for localization to the medullary regions in addition to the IFRs in response to immunization with TLR ligands (Groom et al., 2012). In our experimental model, we did not observe an increase in transferred OTII cell localization to the medullary region after

OVA/LPS/IFA immunization compared to OVA/LPS or OVA immunization. However, other studies have examined immune responses initiated in this region of the LN. CD11b⁺SIGNR1⁺ DCs in the medullary region were able to bind inactivated PR8 injected in the footpad, transfer antigen to LN follicles and initiate humoral responses, and *in vitro* could induce CD4⁺ T cell proliferation (Gonzalez et al., 2010). A subsequent study found that in response to influenza vaccination, LN-resident CD11b⁺ DCs were capable of intranodal migration to the medullary IFR more distally located from the cortical IFR, and activating CD4⁺ T cells localizing there in a CXCR3-dependent manner resulting in clustering and upregulation of early activation surface molecules (Woodruff et al., 2014). The technical limitations in our study did not allow for the distinction between cortical and medullary IFR, and whether IFA-emulsification favors activation of cortical versus medullary IFR requires further study.

Another interesting possibility raised by these studies is whether IFA-emulsified immunization induces signals that specifically promote intranodal DC2 migration to IFR. Our results suggest that IFA-emulsified immunization promotes the accumulation of IL-12⁺ DCSIGN⁺ DC2s, although we were not successful in specifically staining DCSIGN⁺ cells *in situ*, and we did not perform an extensive analysis of chemokine receptors expressed on the DC populations in our study. Some direct evidence exists for specific inflammation-induced chemoattractants directing DC positioning to IFR within the LN. After infection with the intestinal nematode *H. polygyrus*, DCs were observed accumulating in IFR around B cell areas, which was dependent on CXCL13 (León et al., 2012). These findings agreed with prior studies that examined CXCR5⁺ DC localization in the spleen in response to LPS (Yu et al., 2002) and CXCR5⁺ DCs isolated *ex vivo* and transferred into the footpad (Saeki et al., 2000).

Other chemoattractants aside from chemokines may also be responsible for intranodal DC migration. In the spleen, interfering with S1P₁ signaling using FTY720 treatment resulted in immature 33D1⁺ DC2 redistribution from the bridging channels to the marginal zone, although

no effect was seen in mature DCs after LPS administration and DEC205⁺ DCs were unaffected (Czeloth et al., 2007). The G α i-coupled chemoattractant receptor EBI2 (GPR183) has been shown to guide the positioning of activated B cells to the outer follicle to promote encounter with T cells during inflammation and regulate humoral responses (Gatto et al., 2009; Kelly et al., 2011; Pereira et al., 2009). Oxysterols were identified as ligands for EBI2, which were capable of directing EBI2⁺ cell migration (Hannedouche et al., 2011; Liu et al., 2011). Splenic-resident DCs were found to express EBI2 mediating their migration to bridging channels, which could promote CD4⁺ T cell responses (Gatto et al., 2013; Yi and Cyster, 2013). The EBI2-oxysterol axis was necessary for splenic DCIR2⁺ DC2 positioning to the outer T cell zone in response to sheep red blood cell (SRBC) immunization (Lu et al., 2017). Interestingly, LPS but not poly(I:C) induced upregulation of EBI2 and a pattern of DCIR2⁺ DC positioning similar to that seen after SRBC immunization, and type I interferon disrupted EBI2 ligand production required for DC positioning to the outer T cell zone.

The ability of EBI2 to direct immune cell positioning to T-B borders is of particular interest and requires additional study. Emulsion in squalene oil has been shown to disrupt lipid metabolism *in vitro* (Kalvodova, 2010), and introduces the idea that metabolizable oil emulsions could affect oxysterol production. A recent study used a selective gene deletion approach to identify the changes to lipid metabolism pathways induced by oil-emulsion adjuvant AS03 and found that the IL-6 pathway was crucial for the response to AS03 and the promotion of Tfh responses (Givord et al., 2018). In light of the studies examining EBI2 and continued development of tools to directly study EBI2 and its ligands, testing the capability of vaccine adjuvants and especially oil emulsions to modulate EBI2 ligand production is an attractive avenue for future research. The coordination between this chemoattractant axis and chemokine receptor regulation of immune cell positioning within secondary lymphoid organs remains unclear. Most of the studies examining EBI2-mediated DC positioning were performed in the

spleen, and while similarities exist between spleen and lymph node responses, the organs have distinct anatomy and microenvironments, and these findings should be verified in models of infection and immunization in the peripheral LNs. It's possible that the EBI2 axis preferentially influences LN-resident cells versus the mechanism observed regulating CXCR5-mediated positioning of migratory DCs to the follicular border, and whether one axis (or others) dominates during type 1 or type 2 responses still needs to be explored. The factors regulating the positioning of myeloid and DC subsets to IFR, and whether these can be stimulated or enhanced through the use of oil adjuvants, are important subjects to better understand and could identify exciting potential therapeutic targets.

Innate lymphocyte and stromal cell contribution to early inflammation in the LN IFR

Populations of innate and innate-like lymphocytes also can be found in the IFR and medullary regions of the LN. Although they are grouped together here, they are distinct cell types that vary in the types of ligands they recognize as well as their function. These cells are smaller in number compared to classical lymphocytes, and relatively less is known about their intranodal migration dynamics. However, they share the ability to localize to IFR and medullary regions and produce IFN γ upon activation, suggesting a role in supporting early inflammation in these microenvironments (Kastenmüller et al., 2012).

NK cells play an important role in immune system surveillance and use a set of germline encoded receptors to recognize and eliminate abnormal cells, either foreign or altered-self, displaying a lack or improper expression of MHC or virally-induced ligands (Arase et al., 2002; Mandelboim et al., 2001). Under steady-state conditions NK cells are a relatively rare population in the LN but some can be found in the cortical regions near the T-B border with a greater presence in the LN medulla (Bajénoff et al., 2006a). NK cells have been shown to express CXCR3 during inflammation, potentially mediating their migration to inflamed LNs where their

IFN γ production could contribute to Th1 responses (Martín-Fontecha et al., 2004). However, whether CXCR3 dictates their intranodal localization, and whether these cells represent a similar cohort as those residing in the LN remains to be determined. IL-12 and type I interferons are potent activators of NK cells (Newman and Riley, 2007), and since we have found both cytokine families to play a crucial role in augmenting the immune response to oil emulsion vaccine, a further exploration of the cues regulating their positioning and their role in Th1 differentiation within IFR is required.

iNKT cells express an invariant TCR that recognizes lipid antigens presented on CD1d and are capable of rapidly producing high levels of effector cytokines. Although potent TCR activation can lead to cytokine production and changes to lipid metabolism are highly relevant in this regard, their response *in vivo* is highly sensitive to exposure to IL-12 induced by microbial inflammation (Brigl et al., 2003, 2011). IL-18 produced by LN-resident macrophages may also be capable of driving IFN γ production by NK, NKT, and also $\alpha\beta$ T cells (Kastenmüller et al., 2012). iNKT cells have recently been shown to localize within LN IFR, in a manner possibly dependent on CXCR3, and contribute to B cell responses following viral infection through IL-4 production (Gaya et al., 2018). The role of innate lymphocytes in early IFN γ production, particularly in light of findings that this response can be driven by IL-18 and/or IL-12 produced during microbial and viral infection, suggests that oil emulsions activating LN macrophages may potentiate an early IFN γ release in LN IFR by these cells. Although relatively few in number, this early cytokine production could be important to act in tandem with LN macrophages to support the activation of infiltrating monocytes observed in our study.

There is relatively little data on the migrational cues used by these cells within the LN. Whether oil adjuvants can directly activate these cells is also unknown. Although we have observed inflammatory monocytes present in these areas in large numbers after OVA/LPS/IFA,

innate lymphocytes are likely also contributing to the local cytokine milieu. The ability to trigger interferon production by these cells through vaccination could take advantage of their areas of residence within the LN to support LN niche activation.

LN stromal cells provide structure to the organ, and together provide a backbone to support immune cell survival and intranodal cellular migration. Genetic profiling performed in a recent study has revealed a significant heterogeneity within this network and allowed for the identification of FRC specific subsets located in LN IFR (Rodda et al., 2018). RNA sequencing of isolated LN stromal cells identified three populations of T-zone reticular cells (TRCs): the relatively more well studied Ccl19^{hi} TRCs, and two previously unidentified subsets of Ccl19^{lo} and CXCL9⁺ TRCs. The Ccl19^{lo} population expressed cholesterol-25-hydroxylase (Ch25h), which is important for expression of the ligand for EBI2 (Cyster et al., 2014). Staining of PDGFR α and CD11c alongside Ch25h or in the REX3 reporter mouse revealed that CCL19^{lo} and CXCL9⁺ TRCs populate the T-B interface and IFRs. This study introduces the intriguing possibility that these particular stromal cell subsets can initiate early chemoattractant-guided cell positioning, and requires further exploration. A new technique called “niche-seq” combines the use of mice with photoconvertible GFP to isolate cells from specific niches of the LN for downstream sequencing assays and could be particularly useful for better study of these niche-specific cell populations that are relatively rare in number (Medaglia et al., 2017).

Rational vaccine design looking forward and concluding remarks

Informing rational vaccine design through basic science research is critical for the expansion of our toolbox to confront the constantly evolving challenges posed to the immune system. While identifying the best antigenic determinants remains a challenge particularly for highly mutating viruses and tumors, identifying the mechanisms underlying robust LN activation will allow for determining the best strategies for formulating adjuvants and building engineered

vaccines such as nanoparticles and virus-like particles. Alongside emerging technologies with the ability to control the kinetics and biodistribution of vaccine delivery through microneedle injection and vaccine scaffolds (Moyer et al., 2016), there exist many exciting directions for next-generation vaccine development. Moving forward in a safe and efficient manner, away from a reliance on empirical approaches, requires continued study of the fundamental mechanisms underlying protective immunity. One such area is highlighted by advances in our understanding of the high degree of diversity among different compartments within the LN, and the spatiotemporal regulation of adaptive immunity that requires the navigation of these microenvironments.

In this study, we have examined factors shaping the LN response to immunization associated with enhanced type 1 responses. The chemokine regulation of intranodal immune cell positioning is one critical aspect of dictating the quality of the immune response. We have demonstrated that antigen targeting in the dLN can be altered by emulsification of immunization in oil, and corresponds with the establishment of inflammatory niches important for Th1 differentiation and chemokine-directed immune cell localization to these niches. In addition, we found that tissue-derived migratory cells and cells infiltrating the dLN from circulation play potentially distinct roles, and a cooperation between both was needed for optimal Th1 cell differentiation. Rather than trying to identify a single specific potentiator for a given immune response outcome, the best vaccines and adjuvants will be able to stimulate activation through multiple sites and stages, including maturation of APCs at the injection site, and also activation of cells resident in the dLN and infiltrating from circulation capable of initiating important early responses. Identifying adjuvants that can mimic the results seen here using oil emulsion that can target the dLN IFR in a way that leverages the natural host response cascade could provide opportunities for increasing vaccine efficacy.

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