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Bronchus-Associated Lymphoid Tissue (BALT): Structure and Function

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Abstract

Bronchus-associated lymphoid tissue (BALT) is a constitutive mucosal lymphoid tissue adjacent to major airways in some mammalian species, including rats and rabbits, but not humans or mice. A related tissue, inducible BALT (iBALT), is an ectopic lymphoid tissue that is formed upon inflammation or infection in both mice and humans and can be found throughout the lung. Both BALT and iBALT acquire antigens from the airways and initiate local immune responses and maintain memory cells in the lungs. Here, we discuss the development and function of BALT and iBALT in the context of pulmonary immunity to infectious agents, tumors, and allergens as well as autoimmunity and inflammatory diseases of the lung.

1. INTRODUCTION

Bronchus-associated lymphoid tissue (BALT) is considered by early investigators to be a mucosal secondary lymphoid tissue embedded in the walls of the large airways (Sminia *et al.*, 1989), similar to Peyer's patches in the small intestine. However, it is clear that BALT is not constitutively present in all mammalian species, notably mice and humans (Pabst, 1992; Pabst and Gehrke, 1990), and is induced in response to microbial exposure or other types of pulmonary inflammation (Tshering and Pabst, 2000). These inducible tissues may be more properly referred to as a tertiary or ectopic lymphoid tissue and we have coined the term inducible BALT (iBALT) to

describe them (Moyron-Quiroz *et al.*, 2004). In the context of this review, however, we do not attempt to make a distinction between BALT and iBALT and refer to both types of tissues as BALT.

The development of secondary lymphoid organs (Drayton *et al.*, 2006; Randall *et al.*, 2008) and tertiary lymphoid tissues (Carragher *et al.*, 2007) has been recently reviewed. Thus, this review focuses primarily on features and functions specific to BALT and the role of BALT in pulmonary immunity. BALT was previously reviewed by Bienenstock and McDermott (2005) and by us in 2007 (Moyron-Quiroz *et al.*, 2007). However, there have been significant advances since then, particularly in the way particular types of immune responses contribute to BALT formation. Therefore, this comprehensive review focuses first on the architecture and development of BALT, then on the functional properties of BALT, and finally on the role of BALT in response to respiratory infections, allergens, and autoantigens and the role of BALT in pulmonary malignancies.

2. LYMPHOID ARCHITECTURE OF BALT

2.1. Basic architecture and placement in the lung

As originally described in rabbits and rats, which develop BALT independently of microbial stimulation (Pabst and Gehrke, 1990; Sminia *et al.*, 1989), BALT is a densely packed cluster of lymphocytes with follicular structures enmeshed in a reticular network of stromal cells and underlying a specialized airway epithelium that lacks cilia (Sminia *et al.*, 1989), much like Peyer's patches in the small intestine or the nasal-associated lymphoid tissue (NALT) in the nose (Kiyono and Fukuyama, 2004). These structures are described to be located along major bronchial airways embedded in the airway wall with extensive lymphocytic infiltration of the epithelial layer creating a classic dome epithelium (Sminia *et al.*, 1989; van der Brugge-Gamelkoorn *et al.*, 1986b). BALT is also described to occur at airway bifurcations, where it is placed to trap inhaled antigens. However, in other species, in which BALT is not constitutively present in the lung and instead develops in response to microbial stimulation or inflammation, BALT does not always have such a defined structure or rigorous placement in the lung. Instead, iBALT can be located throughout the lung, typically adjacent to small pulmonary arteries (Moyron-Quiroz *et al.*, 2006).

In fact, an important site for the formation of iBALT is the perivascular space, which is filled with periarterial capillaries (Pabst and Tschernig, 2002). This space often becomes densely packed with lymphocytes upon pulmonary inflammation and is often classified as perivascular cuffing. Perivascular cuffing is not necessarily equivalent to BALT, as BALT requires additional architectural changes, including the development of

a stromal cell network, separation of B and T cell areas, the formation of follicular dendritic cells (FDCs) in the B cell follicles, and the development of high endothelial venules (HEVs) as well as lymphatics to facilitate leukocyte entry and exit from BALT. Since pulmonary arteries are typically parallel to bronchial airways, the formation of BALT in the perivascular space also tends to place BALT next to airways. However, in many instances, the lymphocytes in BALT do not infiltrate the airway epithelium and a classic dome epithelium is not necessarily present. Thus, these structures are not absolutely analogous to classic mucosal lymphoid tissues, like Peyer's patches or NALT. In addition, lymphoid clusters can be found even in the small airspaces that are apparently not adjacent to an artery or airway. Some of these clusters can be organized with B and T cell areas and specialized stromal cells, whereas others are simply small clusters of mostly B cells with little obvious organization. Thus, the term BALT is often used to encompass a wide range of tissues in various locations throughout the lung. However, we feel that minimally a pulmonary lymphoid cluster should have a B cell follicular structure with histologically identifiable FDCs in order to be termed BALT (Pabst, 2007).

2.2. Organization of B cell follicles in BALT

The B cell follicle is the most prominent feature of BALT (Fig. 7.1), and in many cases, BALT consists almost exclusively of B cell follicles without adjacent T cell areas (Rangel-Moreno *et al.*, 2006). Many B cell areas in BALT consist of follicles composed primarily of IgD^{hi}IgM^{lo} mature

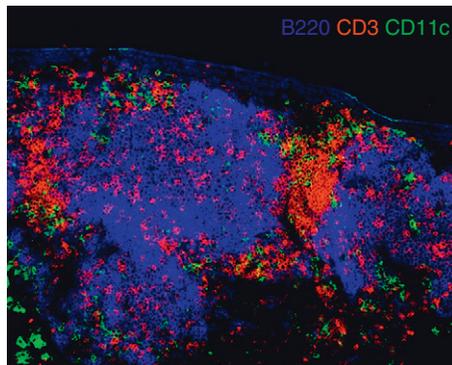


FIGURE 7.1 Organization of murine BALT. Lungs from mice infected with influenza 12 days previously were frozen in OCT medium and sectioned on a cryostat. Sections were probed with antibodies to B220 (blue), CD3 (red), and CD11c (green). Note the presence of three B220⁺ B cell follicles, two of which are underneath a major airway. CD3⁺ T cell zones are between the follicles and some T cells are scattered in the follicles. CD11c⁺ DCs are located primarily in the T cell zones.

resting B cells (Kocks *et al.*, 2007). However, B cell areas of BALT responding to infection or other antigens often contain germinal centers, which can be identified histologically as large blast cells in the center of the follicle that express Ki-67 or PCNA (Rangel-Moreno *et al.*, 2006), and rapidly incorporate BrdU and bind peanut agglutinin (PNA) or the antibody GL7 (Moyron-Quiroz *et al.*, 2004). Germinal center B cells also lose IgD and may have switched to alternative isotypes, such as IgG, IgA, or IgE. In classic BALT tissues that have a dome epithelium, the B cell follicle is found immediately below the epithelium (Sminia *et al.*, 1989). B cell follicles also contain CD4 T cells (Fig. 7.1), particularly in reactive follicles with germinal centers (Woodland and Randall, 2004), but they rarely contain CD8 T cells. In addition, some dendritic cells (DCs) and macrophages are found within the follicle (Fig. 7.1), where they may present antigen or facilitate the clearance of apoptotic germinal center B cells. Plasma cells are not often found within the follicle and are instead located around the edge of the follicle or in the T cell zone (GeurtsvanKessel *et al.*, 2009; Rangel-Moreno *et al.*, 2006). Not all B cell clusters in the lung—even those that form in the perivascular space adjacent to airways—are follicles. Some of these areas lack FDCs and are not separated from T cells or other cell types (Fig. 7.2). These areas may eventually develop into follicles or may simply be loose clusters of lymphocytes. However, it is still not clear whether these different types of tissues represent a morphological spectrum of functionally similar structures or whether the different structures imply different functions.

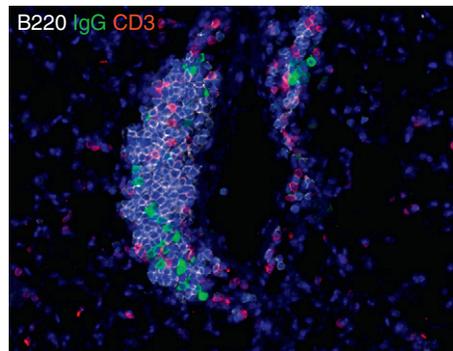


FIGURE 7.2 IgG-secreting cells in murine BALT. Lungs from mice infected with influenza 10 days previously were frozen in OCT medium and sectioned on a cryostat. Sections were probed with antibodies to B220 (white), IgG (green), and CD3 (red) and were counterstained with DAPI (blue). Note that the architecture is not well developed and the B220+ B cells, CD3+ T cells, and IgG+ plasma cells are intermixed.

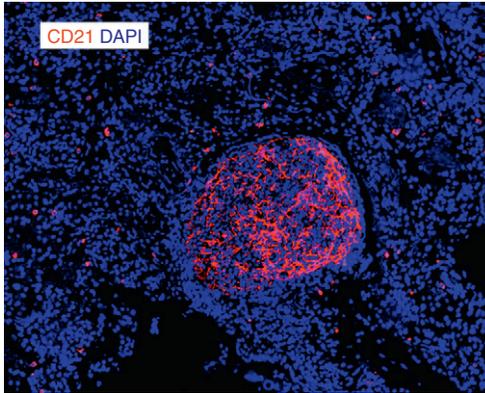


FIGURE 7.3 FDCs in human BALT. A formalin-fixed, paraffin-embedded lung biopsy from a patient with hypersensitivity pneumonitis was sectioned on a microtome and probed with an antibody to CD21 (red) and counterstained with DAPI (blue). Note the robust FDC network and the way the follicular areas are partially separated from the rest of the tissue.

Areas of BALT contain FDCs in the center of the B cell follicles (Fig. 7.3). FDCs in mice are identified by binding to antibodies against CD21/CD35 (Moyron-Quiroz *et al.*, 2004), FDCM1, or FDCM2 (Chvatchko *et al.*, 1996) and their ability to retain immune complexes (Haberman and Shlomchik, 2003). FDCs are dependent on the lymphotoxin (LT) signaling pathway for their differentiation in conventional lymphoid tissues as well as in BALT. Thus, although extensive areas of perivascular cuffing are often observed in the lungs of *Lta*^{-/-} and *LTβR*^{-/-} mice (Futterer *et al.*, 1998; Moyron-Quiroz *et al.*, 2004), these areas lack FDCs and fail to develop proper B cell follicles. FDCs also express CXCL13, which is responsible for the organization of the follicle and for recruiting B cells and some types of T cells to the B cell area (Ansel *et al.*, 2000). In the absence of CXCL13, B cell areas are still formed, but they lack the densely packed structure of true follicles and fail to develop FDCs (Rangel-Moreno *et al.*, 2007). Therefore, the major hallmark of true B cell follicles in BALT is the formation of FDCs.

2.3. T cell zones

T cell zones in BALT are typically found surrounding B cell follicles or between B cell follicles (Fig. 7.1; Woodland and Randall, 2004). T cell areas are also home to DCs (Fig. 7.1; Woodland and Randall, 2004), plasma cells (GeurtsvanKessel *et al.*, 2009), macrophages (Hiramatsu *et al.*, 2003), and occasionally eosinophils (Lee *et al.*, 1997) and neutrophils. HEVs and lymphatics are also found in the T cell zone or sometimes at the boundary

between the B cell follicle and the T cell area (Moyron-Quiroz *et al.*, 2004; Rangel-Moreno *et al.*, 2006). In some cases, the T cell zone is minimally populated with T cells and those that are present are widely scattered (Rangel-Moreno *et al.*, 2006). In these cases, the structure of BALT consists almost entirely of the B cell follicle. However, the T cell zone is often densely packed with T cells and DCs and appears similar to the intrafollicular T cell zone in Peyer's patches.

Reticular cells are found in T cell areas of BALT (Sminia *et al.*, 1989) and given the separation of B and T cell areas, are likely to express T cell attracting chemokines, like CCL21 and CCL19. Both of these chemokines are expressed in the lung (Moyron-Quiroz *et al.*, 2004; Rangel-Moreno *et al.*, 2007), and in species like mice and humans, the mRNA expression of CCL19 in particular is increased following infection or inflammation (Khader *et al.*, 2009). However, histological evidence for the stromal cell expression of CCL19 is lacking and CCL21 seems most prominently expressed on HEVs and lymphatics in BALT (Rangel-Moreno *et al.*, 2007). In *plt/plt* mice, which lack CCL19 and CCL21 (Nakano and Gunn, 2001; Nakano *et al.*, 1997), the T cell areas of BALT are significantly reduced (Rangel-Moreno *et al.*, 2007). However, BALT is still formed and still contains some B and T cells, which are separated into B and T cell areas (Rangel-Moreno *et al.*, 2007). Thus, CCL19 and CCL21 cannot be the only chemokines that promote BALT organization. Similarly, *Ccr7*^{-/-} mice also develop BALT and, according to several reports (Kahnert *et al.*, 2007; Kocks *et al.*, 2007), have more extensive and numerous areas of BALT than do normal mice. Again, B and T cells are separated into distinct regions, suggesting that additional chemokines, such as CXCL12 or others, help to define B and T cell areas in BALT.

2.4. HEVs and lymphocyte homing to BALT

A typical feature of BALT is the development of HEVs (Moyron-Quiroz *et al.*, 2004; Rangel-Moreno *et al.*, 2007), which are thought to recruit recirculating lymphocytes from the blood. HEVs in BALT are located around and between the B cell follicles in the T cell areas (Moyron-Quiroz *et al.*, 2004). Interestingly, HEVs in BALT express peripheral lymph node addressin (PNAd) rather than mucosal addressin cell adhesion molecule (MAdCAM) (Moyron-Quiroz *et al.*, 2004; Xu *et al.*, 2003), suggesting that BALT recruits naïve peripheral lymphocytes rather than memory B and T cells that have been primed in mucosal sites. Interestingly, HEVs in BALT areas of mice express much higher levels of VCAM-1 than HEVs in other secondary lymphoid organs (Xu *et al.*, 2003). Consistent with their expression patterns, L-selectin, PNAd, and LFA-1 are the most important homing molecules for the recruitment of B and T cells to BALT, whereas $\alpha 4\beta 7$ integrin and MAdCAM are not involved (Xu *et al.*, 2003).

Pulmonary inflammation or immunization appears to facilitate lymphocyte homing to BALT, as CFSE-labeled thoracic duct lymphocytes from TNP-KLH-immunized rats intravenously transferred to recipient rats that had also been intratracheally immunized with TNP-KLH were recruited more efficiently to the BALT than those transferred to nonimmunized rats (Sato *et al.*, 2000). Interestingly, labeled cells were observed inside HEVs as well as in T cell areas within 12 h after injection (Sato *et al.*, 2000), suggesting that T cells trafficked through the HEVs to enter BALT and subsequently entered the T cell area or follicles. Consistent with the idea that microbial stimulation promotes the homing of T cells to BALT, OTI TCR transgenic T cells home to BALT induced either by MTB infection (Day *et al.*, 2010) or by infection with modified vaccinia Ankara virus (Halle *et al.*, 2009) and proliferate in response to antigen. In both cases, BALT had to be generated by prior infection in order for naïve T cells to be recruited to the lung.

Prior inflammation is also important for T cell homing to BALT areas in a rat model of asthma (Schade *et al.*, 2010). In these experiments, CFSE-labeled T cells were transferred to normal or CD26-deficient rats either before or after sensitization with aerosolized ovalbumin (OVA). Prior to pulmonary sensitization, there was no difference in T cell homing to the BALT areas of normal or CD26-deficient lungs (Schade *et al.*, 2010). However, after sensitization, T cells preferentially homed to the BALT of CD26-deficient animals (Schade *et al.*, 2010). Given the peptidase activity of CD26, the authors concluded that CD26 normally degrades a T cell attracting chemokine that is normally induced after pulmonary inflammation and that in the absence of CD26, T cells were attracted to the BALT areas more efficiently (Schade *et al.*, 2010). Thus, despite the fact that the chemokines involved in homeostatic and inflammatory homing to BALT via HEVs are not completely understood, it is clear that they play an important role in lymphocyte trafficking to this tissue.

The current model of lymphocyte recruitment to secondary lymphoid tissues involves multiple steps, including addressin-mediated rolling, integrin activation by chemokines, which stops rolling and mediates firm adhesion, and finally, the transendothelial migration of lymphocytes into the tissue following a chemokine gradient (Campbell *et al.*, 1998). Thus, the expression of chemokines on HEVs is critical to proper homing of lymphocytes to lymphoid tissues. In the case of BALT, CCL21 is observed on HEVs (Rangel-Moreno *et al.*, 2007) and probably serves to attract naïve or central memory T cells as well as B cells. Consistent with this idea, we found that mice lacking CCL19 and CCL21 poorly recruited T cells to BALT areas (Rangel-Moreno *et al.*, 2007). However, BALT was still formed and still contained some B and T cells, suggesting that CCL21 is not the only chemokine that plays a role in recruiting T cells from the blood. Moreover, *Ccr7*^{-/-} mice also develop BALT and, according to several reports

(Demoor *et al.*, 2009b; Kahnert *et al.*, 2007; Kocks *et al.*, 2007), have more extensive BALT than do normal mice. In part, this may be due to the failure of regulatory mechanisms (Kocks *et al.*, 2007). However, the data still suggest that signaling through CCR7 is not absolutely required for entry to BALT.

2.5. DCs in BALT

As mentioned above, DCs are present in BALT and are found in highest concentrations in the T cell zone (Fig. 7.1; Woodland and Randall, 2004), but can be scattered throughout the B cell follicle as well. These cells are easily identified in the mouse using antibodies to CD11c (GeurtsvanKessel *et al.*, 2009; Halle *et al.*, 2009; Woodland and Randall, 2004) or CD205 (Kocks *et al.*, 2007). In humans, DCs have been identified using antibodies to DC-LAMP and DC-SIGN (Marchal-Somme *et al.*, 2007), S100 (Sarradell *et al.*, 2003; Yoshinouchi *et al.*, 1999), and MHC class II. Although numerous DC subsets have been identified in the lung, the DC subsets present in BALT have not been rigorously examined due to the difficulties in separating BALT areas from the rest of the lung tissue during preparation for flow cytometric analysis. However, plasmacytoid DCs (pDCs) are known to accumulate in the T cell areas of BALT and are more prevalent in patients with mild chronic obstructive pulmonary disease (COPD) than in patients with advanced disease (Van Pottelberge *et al.*, in press). The ability of DCs to present antigen in BALT and to maintain BALT architecture is discussed in later sections.

3. DEVELOPMENT AND MAINTENANCE OF BALT

3.1. Role of homeostatic chemokines and LT

The field of lymphoid organ development has progressed significantly since the discovery that *Lta*^{-/-} mice do not develop LNs or Peyer's patches (Banks *et al.*, 1995; de Togni *et al.*, 1994). The critical signal for lymphoid organ development comes through the LTβR on mesenchymal cells, which leads to the production of homeostatic chemokines, such as CCL19, CCL21, and CXCL13, that in turn recruit lymphocytes and promote lymphoid organ development (Mebius, 2003; Randall *et al.*, 2008). The expression of these chemokines by stromal cells and the expression of LTαβ on recirculating lymphocytes, particularly B cells, are reinforced by a positive feedback loop (Ngo *et al.*, 1999), in which LT controls chemokine expression by stromal cells and chemokine signaling on lymphocytes maintains surface expression of LTαβ on lymphocytes. Thus, the disruption of any part of this loop leads to impaired lymphoid organ development and architecture.

Similar events occur in the development and maintenance of BALT, although details are slightly different. For example, LT signaling is important for the maintenance of BALT (GeurtsvanKessel *et al.*, 2009) and is important for the expression of homeostatic chemokines in models of chronic inflammation, such as in pulmonary exposure to cigarette smoke (Demoor *et al.*, 2009a). The LT-dependent expression of CXCL13 and CCL19 in the lungs of smoke-exposed mice is consistent with data showing that CXCL13 and CCL19 are controlled by LT signaling in conventional lymphoid organs like the spleen (Ngo *et al.*, 1999). However, it is not consistent with our data showing that these chemokines are induced to similar levels in normal and *Lta*^{-/-} mice upon acute infection with influenza virus (Moyron-Quiroz *et al.*, 2004). Thus, the expression of the so-called homeostatic chemokines seems to be controlled differently during acute and chronic inflammation of the lung.

Given the induced expression of CXCL13 and CCL19 (Moyron-Quiroz *et al.*, 2004), we examined the role for these chemokines in the development of BALT (Rangel-Moreno *et al.*, 2007). Interestingly, BALT was still formed in *Cxcl13*^{-/-} mice after influenza infection and had separated B and T cell areas, HEVs, and lymphatics (Rangel-Moreno *et al.*, 2007). However, the B cell areas contained only loose collections of B cells and FDCs did not develop. Thus, CXCL13 is necessary for proper B cell follicle formation in BALT. In contrast, *plt/plt* mice, which lack CCL19 and CCL21, formed BALT with large B cell follicles, but much smaller T cell zones (Rangel-Moreno *et al.*, 2007). HEVs were also smaller in the animals, possibly due to the lack of CCL21 expression and reduced traffic through the endothelium (Rangel-Moreno *et al.*, 2007). Interestingly, *Cxcl13*^{-/-} × *plt/plt* mice failed to form detectable BALT following influenza infection (Rangel-Moreno *et al.*, 2007). Thus, both B- and T cell attracting chemokines are important for BALT formation and play different roles in its architecture. Similar studies used these same mice to examine the formation and function of BALT surrounding tuberculosis granulomas and came to similar conclusions.

Other studies examined BALT formation using *Ccr7*^{-/-} mice (Demoor *et al.*, 2009b; Kahnert *et al.*, 2007; Kocks *et al.*, 2007). Surprisingly, these animals developed BALT spontaneously and formed larger areas of BALT after infection than their normal counterparts. The overall architecture of BALT was apparently normal in *Ccr7*^{-/-} mice, with separated B and T cell zones, lymphatics, HEVs, and DCs (Kocks *et al.*, 2007). The BALT hypertrophy in these mice was attributed to a failure of Tregs to home to conventional lymphoid organs and prevent autoimmune reactions (Kocks *et al.*, 2007). Thus, BALT in *Ccr7*^{-/-} mice may be partly supporting autoreactive T and B cell responses, but CCR7 is not required for BALT development or organization.

CCR7 is also important for the recovery of CCR7⁺ T cells from peripheral nonlymphoid tissues via attraction to CCL21-expressing lymphatic

vessels (Bromley *et al.*, 2005; Debes *et al.*, 2005). Therefore, in the absence of CCL21 or CCR7, CCR7-expressing cells, including T cells, B cells, and DCs, are unable to return to the circulation and may simply pile up in peripheral tissues (Bromley *et al.*, 2005; Debes *et al.*, 2005). If enough T and B cells are stuck in peripheral tissues, they may begin to spontaneously form organized lymphoid tissues. This possibility is proposed by some investigators to explain the development of ectopic follicles, such as BALT, in *Ccr7*^{-/-} mice (Hopken *et al.*, 2007). In fact, a similar mechanism is proposed to explain the formation of BALT in normal animals at sites of inflammation or infection in normal individuals (Carragher *et al.*, 2007; Thauinat *et al.*, 2006). In this model, infection or inflammation leads to rerouting of the lymphatic drainage toward newly formed tertiary lymphoid tissues (Thauinat *et al.*, 2006). As a result, the developing lymphoid tissue is maintained by the continuous influx of antigen and DCs, which leads to sustained local immune activation. Regardless of the final explanation, altered draining of pulmonary lymphatics is likely to play a role in the development and maintenance of BALT.

In addition to its role in the expression of homeostatic chemokines, LT signaling also controls the differentiation of HEVs (Browning *et al.*, 2005) and is important for the expression of various adhesion molecules on vascular endothelial cells and stromal cells. For example, the expression of MAdCAM, PNA^d, VCAM-1, and ICAM-1 is controlled, in part, by the activities of LT α , LT α β , and TNF signaling through LT β R and TNFR1 (Browning *et al.*, 2005; Cuff *et al.*, 1998, 1999). Similar pathways control the expression of the sulfotransferases that generate the PNA^d epitope as well as the expression of GlyCAM and MAdCAM (Drayton *et al.*, 2004; Pablos *et al.*, 2005). Moreover, the development of FDCs is controlled by LT signaling (Endres *et al.*, 1999) and some DC subsets are dependent on LT signaling for their homeostatic maintenance (Kabashima *et al.*, 2005). Given that the LT signaling pathway controls so many aspects of lymphoid tissue architecture and the differentiation of so many cell types important for the function of lymphoid tissues, it is difficult to imagine that BALT can develop normally in the absence of LT signaling. Nevertheless, many publications claim to observe BALT and B cell follicles in *Lta*^{-/-} mice (Day *et al.*, 2010; Demoor *et al.*, 2009a; Kashino *et al.*, 2010). In fact, we also find perivascular clusters of B cells and even T cells in *Lta*^{-/-} mice (Moyron-Quiroz *et al.*, 2004). However, these clusters have very little organization and lack FDCs and HEVs. Despite this lack of organization, however, pulmonary immune responses can be generated in splenectomized *Lta*^{-/-} mice (Constant *et al.*, 2002; Day *et al.*, 2010; Demoor *et al.*, 2009a; Kashino *et al.*, 2010; Lund *et al.*, 2002), suggesting that lymphoid clusters in the lung do have some function, even in the absence of apparent organization.

Lymphoid tissue inducer (LTi) cells are instrumental for the initiation of conventional lymphoid organ development (Cupedo *et al.*, 2002;

Eberl and Littman, 2003). LT_i cells are first generated in the fetal liver and although they express CD4, they do not express CD3 and are not T cells or B cells (Mebius *et al.*, 2001). LT_i cells express LT $\alpha\beta$ and are the first cells to arrive at the developing LN anlagen, where they initiate LT signaling and promote the first stages of lymphoid organ development (Randall *et al.*, 2008). Given their role in the development of conventional lymphoid organs, one might assume that they would be involved in the development of ectopic lymphoid tissues, such as BALT. In fact, transgenic mice that overexpress IL-7 have abnormally high numbers of LT_i cells and develop a much higher number of LNs and Peyer's patches than normal mice and also develop ectopic lymphoid tissues in pancreas and salivary gland (Meier *et al.*, 2007). We have also observed the development of BALT in IL-7 transgenic mice (J. Rangel-Moreno, T. D. Randall, and D. Finke, unpublished data), suggesting that LT_i cells can lead to the development of BALT. Importantly, IL-7 transgenic mice crossed to *Rorc*^{-/-} mice, which lack LT_i cells (Sun *et al.*, 2000), do not develop any secondary lymphoid organs or ectopic lymphoid tissues (Meier *et al.*, 2007). Thus, in this model, the development of ectopic follicles is dependent on LT_i cells. However, we have also observed that nontransgenic *Rorc*^{-/-} mice and *Id2*^{-/-} mice infected with influenza do develop BALT (J. Rangel-Moreno and T. D. Randall, unpublished data). Since these mice fail to develop LT_i cells, it seems unlikely that they play an essential role on BALT development. Thus, LT_i cells may be sufficient, but not necessary for the development of ectopic tissues like BALT.

3.2. Dendritic cells

In addition to their antigen-presenting ability, DCs are also implicated in the maintenance of BALT (GeurtsvanKessel *et al.*, 2009; Halle *et al.*, 2009). For example, CD11b^{hi}MHCII^{hi} DCs accumulate in the lungs after influenza infection and continue to increase in numbers after infection is cleared (GeurtsvanKessel *et al.*, 2009), correlating with the formation of BALT. Importantly, the depletion of pulmonary DCs triggers the dissolution of BALT (GeurtsvanKessel *et al.*, 2009). A similar study showed that pulmonary infection with modified vaccinia Ankara infection leads to the development of long-lasting BALT areas (Halle *et al.*, 2009). As in the previous study, the depletion of DCs from the lungs led to a significant reduction in BALT size, although the numbers of BALT areas remained similar. Thus, it seems that DCs are required to support the ongoing maintenance of BALT. This is an intriguing idea, since it suggests that unlike conventional lymphoid organs, BALT areas must be actively maintained by immune-stimulating DCs.

How might DCs be important for BALT maintenance? One possibility is that DCs maintain BALT by expressing homeostatic chemokines,

including CXCL12 and CXCL13, as well as the cytokine LT β (GeurtsvanKessel *et al.*, 2009). In fact, LT β R signaling is required for the maintenance of BALT (GeurtsvanKessel *et al.*, 2009), similar to its role in the maintenance of other ectopic lymphoid tissues (Gatumu *et al.*, 2009). However, given that the depletion of pulmonary DCs only modestly reduces LT β expression and has no effect on either CXCL13 or CCL21 expression (GeurtsvanKessel *et al.*, 2009), it seems that DCs are performing some other function, such as presenting IL-15 to CD8 T cells (McGill *et al.*, 2010) that leads to the maintenance of BALT. Since CD8 T cells are essential for the maintenance of germinal centers in ectopic follicles in other locations (Wagner *et al.*, 1998), they may also support the maintenance of BALT.

3.3. Neuromodulation of BALT

New data suggest that neuronal stimulation is important for secondary lymphoid organ development via the retinoic acid-dependent production of CXCL13 by nerve fibers adjacent to the developing LN anlagen (van de Pavert *et al.*, 2009). Interestingly, areas of BALT contain numerous cholinergic neurons (Cavallotti *et al.*, 2004, 2005), suggesting a possible role for neuromodulation in BALT development or function. Consistent with this idea, one study showed that sensory nerve stimulation in the airways following respiratory syncytial virus (RSV) infection in rats leads to leukocyte recruitment to the airways and BALT hyperplasia (Auais *et al.*, 2003). Interestingly, T cells in BALT expressed high levels of the high-affinity substance P receptor, neurokinin 1. Moreover, inhibitors of neurokinin 1 blocked lymphocyte recruitment to the airways (Auais *et al.*, 2003). Based on these data, the authors of this study concluded that cells from BALT were directly being recruited to the airways, because lymphocytes in peripheral blood lack the neurokinin 1 receptor. However, neuronal stimulation could be indirectly promoting the recruitment of lymphocytes by increasing the production of CXCL13 or other chemokines that in turn recruit lymphocytes in areas of BALT (van de Pavert *et al.*, 2009). Further study is required to clarify any potential role of neuronal stimulation in BALT development or function.

4. ANTIGEN ACQUISITION

4.1. Microfold cells

Most mucosal lymphoid tissues are located directly beneath a specialized epithelial layer that is infiltrated by lymphocytes and DCs and contains microfold cells (M cells) (Man *et al.*, 2004; Miller *et al.*, 2007). M cells are

specialized mucosal epithelial cells that lack cilia, do not express the polymeric Ig receptor, and appear flattened with surface microfolds on the luminal surface (Man *et al.*, 2004; Miller *et al.*, 2007). M cells are thought to transport antigens from the mucosal lumen to DCs that are in close contact with the dome epithelium. M cells are easily observed in NALT and Peyer's patches and are also observed on villous epithelium in the small intestine that is distant from organized lymphoid tissues (Jang *et al.*, 2004) and in the nasal passages away from NALT (Kunisawa *et al.*, 2005). Importantly, some studies provide clear evidence of M cells in the epithelial layer over BALT using electron microscopy (Tango *et al.*, 2000), standard microscopy (Sminia *et al.*, 1989), staining with the lectin, *Ulex europaeus* agglutinin 1 (UEA1) (Tango *et al.*, 2000), and functionally by the transport of antigens (Gregson *et al.*, 1982; van der Brugge-Gamelkoorn *et al.*, 1985c). Changes in the epithelial layer over BALT consistent with the differentiation of M cells also occur upon pulmonary inflammation or antigen stimulation (van der Brugge-Gamelkoorn *et al.*, 1986a,b). Thus, it seems that epithelial M cells are one way in which BALT acquires antigen from the lumen of the airways. However, not all areas of BALT have a well-defined dome epithelium. Moreover, some areas of BALT appear to be exclusively perivascular or are found in the lower airspaces and lack an association with either airways or arterioles. Thus, these areas of BALT seem to lack M cells and must acquire antigens locally (such as autoantigens or infectious agents) or acquire pulmonary antigens via some other mechanism, such as afferent lymphatics.

4.2. Lymphatics and DC migration

BALT areas have lymphatic vessels that are identified using the antibodies LyVE1 in mice (Carragher *et al.*, 2007) or M2A in humans (Rangel-Moreno *et al.*, 2006). Lymphatics are observed surrounding the B cell follicle and in the T cell area (Carragher *et al.*, 2007; Kocks *et al.*, 2009; Rangel-Moreno *et al.*, 2006). However, it is difficult to assess histologically whether these are afferent lymphatics that bring antigens and cells from distal regions of the lung to BALT or whether they are efferent lymphatics that collect lymphocytes from BALT and return them to the circulation. The idea that DCs acquire antigen in the airways and then migrate to BALT via afferent lymphatics is supported by evidence of DCs and possibly macrophages filled with diesel exhaust particles (DEPs) (Hiramatsu *et al.*, 2003), silica particles (Lee and Kelly, 1993), cigarette smoke particles (van der Strate *et al.*, 2006), and other antigens (Halle *et al.*, 2009) in BALT. In each of these cases, the antigen-bearing DCs are located in the T cell zone of BALT (Fig. 7.1) near lymphatics. However, it is not clear whether antigen gets to BALT via some undefined mechanism,

where it is engulfed by resident DCs or whether the DCs obtained the antigens in the airways.

An exciting new study resolves this issue using live imaging of *ex vivo* lung tissue to show that DCs can migrate from the lung airways into BALT (Halle *et al.*, 2009). This study implies that DCs either migrate directly across the epithelium or enter afferent lymphatics that lead to BALT (Halle *et al.*, 2009). In either case, these data demonstrate that pulmonary antigens can be acquired by BALT via mechanisms other than transfer across the epithelium by M cells (Kiyono and Fukuyama, 2004). Importantly, this study also showed that antigen-pulsed DCs transferred to the airways prime T cell responses in the BALT. Thus, DC migration to BALT is an important mechanism by which antigen is acquired for local immune responses.

4.3. *In situ* antigens

In cases in which BALT is formed in response to autoantigens, such as in rheumatoid arthritis (RA) (Rangel-Moreno *et al.*, 2006), antigens could be expressed directly in the follicle—perhaps by DCs or other cell types. In addition, in cases where BALT is formed in response to infection, the infectious agent may actually be present in BALT. For example, BALT may form at sites of influenza virus infection and may encompass virally infected cells (Moyron-Quiroz *et al.*, 2004). Thus, antigens would be present in BALT with no need to acquire antigen via M cells or lymphatics. Similarly, some viruses, such as Epstein Barr virus (EBV) directly infect B cells and may be present in the B cell follicles of BALT (Kocks *et al.*, 2009). Moreover, in the case of MTB, BALT essentially forms around the tuberculosis granuloma (Kahnert *et al.*, 2007). Again, MTB antigens may directly incorporated into BALT with the need for specialized mechanisms of antigen transport. Thus, there are many ways for BALT to acquire or be exposed to pulmonary antigens.

5. B CELL RESPONSES IN BALT

5.1. B cell responses and germinal centers

Germinal centers are oligoclonal clusters of B cells responding to antigen, typically in T cell-dependent immune responses (Thorbecke *et al.*, 1994). B cells undergo intense clonal expansion in germinal centers, where they ultimately differentiate into long-lived plasma cells or memory B cells. Germinal centers are commonly observed in BALT and can be visualized by staining for markers of proliferating cells (PCNA or Ki-67) (Rangel-Moreno *et al.*, 2006) or for cell surface markers of germinal center B cells, such as PNA and GL7 (GeurtsvanKessel *et al.*, 2009; Moyron-Quiroz *et al.*, 2004). We have also used BrdU labeling to identify germinal centers in

mouse BALT and showed that nearly half of the B cells in the germinal centers can be labeled with BrdU in a few hours (Moyron-Quiroz *et al.*, 2004). This rate of proliferation is consistent with B cell proliferation in the germinal centers of conventional lymphoid organs.

The presence of germinal centers in BALT suggests that B cell responses can be initiated and sustained locally. Consistent with this idea, antigen-specific antibody secreting cells are often observed in BALT following pulmonary immunization (GeurtsvanKessel *et al.*, 2009; van der Brugge-Gamelkoorn *et al.*, 1985b, 1986a,b). Moreover, we find that preexisting BALT accelerates B cell responses to influenza in mice with preexisting BALT and leads to reduced morbidity and mortality in response to a number of viruses, including influenza, SARS corona virus, and mouse pneumovirus (Wiley *et al.*, 2009). We observe similar results using mice lacking conventional lymphoid organs (Moyron-Quiroz *et al.*, 2004), suggesting that on its own, BALT is able to initiate pulmonary immune responses that are faster and more protective than those initiated in systemic sites. Another study shows that influenza nucleoprotein-specific plasma cells as well as germinal centers are found in BALT after influenza infection (GeurtsvanKessel *et al.*, 2009). This response is dependent on DCs, as germinal centers are reduced in size and IgA-secreting plasma cells are reduced in number when CD11c⁺ cells are depleted. Interestingly, long-lived nucleoprotein-specific plasma cells in the bone marrow are also reduced by the elimination of iBALT via DC depletion in the lung (GeurtsvanKessel *et al.*, 2009), suggesting that many of the long-lived plasma cells in the bone marrow are derived from precursors in the lungs rather than in the LN. Moreover, the dissolution of iBALT following DC depletion results in reduced IgA responses in the lung and lower hemagglutinin inhibiting activity in the serum following challenge infection (GeurtsvanKessel *et al.*, 2009). Thus, both local and systemic antibody responses are enhanced by the presence of BALT.

Interestingly, despite the fact that CXCL13 is responsible for organizing the B cell follicle, B cell responses in BALT are not dramatically disrupted in *Cxcl13*^{-/-} mice (Rangel-Moreno *et al.*, 2007), since germinal centers are formed and antibody production appears normal following influenza infection. However, germinal centers are severely disrupted and antibody is dramatically reduced in the absence of CCL19 and CCL21, possibly due to defects in T cell responses or to poor DC recruitment to BALT. Thus, the homeostatic chemokines are important for BALT-dependent B cell responses in unexpected ways.

5.2. Isotype switching and somatic mutation

Mature resting B cells initially express surface IgM and IgD. However, when stimulated by antigen, particularly in T cell-dependent responses, B cells undergo isotype switching to various IgG isotypes, IgA and IgE

(Stavnezer *et al.*, 2008). In conventional lymphoid organs, switching can take place in extrafollicular foci or in germinal centers (Jacob and Kelsoe, 1992). Switching to particular isotypes depends on the type of immune response. For example, systemic Th1 responses typically trigger switching to IgG2a, whereas Th2 responses promote switching to IgG1 and IgE (Stavnezer *et al.*, 2008). In contrast, mucosal immune responses typically lead to switching to IgA (Briere *et al.*, 1995; Shikina *et al.*, 2004), which is easily transported across mucosal epithelium via the polymeric Ig receptor (Johansen *et al.*, 1999; Shimada *et al.*, 1999). Based on the concept that BALT is a mucosal lymphoid tissue, one might expect that isotype switching in BALT would result in IgA-producing B cells. However, B cells in BALT appear to switch to the full spectrum of isotypes, depending on the type of immune response. For example, we find that immune responses to influenza lead to IgG-secreting cells in BALT (Fig. 7.2). Many studies find that IgG-secreting cells predominate over IgA-secreting cells in BALT (Plesch *et al.*, 1983; Sminia *et al.*, 1987, 1989), suggesting that BALT does not act like a typical mucosal lymphoid tissue. However, IgA can be easily observed under a variety of conditions (Kolopp-Sarda *et al.*, 1994), including after influenza infection (GeurtsvanKessel *et al.*, 2009). In addition, IgE is often found in BALT areas of patients with allergies (Slavin *et al.*, 1992) or in experimental animals that have been sensitized to antigens under Th2 inducing conditions (Chvatchko *et al.*, 1996; Gajewska *et al.*, 2001). Thus, BALT does not preferentially promote isotype-switching to IgA, but does promote switching to isotypes appropriate for the type of immune response.

Another function of germinal centers is to support somatic hypermutation and affinity selection of B cells (Kelsoe, 1996). Current thinking suggests that B cells proliferate in the dark zone of the germinal center and are selected for high-affinity antigen receptors by immune complex-bearing FDCs in the light zone (Allen *et al.*, 2007; Schwickert *et al.*, 2007). Most areas of BALT in patients or animals with active immune responses have germinal centers and FDCs (Fig. 7.3). Moreover, we find that germinal centers in patients with RA had distinct light and dark zones (Rangel-Moreno *et al.*, 2006). Since extensive FDC networks are observed in the RA patients and since the FDCs in BALT are capable of binding immune complexes, it stands to reason that high-affinity B cells are being selected in BALT germinal centers and will likely produce both long-lived plasma cells and memory B cells. In the case of B cell responses to pulmonary infections, this type of response is probably beneficial. However, in response to autoantigens in patients with RA, this response is likely to be pathologic.

Additional evidence for affinity selection in the germinal centers of BALT comes from patients with COPD. BALT from these patients contains B cells that predominantly expressed CD27, which is a memory marker on B cells (van der Strate *et al.*, 2006). BALT from these patients also contains numerous germinal centers and a molecular analysis of

B cells from these germinal centers shows evidence of ongoing somatic hypermutation (van der Strate *et al.*, 2006). Thus, it is likely that high-affinity B cells are being selected in these patients, although the antigen specificity of these B cells is unknown.

5.3. Maintenance of plasma and memory B cells

As mentioned in previous sections, isotype-switched plasma cells are often found in the T cell zone of BALT, similar to the placement of plasma cells in the T zone of the spleen. Given the dependence of BALT plasma cells on DCs (GeurtsvanKessel *et al.*, 2009), it is possible that DCs or other myeloid cells in the T zone are producing plasma cell survival factors, such as IL-6 and APRIL (Mohr *et al.*, 2009). Thus, the depletion of DCs may lead to reductions in local plasma cell numbers via impairment of the plasma cell niche. Moreover, CXCL12, which is thought to be important for the recruitment of long-lived plasma cells to the bone marrow (Hargreaves *et al.*, 2001; Hauser *et al.*, 2002), is also expressed in the T zones of BALT. Therefore, BALT may serve as a reservoir of long-lived plasma cells that were generated locally in response to pulmonary antigens.

6. T CELL RESPONSES

6.1. T cell priming and maintenance of memory

Despite years of accumulating evidence that reactive BALT can be observed in experimentally immunized or environmentally exposed animals and the observations that antigen-specific B cell responses can occur in BALT, direct evidence for antigen-specific T cell responses in BALT has been lacking. We showed that in mice lacking conventional secondary lymphoid organs, CD8 T cell responses to influenza are primed with normal kinetics (Moyron-Quiroz *et al.*, 2004) and that antigen-specific CD8 T cells can be found in regions of BALT (Moyron-Quiroz *et al.*, 2006). Thus, we concluded that CD8 T cells could be primed in BALT, but we could not definitively exclude the (unlikely) possibility that they were actually primed in systemic sites like the bone marrow. Similar studies using splenectomized *Lta*^{-/-} mice show that immune responses to pulmonary infection with MTB (Day *et al.*, 2010; Kashino *et al.*, 2010) and *Leishmania major* antigens (Constant *et al.*, 2002) are intact in the absence of conventional lymphoid organs and antigen presentation appears to occur directly in the lung. These studies suggest that BALT is likely to be a priming site for naïve T cells responding to pulmonary antigens.

However, more recent studies directly demonstrate that BALT can recruit and prime naïve T cells. For example, following the induction of BALT by infection with MTB, adoptively transferred OVA-specific OTII TCR transgenic T cells are primed in the lungs by pulmonary exposure to OVA (Day *et al.*, 2010). Importantly, T cell priming is intact in the lungs of MTB-infected mice that lack conventional secondary lymphoid organs, but does not occur if BALT is not previously induced by infection with MTB (Day *et al.*, 2010). Thus, T cell priming in the lung to pulmonary antigens requires the presence of BALT. Similar studies showed that after BALT is induced in response to modified vaccinia virus, adoptively transferred OVA-specific naïve OTI cells are recruited to BALT and proliferate locally in response to intratracheally administered OVA-pulsed DCs (Halle *et al.*, 2009). This study also examined the dynamics of T cells in BALT explants and showed that long-lasting interactions between T cells and antigen-bearing DCs occur in the T cell zone of BALT between 24 and 48 h after transfer (Halle *et al.*, 2009). By 48 h after transfer, the T cells appeared blast-like and had probably begun to proliferate. In contrast, antigen-nonspecific T cells did not exhibit stable interactions with DCs and had much higher motility coefficients (Halle *et al.*, 2009). Thus, antigen presentation and T cell priming can occur directly in BALT in response to antigens derived from the airways.

Memory T cells can also be maintained in BALT. We found that memory T cell responses to influenza were maintained in the lungs of mice lacking conventional secondary lymphoid organs (Moyron-Quiroz *et al.*, 2006). BALT was present in these animals and was maintained for at least 3 months after infection (Moyron-Quiroz *et al.*, 2006). Using *in situ* staining with influenza-specific tetramers, we showed that BALT had antigen-specific CD8 memory T cells and that these cells could react to secondary influenza infections *in situ* (Moyron-Quiroz *et al.*, 2006). Importantly, T cell-dependent memory responses to influenza were intact in these mice and virus clearance was accelerated. Thus, BALT supports T cell priming and expansion as well as memory T cell maintenance.

6.2. Tregs in BALT

Mucosal lymphoid organs, such as the Peyer's patches, tend to elicit T cell responses with a mucosal phenotype that produce IL-5, TGF β , or IL-10 rather than IFN γ or IL-4 (Kiyono and Fukuyama, 2004). In addition, mucosal immune responses to commensal or food antigens often elicit Tregs (Fujihashi *et al.*, 2001). These data suggest that BALT may bias CD4 T cell differentiation toward mucosal type responses or Treg responses. Consistent with this idea, we find that in mice lacking conventional lymph nodes (Moyron-Quiroz *et al.*, 2004) and in mice that have BALT preinduced with protein cage nanoparticles (Wiley *et al.*, 2009), immune

responses to subsequent pulmonary infection with influenza and other viruses lead to substantially reduced morbidity and mortality. Since morbidity in response to viral infection is often due to overexuberant T cell responses, it is possible that BALT primes T cells with an anti-inflammatory or mucosal phenotype. Unfortunately, the presence of Tregs was not examined in these studies.

Interestingly, IgA responses are often observed in BALT (Breel *et al.*, 1988; GeurtsvanKessel *et al.*, 2009; Sminia *et al.*, 1987; Suda *et al.*, 1999), and based on other data, may be linked to Tregs. For example, the transfer of naturally occurring Tregs to recipient mice results in their recruitment to Peyer's patches and their differentiation into T follicular helper (Tfh) cells that promote IgA production (Tsuji *et al.*, 2009). Similarly, other studies report that Tregs facilitate germinal center reactions (Marinova *et al.*, 2007). Thus, the presence of Tregs in BALT may reflect the presence of IgA-helper activity rather than suppressor activity.

Two studies have looked at the role of Tregs suppressor activity in BALT. In the first study, FOXP3⁺ Tregs were preferentially found in areas of BALT, rather than in nonlymphoid areas of the lungs (Heier *et al.*, 2008). However, the presence or frequency of Tregs in BALT did not correlate with either atopy or clinical symptoms of asthma in a cohort of children less than 2 years of age. Another study showed a more direct effect of Tregs on BALT and demonstrated that Tregs normally suppressed the development of BALT (Kocks *et al.*, 2007). This study showed that *Ccr7*^{-/-} mice have very few Tregs in their LNs and also develop iBALT shortly after birth. Furthermore, the development of iBALT is prevented by the adoptive transfer of *Ccr7*^{+/+} Tregs to *Ccr7*^{-/-} recipients (Kocks *et al.*, 2007). Surprisingly, *Ccr7*^{-/-} mice actually have higher numbers of Tregs in peripheral tissues, including the lung, suggesting that it is the activity of Tregs in the LNs rather than in the lungs that prevents iBALT formation (Kocks *et al.*, 2007). Thus, Tregs normally suppress the formation of BALT, probably by suppressing the activity of autoreactive T cells. Given these data, then it should be instructive to examine the numbers and functions of Tregs in the BALT of patients with RA, as these patients often develop extensive areas of BALT.

7. ROLE OF BALT IN RESISTANCE TO INFECTIOUS DISEASE

7.1. Viral infections

The respiratory tract is a common portal of entry for viruses and it is a challenge for the immune system to effectively eliminate viruses and virally infected cells, without causing so much damage and inflammation that pulmonary function is compromised. This balancing act is achieved

through an intricate network of innate and adaptive immune mechanisms as well as through immunoregulatory and anti-inflammatory mechanisms. BALT is likely to be one of the mechanisms that both helps to facilitate viral clearance by initiating immune responses and by reducing inflammatory responses. For example, we found that *Lta*^{-/-} mice, which lack LNs and Peyer's patches, are more sensitive to influenza virus and, although they do generate immune responses, both B and T cell responses are delayed (Lund *et al.*, 2002). At the time, we questioned where immune responses might be initiated in *Lta*^{-/-} mice and concluded that both B and T cell responses were probably generated in the lungs based on the flow cytometric identification of germinal center B cells in the lung (Lund *et al.*, 2002). Since germinal centers are exclusively formed in secondary lymphoid tissues, we suggested that BALT was formed in the lungs of *Lta*^{-/-} mice and initiated immune responses to influenza locally.

We later tested this hypothesis by generating mice that lacked all secondary lymphoid organs, but retained the LT signaling pathway (splenectomized *Lta*^{-/-} mice that had been irradiated and reconstituted with normal bone marrow—SLP mice; Moyron-Quiroz *et al.*, 2004). Upon influenza infection, SLP mice rapidly formed BALT with easily identified germinal centers, separated B and T cell areas, HEVs, and other hallmarks of secondary lymphoid tissues (Moyron-Quiroz *et al.*, 2004). Interestingly, homeostatic chemokines such as CCL21 and CXCL13 were induced in the lungs following influenza infection independently of the LT or TNF signaling pathways (Moyron-Quiroz *et al.*, 2004). Given that LT signaling is essential for homeostatic chemokine expression in conventional lymphoid organs (Ngo *et al.*, 1999), it seems that acute infection leads to an alternative pathway for the expression of these chemokines.

SLP mice also generated B and T cell responses with no delay and cleared virus with the same kinetics as normal mice (Moyron-Quiroz *et al.*, 2004). Surprisingly, the SLP mice exhibited less morbidity in response to influenza infection and could survive doses of virus that killed normal mice (Moyron-Quiroz *et al.*, 2004). Thus, it seems that immune responses initiated in BALT are less inflammatory than systemic immune responses. In fact, a more recent study showed that BALT can be induced by exposure to inert protein cage nanoparticles that are assembled from 24 subunits of the small heat shock protein (sHsp 16.5) of *Methanococcus jannaschii* (Wiley *et al.*, 2009). Once induced, the presence of BALT led to accelerated immunity in response to influenza, mouse-adapted SARS coronavirus, and mouse pneumovirus (Wiley *et al.*, 2009). Reduced morbidity and increased survival were also observed (Wiley *et al.*, 2009). Thus, BALT seems to be generally protective against respiratory viruses.

Once BALT is formed, it seems to persist in the lungs for months following viral clearance (Moyron-Quiroz *et al.*, 2006). Although the areas of BALT became smaller overtime, they can be easily identified for

up to 3 months (Moyron-Quiroz *et al.*, 2006). The long-lived areas of BALT harbored influenza-specific memory CD8 T cells that were maintained locally by homeostatic proliferation. Long-lived BALT was also home to influenza-specific plasma cells that probably contributed to the persistence of influenza-specific neutralizing IgG in the serum and BAL. Importantly, both long-lived antibody production and memory T cells were functional and could neutralize a viral challenge with the same strain of influenza (antibody) or accelerate the clearance of a different serotype of influenza (T cells) (Moyron-Quiroz *et al.*, 2006). Thus, once formed, BALT probably plays an important role in combating successive rounds of the same infection and may even help initiate local immunity to unrelated viruses or pathogens.

The idea that successive viral infections alter subsequent immune responses is not new (Goulding *et al.*, 2007). Some investigators have shown that the specific order of different viral infections leads to different types of pulmonary pathology, including the formation of BALT (Chen *et al.*, 2003). Interestingly, in this study, acute infection with lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV), or influenza led to moderate to severe interstitial pneumonia, but no discernable BALT areas. However, large areas of BALT developed following subsequent infection with vaccinia virus (Chen *et al.*, 2003). Moreover, whereas influenza immune mice developed small areas of BALT even after challenge with LCMV, they developed extensive areas of BALT after challenge with MCMV (Chen *et al.*, 2003). Interestingly, viral titers to secondary infection were decreased in the mice that developed BALT during primary infection (Chen *et al.*, 2003), consistent with our observations that preexisting BALT facilitate the clearance of multiple respiratory viruses (Moyron-Quiroz *et al.*, 2004; Wiley *et al.*, 2009).

Two studies shed light on how viral infection leads to the formation and persistence of BALT (GeurtsvanKessel *et al.*, 2009; Halle *et al.*, 2009). The first study showed that pulmonary influenza virus infection leads to DC recruitment to the lungs and that DCs are required to maintain BALT structures (GeurtsvanKessel *et al.*, 2009). When DCs are depleted using CD11c-diphtheria toxin receptor transgenic (CD11c-DTR) mice, BALT areas are dramatically reduced in size. This study also demonstrated the involvement of BALT areas in local immune responses to influenza as they showed that nucleoprotein-specific plasma cells were formed in BALT and that influenza-specific antibody titers were decreased upon DC depletion (GeurtsvanKessel *et al.*, 2009). The second study used infection with modified vaccinia Ankara virus to trigger BALT formation (Halle *et al.*, 2009). Again, the maintenance of iBALT was dependent on CD11c⁺ cells, as depletion of CD11c⁺ cells from the lungs of infected CD11c-DTR mice lead to the rapid dissolution of iBALT. Functionally, this study showed that pulmonary DCs transferred to the airways of

recipient mice rapidly accumulate in BALT areas where they interact with T cells and present antigen (Halle *et al.*, 2009). Thus, DCs are an important component of BALT, not only for the presentation of antigen to T cells, but also for the maintenance of BALT integrity.

In addition to acute viral infection, some viruses, such as those in the herpesvirus family, become latent in a variety of cells types. For example, murine γ -herpesvirus 68 (γ HV-68) is a natural rodent pathogen that is related to the human pathogens, EBV, and Kaposi's sarcoma virus (KSHV) (Efstathiou *et al.*, 1990). Pulmonary infection of mice with γ HV-68 leads to the development of BALT, which is exacerbated in $Ccr7^{-/-}$ mice (Kocks *et al.*, 2009). Despite the extensive areas of BALT in $Ccr7^{-/-}$ mice, peak viral titers are higher and the clearance of lytic infection from the lungs is delayed (Kocks *et al.*, 2009). These changes are likely due to poor priming of γ HV-68-specific T cells in $Ccr7^{-/-}$ mice. Interestingly, latent virus was detected in BALT areas of both normal and $Ccr7^{-/-}$ mice. This is not too surprising since latent gHV-68 is known to be harbored in B cells (Flano *et al.*, 2000). However, the authors of this study showed that genes from both lytic and latent phase of the virus were expressed in areas of BALT even at late timepoints after infection (Kocks *et al.*, 2009), suggesting that virus was continuously undergoing reactivation in areas of BALT. If so, then it is possible that the reactivity of BALT to environmental antigens is leading to viral reactivation or conversely that viral reactivation is promoting immune activity in BALT and promoting BALT persistence. A similar possibility has been suggested for both EBV and KSHV in ectopic follicles in the CNS of patients with multiple sclerosis (Franciotta *et al.*, 2008).

7.2. Tuberculosis

Mycobacterium tuberculosis (Mtb) remains one of the major health threats throughout the world and accounts for the deaths of over two million people per year (North and Jung, 2004). *M. tuberculosis* infection is typically restricted to the lungs and local immune mechanisms are primarily responsible for *M. tuberculosis* containment (North and Jung, 2004). Once Mtb is established in the lungs, it is difficult to eradicate through immune mechanisms alone and drug treatment is required for sterilizing clearance. Mtb becomes established in granulomas, which, in humans, consist of a central core that is often necrotic, surrounded by macrophages, multinucleate giant cells, and lymphocytes (Actor *et al.*, 1999). CD4 T lymphocytes, particularly Th1 cells are essential for containment (Flynn and Chan, 2001). These cells are required to produce IFN γ and to activate macrophages, which are the main effector cell type that kills Mtb (North and Jung, 2004). Surrounding the granulomas in humans are clusters of lymphocytes, particularly B cells, which form structures similar to BALT

(Ulrichs *et al.*, 2004). These granuloma-associated BALT areas have B cell follicles with occasional Ki-67⁺ germinal center-like foci (Ulrichs *et al.*, 2004). T cell areas are also observed around the outside edge of the follicles. Macrophages and DCs are also observed in the T cells, although not to the extent that they are found in the granuloma itself. Interestingly, granulomas had to reach a minimal size to trigger the formation of BALT areas, but the BALT areas did not expand as the granuloma size increased (Ulrichs *et al.*, 2004).

Similar areas of BALT were found in murine models of *Mtb* infection, in which multiple B cell clusters were also observed surrounding the granuloma (Kahnert *et al.*, 2007). Well-defined B cell areas with FDCs were formed as early as day 42 after pulmonary infection and were maintained to at least day 90 postinfection (Kahnert *et al.*, 2007). *Mtb* infection progressively induced the expression of both CCL19 and CXCL13 (Kahnert *et al.*, 2007; Khader *et al.*, 2009), homeostatic chemokines implicated in the structural integrity of conventional secondary lymphoid organs (Ansel *et al.*, 2000; Forster *et al.*, 1999) as well as ectopic lymphoid tissues like BALT (Moyron-Quiroz *et al.*, 2004; Rangel-Moreno *et al.*, 2005). This study also examined *Ccr7*^{-/-} mice, which are unable to respond to the homeostatic chemokines CCL21 and CCL19 (Forster *et al.*, 1999). Although the mycobacterial load in the lungs of *Ccr7*^{-/-} mice was no different than that in normal mice, the follicular structure of the B cell areas was disrupted and the overall size of the inflammatory lesions in the lung were increased (Kahnert *et al.*, 2007). Moreover, the burden of *Mtb* in the spleen was increased in *Ccr7*^{-/-} mice (Kahnert *et al.*, 2007), suggesting that the follicular structures in the lung may facilitate containment of *Mtb*.

Given the link between B follicular structures surrounding the granuloma and *Mtb* containment, another study examined the role for B cells in response to *Mtb* (Maglione *et al.*, 2007). Although previous studies showed that B cells played a limited role in resistance to *Mtb* in mice (Turner *et al.*, 2001), a newer study demonstrated that the B cell follicles formed around tuberculosis lesions in mice developed extensive germinal centers (Maglione and Chan, 2009), suggesting that the B cells were responding to antigen. In addition, B cell-deficient animals displayed more extensive lung lesions and higher mycobacterial burdens in the lung, and ultimately died earlier than their intact counterparts (Maglione and Chan, 2009), despite apparently normal Th1 responses. Thus, B cells do contribute to immunity to *Mtb*, possibly via their ability to form local immune aggregates in the lung.

Homeostatic chemokines also play a role in the containment of *Mtb* as shown in a recent study that examined pulmonary immune responses to *Mtb* in *Cxcl13*^{-/-} mice and in *plt/plt* mice as well as in *Cxcl13*^{-/-} × *plt/plt* mice (Khader *et al.*, 2009). All three mutant strains of mice exhibited delayed granuloma formation and severely disrupted BALT architecture,

despite clusters of iNOS-expressing activated macrophages in granulomatous lesions (Khader *et al.*, 2009). Interestingly, the *Cxcl13*^{-/-} mice and *plt/plt* mice displayed slightly different immunological abnormalities. For example, the spatial arrangement of granulomas was disrupted in *Cxcl13*^{-/-} mice and T cells tended to accumulate in perivascular areas rather than near infected macrophages. In contrast, the generation of Th1 cells was delayed in *plt/plt* mice (Khader *et al.*, 2009). Importantly, mycobacterial titers were higher in the lungs of both *Cxcl13*^{-/-} mice and in *plt/plt* mice and were higher still in the lungs of *Cxcl13*^{-/-} × *plt/plt* mice, demonstrating that the expression of homeostatic chemokines in the lungs were functionally important for priming local immune responses to Mtb (Khader *et al.*, 2009).

Although the studies above suggested that BALT areas, B cells, and pulmonary expression of homeostatic chemokines were important for immunity to Mtb, it is often difficult to distinguish the effects of these mutations on local events in the lung, from their well-established effects in conventional secondary lymphoid organs. Thus, in order to convincingly demonstrate that BALT areas in the lung can play a role in initiating local immune responses to Mtb, two studies involved infected mice that lacked conventional secondary lymphoid organs and examined pulmonary immunity (Kashino *et al.*, 2010). The first study showed that splenectomized *Lta*^{-/-} mice could generate IFN γ -producing CD4 T cells in the lungs in response to pulmonary challenge (Kashino *et al.*, 2010), although it was not clear whether this response was similar or reduced compared to that in normal mice. In addition, BALT was much more rapidly formed in splenectomized *Lta*^{-/-} mice than in normal mice following Mtb infection (Kashino *et al.*, 2010), similar to what we have observed in response to influenza virus.

In the second, much more detailed study, the authors found that splenectomized *LT β R*^{-/-} mice could generate immune responses to Mtb, but that the CD4 T cell response was slower to get started and was reduced in magnitude in the absence of conventional lymphoid organs (Day *et al.*, 2010). CD8 T cell responses were also slower to get started in mice lacking conventional lymphoid organs. Delayed and reduced immune responses resulted in high mycobacterial titers in the lungs (Day *et al.*, 2010). Granulomatous lesions with associated BALT areas were observed in all groups of mice, although they were slower to appear in the lungs of mice lacking conventional lymphoid organs (Day *et al.*, 2010). Again these data are suggestive that immune responses can be initiated in the BALT areas of the lungs. Importantly, the authors went on to show that adoptively transferred OVA-specific OTII TCR transgenic cells could be primed directly in the lungs of mice lacking conventional lymphoid organs, but only after the granulomatous response had initiated BALT development (Day *et al.*, 2010). Thus, these results clearly

demonstrate the utility of BALT initiating local pulmonary immune responses. The major drawback with both of these studies is that they used mice with defective LT signaling pathways and they did not look at BALT organization (Day *et al.*, 2010; Kashino *et al.*, 2010). Given the importance of LT signaling in the organization of BALT (Moyron-Quiroz *et al.*, 2004), the development of FDCs (Endres *et al.*, 1999) and the expression of PNAd on HEVs (Browning *et al.*, 2005), it is very possible that immunity to Mtb may not be impaired at all in mice lacking conventional lymphoid organs and that BALT may be entirely sufficient to initiate local pulmonary immune responses.

7.3. Other bacterial infections

The presence of BALT in some species, such as mice and humans, is typically associated with infection (Gould and Isaacson, 1993). In other species, such as rats and rabbits, BALT seems to be constitutively present (Pabst and Gehrke, 1990), but is increased in number, size, and architectural complexity in response to microbial stimulation. In pigs, BALT is not present at birth or under germ-free conditions (Pabst and Gehrke, 1990), but is present in about 50% of pigs housed under conventional conditions by 2 months after birth (Pabst and Gehrke, 1990). In another study, 100% of 4-month-old pigs had BALT even prior to infection. However, infection with *Actinobacillus pleuropneumoniae* by aerosol leads to increases in the number of BALT areas and dramatic increases were observed if the pigs had been previously immunized against the bacteria (Delventhal *et al.*, 1992). Moreover, pigs naturally infected with *Mycoplasma hyopneumoniae* have extensive BALT areas that are considerably expanded compared to controls (Sarradell *et al.*, 2003). IgA-producing plasma cells are also associated with BALT expansion in response to infection with *M. hyopneumoniae* (Sarradell *et al.*, 2003). In addition, IL-2, IL-4, and TNF α -producing cells were observed in BALT areas of *M. hyopneumoniae*-infected pigs (Rodriguez *et al.*, 2004), suggesting that ongoing immune responses were occurring in these sites. Similarly, surfactant protein D (SPD) was observed to accumulate in DCs in the BALT areas of pigs responding to *A. pleuropneumoniae* and *Staphylococcus aureus* (Soerensen *et al.*, 2005), suggesting that either DCs acquired SPD as they migrated from the airways to the BALT or that SPD-coated bacteria were transported by M cells to the underlying DCs in BALT. In either case, these data demonstrate that BALT efficiently collects antigens from the airways.

In humans, chronic bacterial stimulation is a common insult that leads to BALT development or hypertrophy, particularly, during development. For example, BALT was commonly observed in the lungs of fetuses with chorioamnionitis (31%) or chorioamnionitis with intrauterine pneumonia (69%) compared to fetal lungs showing no signs of infection (3%) (Gould

and Isaacson, 1993). However, BALT was also observed at a high frequency (84%) in samples from infants who succumbed to sudden infant death syndrome (SIDS), even though only two of the infants with BALT showed evidence of pulmonary infection (Gould and Isaacson, 1993). Similarly, another study showed that BALT was present in 100% (66/66) of fetuses with chorioamnionitis, whereas BALT was present in only 10% (8/75) fetuses who died as a result of other causes (Ersch *et al.*, 2005). Thus, pulmonary exposure to bacteria often leads to the development of BALT, which facilitates local immune responses.

8. ROLE OF BALT IN PULMONARY RESPONSES TO ALLERGENS AND ENVIRONMENTAL ANTIGENS

8.1. Endotoxin exposure

Endotoxin or lipopolysaccharide (LPS) is a component of gram-negative bacteria and is a classic T cell independent B cell antigen and mitogen. LPS engages the TLR4 signaling pathway (Hoshino *et al.*, 1999; Takeuchi *et al.*, 1999) and triggers B cell activation, proliferation, and differentiation to antibody secreting cells (Pike *et al.*, 1987). TLR4 signaling also activated macrophages and DCs, epithelial cells, and even fibroblasts to produce inflammatory cytokines and chemokines (Kawai *et al.*, 2001; Takeda and Akira, 2001). In the case of DCs, LPS induces them to mature into potent antigen-presenting cells that effectively prime T cells. Endotoxin is also common in the environment and exposure to significant levels of LPS is associated with the development or exacerbation of asthma (Becker *et al.*, 2002; Murakami *et al.*, 2007), bronchitis, and COPD (Droemann *et al.*, 2005; Mizutani *et al.*, 2009). Experimentally, pulmonary exposure of rats to endotoxin leads to the expansion of preexisting BALT and increases in pulmonary plasma cells and ultimately the formation of germinal centers (van der Brugge-Gamelkoorn *et al.*, 1985a). Given that germinal centers are dependent on T cells, LPS in this case is probably acting to activate DCs, which migrate from the airways to BALT and prime B and T cells to environmental antigens. Interestingly, LPS does not have to be administered via the airways in order to lead to BALT hyperplasia (Banfi *et al.*, 2009). In this study, the authors intraperitoneally administered LPS to rats as a model of sepsis-related lung injury and observed BALT hyperplasia, signs of acute respiratory distress syndrome (ARDS), diffuse alveolar damage, and emphysema (Banfi *et al.*, 2009).

In mice, which lack BALT prior to pulmonary inflammation, persistent dosing with LPS leads to the development of BALT along major airways with the accumulation of B cells, T cells, and macrophages in the lungs—even in areas that lacked BALT (Vernooy *et al.*, 2002). Macrophages in the

lungs of LPS-exposed mice expressed very high levels of MHC class II, consistent with an activated phenotype. Marked changes in the airways were also observed with an increase in goblet cell numbers and enlargement of airspaces suggesting of emphysema (Vernooij *et al.*, 2002).

Although the above studies experimentally administered LPS as an inflammatory agent, environmental exposures to LPS also induce changes in BALT (Charavaryamath *et al.*, 2005). In one study, rats were exposed to swine barn air (containing very high concentrations of LPS) or ambient air for up to 20 days. Barn air-exposed mice exhibited an increase in airway hyper-reactivity, which correlated with increases in lymphocytes, neutrophils, and eosinophils (Charavaryamath *et al.*, 2005). BALT was hyperplastic and contained reactive germinal centers and plasma cells in rats exposed to barn air (Charavaryamath *et al.*, 2005). Thus, even environmental exposures to LPS, often with additional antigenic or inflammatory components, lead to BALT reactivity and alterations in pulmonary physiology.

8.2. Allergy and asthma

Given the role of pulmonary inflammation in asthma, one might expect that the development of BALT would correlate with asthma or at least with the severity of asthma. However, several studies suggest that BALT formation is not necessarily associated with asthma. For example, one study examined endobronchial biopsies from nonsmoking cross-country skiers and control subjects and found that although a higher frequency of skiers had areas of BALT than controls, the presence of BALT was not associated with either a history of respiratory allergy or asthma (Sue-Chu *et al.*, 1998). In both groups, the areas of BALT consisted of small B cell follicles surrounded by T cells and macrophages or DCs, but did not appear reactive and did not contain germinal centers (Sue-Chu *et al.*, 1998). Similarly, a study of 45 infants from 4 to 23 months of age concluded that BALT structures were present in about half the samples, but their presence did not correlate with lung function, atopy, or (surprisingly) rhinovirus positivity (Heier *et al.*, 2008). Interestingly, in addition to the standard description of B cell follicles surrounded by T cells and DCs, the authors of this study found that FOXP3⁺ Tregs as well as pDCs were preferentially found in areas of BALT (Heier *et al.*, 2008). Thus, there is no obvious connection between the presence of BALT and the development of asthma.

However, another study suggests that while the presence of BALT may not correlate with asthma, the reactivity of BALT increased in asthma patients (Elliot *et al.*, 2004). In this study, a postmortem comparison of lung tissue from nonsmokers, smokers, nonfatal asthma, and fatal asthma revealed minimal differences in the frequency of patients in each group with BALT, the number of BALT areas, or the location of BALT areas (Elliot *et al.*, 2004). However, the total area of BALT observed in each

group was significantly different, with large BALT observed in both asthma groups compared to either nonsmokers or smokers (Elliot *et al.*, 2004). In addition, there is evidence that exposure to particular allergens, such as *Aspergillus fumigatus*, can lead to pulmonary allergies reminiscent of asthma (Slavin *et al.*, 1992). For example, extensive areas of BALT were observed in a patient with allergic bronchopulmonary aspergillosis, in which germinal centers were prevalent and in every case stained with IgE (Slavin *et al.*, 1992). These data suggested that IgE was being generated locally, presumably in response to *Aspergillus*. Thus, BALT has potential to participate in allergic responses.

Similar results have been observed using experimental systems. For example, using the mouse model of allergic airway disease, in which mice are initially sensitized to OVA via the peritoneal cavity and then repeatedly challenged by intratracheal instillation of OVA, one study demonstrated that BALT was formed in response to OVA and that OVA-specific IgM-, IgG1-, and IgA-producing plasma cells could be found in the lung (Chvatchko *et al.*, 1996). Moreover, germinal centers containing IgE-expressing B cells were observed and IgE was produced locally in the lung (Chvatchko *et al.*, 1996). Thus, pulmonary antigen exposure in the context of a Th2 response can lead to local IgE responses in the BALT areas of the lung.

Eosinophilia is another hallmark of asthma. Eosinophils are recruited by Th2 responses and IL-5, one of the canonical cytokines made by Th2 cells (Maggi, 1998), is a growth and survival factor for eosinophils (Coffman *et al.*, 1989; Sher *et al.*, 1990). To determine a role for eosinophils in asthma, one study generated transgenic mice that expressed IL-5 in lung epithelial cells (Lee *et al.*, 1997). The transgenic mice exhibited pulmonary pathology that was reminiscent of asthma, including a dramatic eosinophilia, goblet cell hyperplasia, and epithelial cell hypertrophy (Lee *et al.*, 1997). However, the mice also developed extensive areas of BALT. Interestingly, eosinophils were clustered around the B cell follicles, in the same location where T cells and DCs are normally located (Lee *et al.*, 1997). These mice also exhibited airway hyper-responsiveness, independently of any antigenic challenge, suggesting that the pathologic changes induced by IL-5 overexpression directly triggered this asthma-like condition (Lee *et al.*, 1997).

The transgenic overexpression of IL-6 in the lung epithelium also induces the formation of BALT (Goya *et al.*, 2003), but without the accumulation of eosinophils. BALT areas in IL-6 transgenic mice consisted primarily of primary B cell follicles without germinal centers. T cells, particularly CD4 T cells, were present surrounding and between B cell follicles and plasma cells were also observed (Goya *et al.*, 2003). Although pulmonary function was not measured, there were no visible changes in the overall lung architecture (other than the accumulation of BALT) that might be suggestive of an asthma phenotype. Thus, the development of BALT *per se* does not directly lead to asthma or even lung disease, but the

presence of BALT may participate in ongoing Th2 responses and exacerbate preexisting asthma.

An interesting question in asthma and allergic airway disease is where does Th2 priming occur? It seems likely that asthmatic individuals are exposed to allergens via the airways, but it is unclear whether T cells can be primed *in situ* in the lung or whether antigen must traffic to draining LNs. Two studies shed light on this issue in the context of Th2 responses and show that mice lacking draining LNs are fully competent to prime Th2 cells and promote allergic airways disease (Constant *et al.*, 2002; Gajewska *et al.*, 2001). Both studies used *Lta*^{-/-} mice, which lack LNs and Peyer's patches and, after splenectomy, also lack the spleen. In the first study, the authors used an adenovirus to overexpress GM-CSF in the lungs prior to repeated aerosol OVA exposure (Gajewska *et al.*, 2001). They observed robust Th2 responses in both normal and *Lta*^{-/-} mice, with Th2 cytokine expression and the accumulation of eosinophils. However, the overall inflammatory response in the lungs of *Lta*^{-/-} mice was dramatically higher than in normal mice (Gajewska *et al.*, 2001), consistent with our observation using influenza infected *Lta*^{-/-} mice (Lund *et al.*, 2002). Interestingly, splenectomy of the *Lta*^{-/-} mice completely abrogated the Th2-induced allergic response, suggesting that conventional secondary lymphoid tissues are essential for priming Th2 responses to pulmonary antigens (Gajewska *et al.*, 2001). The authors of this study did not specifically examine BALT, but perivascular and peribronchiolar lymphoid aggregates could be observed in both normal and *Lta*^{-/-} mice (Gajewska *et al.*, 2001). However, in our experience, the lack of LT signaling in *Lta*^{-/-} mice would have prevented BALT organization and may dramatically impair the ability of BALT to prime local Th2 responses (Lund *et al.*, 2002; Moyron-Quiroz *et al.*, 2004).

In the second study (Constant *et al.*, 2002), the authors intranasally administered *L. major* parasites to normal and splenectomized *Lta*^{-/-} mice and then intranasally challenged the primed mice a week later with another dose of *L. major* (Constant *et al.*, 2002). Robust Th2 responses were observed in both groups, with the production of IL-5 and IL-13 and the accumulation of eosinophils (Constant *et al.*, 2002). The authors did not specifically examine the lungs for BALT, but they did note that large numbers of antigen-bearing DCs remained in the lung after challenge leading to their conclusion that Th2 responses could be primed directly in the lung (Constant *et al.*, 2002). The difference between the conclusions of these studies is striking and is likely due to the differences in the antigens used to prime Th2 responses. Certainly, one would expect that challenge with live *L. major* parasites to be a stronger stimulus than aerosol OVA. Nevertheless, we can conclude that spleen and LNs are not required for some pulmonary Th2 responses, but certainly facilitate normal responses, particularly, in the systemic absence of LT.

8.3. Hypersensitivity pneumonitis

Hypersensitivity pneumonitis is an inflammatory disease of the alveoli caused by hypersensitivity to inhaled organic antigens (Mohr, 2004). Exposure is commonly occupational and farmers, in particular, are often exposed to molds and fungi in barns leading to the term “Farmer’s Lung” (Seal *et al.*, 1968). The most effective treatment is avoidance of exposure, which can cause acute symptoms. Unlike asthma, hypersensitivity pneumonitis targets alveoli rather than airways.

Given that hypersensitivity pneumonitis is driven by chronic pulmonary exposure to antigen, it is not surprising that patients may develop BALT. In fact, we observed well-developed BALT areas with extensive germinal centers and FDC networks in lung biopsies of some hypersensitivity pneumonitis patients (Fig. 7.3; Rangel-Moreno *et al.*, 2006), although other patients showed only smaller B cell clusters that lacked FDCs and germinal centers. Another study showed similar results with three of five patients showing well-defined BALT with FDCs and germinal centers (Suda *et al.*, 1999). This study also showed that some areas of BALT were similar to classic mucosal type tissues with a dome epithelium containing subepithelial DCs and peripheral T cell areas (Suda *et al.*, 1999). Experimentally, hypersensitivity pneumonitis can be mimicked by first immunizing animals with protein antigens and then chronically exposing them to the same antigen via the pulmonary route. For example, chronic pulmonary exposure of rats leads to BALT hypertrophy and histologic parameters of hypersensitivity pneumonitis (de Dios Escobar *et al.*, 1994).

Given the antigen-driven development and exacerbation of hypersensitivity pneumonitis, it seems reasonable to conclude that pulmonary immune responses to the sensitizing antigen will be pathologic and not beneficial in this disease. Thus, one would like to conclude that the development or hyperplasia of BALT would contribute to pathology. However, despite the correlation between chronic hypersensitivity pneumonitis and BALT development, there is no direct causal link in either direction that establishes any functional relationship.

9. ROLE OF BALT IN RESPONSE TO PARTICULATES AND CIGARETTE SMOKE

9.1. Particulate exposure and BALT formation

The lung is exposed to a wide variety of particulates, many of which are intrinsically inflammatory because they cannot be metabolized and persist in phagocytes or because their components engage particular receptors that trigger an inflammatory response. For example, silicosis is a chronic diffuse

parenchymal lung disease that results from prolonged exposure to inhaled crystalline silica particles. Pulmonary silica exposure results in nodules of mononuclear cell infiltration at site of silica accumulation that ultimately leads to pulmonary fibrosis. Alveolar macrophages are the major cells that respond initially by producing inflammatory mediators that recruit additional cells such as cytokine-producing lymphocytes. IFN γ -producing lymphocytes are important in the pathogenesis of silicosis and intriguingly, the majority of IFN γ -producing lymphocytes in the lung are found in regions of BALT that contain silica-laden macrophages, rather than in areas of diffuse inflammation (Davis *et al.*, 1999). Interestingly, the pulmonary exposure of rats to silica prompted silica-laden alveolar macrophages to transmigrate across the epithelium and accumulate in the BALT (Lee and Kelly, 1993), similar to the kinetic observation of virus-activated DCs in the airways transmigration across the epithelium into BALT (Halle *et al.*, 2009). Additional silica-laden macrophages accumulated in the draining LN (Lee and Kelly, 1993). Silicosis also leads to persistent increases in TNF α and IL1 β , both of which are expressed in BALT areas. However, any link between BALT formation or activity and the pathology of silicosis remains to be identified.

Another particulate to which humans are commonly exposed is DEPs. DEPs are a major component of air pollution in urban areas and exposure to these particles is thought to exacerbate asthma (Matsumoto *et al.*, 2006) and COPD (Anderson *et al.*, 1997) and lead to decreased pulmonary function. Interestingly, pulmonary exposure to high levels of DEPs correlated with the formation of BALT with central accumulations of B cells and peripheral accumulations of T cells (Hiramatsu *et al.*, 2003). Cells filled with black DEPs could be clearly observed both in interstitial areas without organized lymphoid tissue as well as in areas of BALT (Hiramatsu *et al.*, 2003), although areas of BALT appeared to accumulate a higher frequency of DEP-laden cells. These cells were claimed to be alveolar macrophages, which is certainly possible. However, they could easily be DEP-containing DCs that migrate into BALT areas as well. In either case, these cells are likely to initiate inflammatory responses and alter local immune responses to other pulmonary antigens and pathogens (Anderson *et al.*, 1997; Matsumoto *et al.*, 2006). Whether BALT has any effect on the pulmonary inflammatory response to DEP remains to be determined.

The exposure to cigarette smoke is another major hazard to pulmonary health and function and is by far the most important risk factor for developing COPD (Pauwels and Rabe, 2004). COPD is the fifth leading cause of death in the world and, due to its chronic and debilitating nature, represents a major burden on the global economy (Pauwels and Rabe, 2004). The global initiative for chronic obstructive lung disease (GOLD) defines COPD as "a disease state characterized by airflow limitation that is not fully reversible, and that is usually both progressive and associated

with abnormal inflammatory response of the lungs to noxious particles or gases” (Pauwels *et al.*, 2001, 2004). Given the association between COPD and cigarette smoke exposure, several studies have examined how smoking might affect the formation of BALT. For example, an early study showed that smokers with significant airway obstruction had increased numbers of B cell areas (BALT) relative to smokers without airway obstruction (Bosken *et al.*, 1992), whereas other studies showed that similar frequencies of smoking and nonsmoking patients had at least some areas of BALT, but that the frequency of BALT within the lungs of smokers was higher than in nonsmokers and the frequency of BALT areas with reactive germinal centers was also higher in smokers than in nonsmokers (Elliot *et al.*, 2004). A study in rats (which typically have preexisting BALT) showed that exposure to cigarette smoke increased the BALT area by nearly fourfold and increased the length of the epithelium that was in contact with BALT by nearly threefold (Escobar Castellon *et al.*, 1992). The measurements of BALT area and length of epithelial contact with BALT were measured in two-dimensional sections. Thus, the actual differences in BALT volume and epithelial surface area in contact with BALT are likely to be of much larger magnitude.

Attention to the possible role of BALT in the lungs of smokers was increased significantly by a study showing that the development of BALT was associated with severe COPD, particularly, patients classified with GOLD stages 3 and 4 disease (Hogg *et al.*, 2004). BALT in these patients consisted of dense B cell follicles, often with germinal centers (Hogg *et al.*, 2004). CD4 T cells were found within and surrounding the follicles and also scattered around the airways in areas that lacked BALT (Hogg *et al.*, 2004). Importantly, changes in the forced expiratory volume in one second (FEV₁) correlated very strongly with the percentage of airways with lymphoid follicles, the percentage of airways with B cells, and the accumulated volume of B cells in the lungs (Hogg *et al.*, 2004), suggesting the possibility that BALT was playing an important role in COPD or that patients with advanced COPD were being colonized by opportunistic pathogens, which were leading to the development of BALT due to immune stimulation.

Given the association of B cells and BALT with advanced COPD, several studies examined how B cells might be involved in disease. One study showed that in surgical sections of lung from COPD patients undergoing either lung transplant or volume reduction surgery, B cell follicles were present and surrounded by T cell areas (van der Strate *et al.*, 2006). Interestingly, this study also showed that cells filled with black pigment—presumed to be from smoke exposure—were clustered around the edge of the T cell area (van der Strate *et al.*, 2006). These cells likely correspond to macrophages or DCs that have phagocytosed cigarette smoke particles in the airways and then migrated to the BALT areas. Importantly, the

B cells in the follicles predominantly expressed CD27, which is a memory marker on B cells and some follicles showed evidence of proliferating germinal center cells (van der Strate *et al.*, 2006). This study also showed that the B cells were likely responding to antigen as the authors found clonal B cell populations in seven of eight patients and also found evidence of ongoing somatic hypermutation (van der Strate *et al.*, 2006). Thus, the authors conclude that the B cells in BALT areas are clearly responding to antigens, even though a direct link with ongoing COPD could not be established. In contrast, another study compared BALB/c and scid mice and showed that although cigarette smoke exposure induced BALT areas in normal mice, scid mice completely lacked identifiable BALT (D'Hulst *et al.*, 2005). Nevertheless, both groups of mice developed pulmonary emphysema to a similar extent and expressed inflammatory cytokines to the same degree (D'Hulst *et al.*, 2005). Thus, B cell reactivity and visible BALT formation do not appear to be necessary for the development of lung disease following cigarette exposure.

Given the well-established role of homeostatic chemokines and LT in the development of conventional lymphoid tissues as well as in BALT (Khader *et al.*, 2009; Randall *et al.*, 2008; Rangel-Moreno *et al.*, 2007), it made sense to test the role of these molecules in mouse models of cigarette smoke-induced COPD. For example, one study found that 4 weeks of cigarette smoke exposure induced the expression of both CXCL13 and CCL19 in the lung tissue, but that this increase was abrogated in *Lta*^{-/-} mice (Demoor *et al.*, 2009a). This is consistent with data showing that CXCL13 and CCL19 are controlled by LT signaling in conventional lymphoid organs like the spleen (Ngo *et al.*, 1999). However, it is inconsistent with data showing that these chemokines are induced to similar levels in mice upon acute infection with influenza virus (Moyron-Quiroz *et al.*, 2004). However, despite the dependence of homeostatic chemokines on LT signaling in this model, more B cells accumulated in the lungs of *Lta*^{-/-} mice and the formation of BALT-like areas were even more extensive, despite the lack of apparent organization. These data are consistent with previous studies showing that B cells accumulate normally in the lungs of *Lta*^{-/-} and *Cxcl13*^{-/-} mice following influenza infection (Rangel-Moreno *et al.*, 2007), but fail to form well-defined follicles. In addition, BALT-like areas were also observed in air-exposed *Lta*^{-/-} mice, but not in air-exposed normal mice (Demoor *et al.*, 2009a). These data are consistent with previous studies showing pulmonary inflammation in unmanipulated *Ltbr*^{-/-} mice (Futterer *et al.*, 1998). The BALT-like areas in *Ltbr*^{-/-} mice were not organized into B cell follicles, did not have FDCs or separated B and T cell areas, and lacked detectable HEVs (Futterer *et al.*, 1998), similar to the ones observed in cigarette smoke-exposed *Lta*^{-/-} mice (Demoor *et al.*, 2009a). IgA production in the BALF of smoke-exposed *Lta*^{-/-} mice was also dramatically decreased compared to

controls (Demoor *et al.*, 2009a) again consistent with the previous data showing that IgA responses are impaired in the absence of LT β R signaling (Kang *et al.*, 2002). Thus, although LT signaling controls homeostatic chemokine expression in the lung in response to cigarette smoke, the lack of LT signaling does not impede the accumulation of lymphocytes in BALT-like clusters in the lung. Unfortunately, there was no evaluation of pulmonary function in these animals so the impact of impaired LT signaling on COPD was not determined (Demoor *et al.*, 2009a).

Similar results were observed using *Ccr7*^{-/-} mice (Demoor *et al.*, 2009b), which lack the receptor for CCL19 and Ccl21 (Forster *et al.*, 1999). In these experiments, the authors found that BALT-like areas were already present in untreated *Ccr7*^{-/-} mice and increased in size upon smoke exposure, whereas untreated normal animals completely lacked BALT and developed smaller areas upon smoke exposure (Demoor *et al.*, 2009b). Interestingly, DC accumulation was impaired in the lungs of *Ccr7*^{-/-} mice 4 weeks after cigarette smoke exposure, but not at later times. However, B and T cell accumulation in the lungs was normal in the absence of CCR7 (Demoor *et al.*, 2009b). Interestingly, the development of cigarette smoke-induced emphysema, as measured by airspace enlargement, was seemingly abrogated in the *Ccr7*^{-/-} mice (Demoor *et al.*, 2009b). However, the airspaces in control *Ccr7*^{-/-} mice were already the same size as those in the cigarette-smoke-exposed normal mice, even though they did not enlarge as much after smoke exposure (Demoor *et al.*, 2009b). Thus, the starting point was already higher in the *Ccr7*^{-/-} mice, perhaps because of the preexisting accumulations of BALT-like areas or perhaps because of other underlying immune defects in *Ccr7*^{-/-} mice, such as autoimmunity (Kocks *et al.*, 2007).

In contrast to the results with *Ccr7*^{-/-} mice and *Lta*^{-/-} mice, cigarette-smoke-exposed *CCR6*^{-/-} mice developed slightly fewer and smaller lymphoid aggregates in their lungs (Bracke *et al.*, 2006). Given that CCR6 is the receptor for CCL20 (Rossi and Zlotnik, 2000), which is a chemokine typically expressed in mucosal epithelium such as the dome epithelium of Peyer's patches (Iwasaki and Kelsall, 2000) or NALT (Rangel-Moreno *et al.*, 2005), it makes sense that CCR6 could be involved in the recruitment or placement of DCs or lymphocytes in areas of BALT. In fact, CCL20 expression is increased on cigarette smoke exposure and the recruitment of DCs and T cells to the lungs of cigarette-smoke-exposed *CCR6*^{-/-} mice is reduced (Bracke *et al.*, 2006). Importantly, the reductions in lymphocytes and DC recruitment to the lungs correlated with less severe COPD in *CCR6*^{-/-} mice, suggesting a possible link between the CCR6-mediated cellular recruitment and COPD (Bracke *et al.*, 2006).

In addition to the role of cigarette smoke-induced homeostatic chemokines and their receptors in the formation of BALT, the role of some inflammatory chemokines has also been investigated. For example,

cigarette smoke exposure of *Ccr5*^{-/-} mice led to decreases in the recruitment of neutrophils, DCs, and T cells to the lung as well as reduced expression of TNF α and IL-2 (Bracke *et al.*, 2007). In addition, there were fewer lymphoid aggregates observed in the lungs of smoke-exposed *Ccr5*^{-/-} mice and those observed were smaller and less organized than those in smoke-exposed control mice (Bracke *et al.*, 2007). Importantly, *Ccr5*^{-/-} mice were partially protected from cigarette smoke-induced emphysema (Bracke *et al.*, 2007). Thus, as with the previous studies, it is difficult to firmly make a connection between the extent of BALT formation and the development of COPD. However, in this case, poor quality BALT correlated with reductions in some aspects of COPD-related pathology.

pDCs were the focus of another study of the role of lymphoid follicles and COPD (Van Pottelberge *et al.*, in press). In this paper, the authors found that pDCs were located in the T cell areas of BALT in COPD patients (Van Pottelberge *et al.*, in press), similar to where conventional DCs have been observed in previous studies (Woodland and Randall, 2004). Interestingly, the number of pDCs goes up in the airway walls and in BALT in patients with GOLD stages I and II disease, but not in GOLD stages III and IV disease (Van Pottelberge *et al.*, in press). In addition, they found that cigarette smoke extract impaired the maturation of pDCs such that they produced less IFN α (Van Pottelberge *et al.*, in press), suggesting that patients with the most severe disease were less capable of dealing with viral infections. However, pDC from patients with COPD produced higher amounts of TNF α and IL-8 compared to pDCs from healthy controls (Van Pottelberge *et al.*, in press), suggesting that cigarette smoke exposure alters the type of inflammatory response triggered by viruses, which may lead to viral exacerbations of COPD (De Serres *et al.*, 2009).

As the studies above clearly demonstrate, exposure to cigarette smoke clearly promotes the formation of BALT in experimental model systems and BALT is associated with severe COPD in human patients. However, the causal link between BALT and COPD remains nebulous. For example, BALT could be formed and COPD induced in response to cigarette smoke, but be independent of one another as suggested by the studies in scid mice (D'Hulst *et al.*, 2005). Alternatively, cigarette smoke could be inducing the formation of lymphoid tissues that promote autoreactive or locally inflammatory responses that contribute to the pathogenesis of COPD (van der Strate *et al.*, 2006). In another scenario, BALT is formed as a consequence of exposure to opportunistic pathogens in patients with severe COPD. In this case, BALT would be presumed to be beneficial due to its immune-promoting function that would facilitate pathogen clearance or containment (Khader *et al.*, 2009; Moyron-Quiroz *et al.*, 2004). Thus, it is not clear whether BALT is beneficial, harmful, or neutral to the progression of COPD (Brusselle *et al.*, 2009).

10. CONTRIBUTION OF BAL T TO AUTOIMMUNITY AND PULMONARY FIBROSIS

10.1. RA and Sjogren's syndrome

RA and Sjogren's syndrome (SS) are two autoimmune diseases that are associated with the development of ectopic lymphoid follicles in affected target tissues. For example, RA patients often exhibit ectopic lymphoid follicles in their joints (Takemura *et al.*, 2001), whereas SS patients often have ectopic follicles in their salivary and lacrimal glands (Gatumu *et al.*, 2009). These follicles contain separated B and T cell areas, germinal centers, FDCs, and HEVs and are thought to be involved in autoimmunity by generating high-affinity autoreactive B cells and by maintaining autoreactive effector T cells. In addition, there is a subset of patients with RA and SS that develop pulmonary disease. Some of these patients develop pulmonary disease prior to other manifestations of RA or SS and are often diagnosed in a pulmonary clinic (Rangel-Moreno *et al.*, 2006). This subset of patients often has much more severe disease with a worse long-term prognosis (Turesson *et al.*, 2002, 2003). We examined lung biopsies from several of these patients and consistently observed BAL T. The BAL T areas varied from rather small isolated lymphoid follicles to large, highly organized clusters of B cell follicles (Rangel-Moreno *et al.*, 2006). Germinal centers were almost always present and often occupied almost the entire follicle and were supported by extensive FDC networks. CXCL13 was expressed in the B cell follicles as expected and CCL21 was observed on both lymphatics and HEVs (Rangel-Moreno *et al.*, 2006). Lymphatics typically surrounded the B cell follicles. Other studies showed similar results and confirmed that both B cell follicles (Atkins *et al.*, 2006) and CD4 T cell infiltrations of lymphoid follicles (Turesson *et al.*, 2005) were much higher in patients with RA than in patients with interstitial pneumonia not related to RA. Additional studies showed that BAL T areas in RA patients looked very much like mucosal lymphoid tissues, with prominent dome epithelium and IgA⁺ B cells (Hayakawa *et al.*, 1998; Sato *et al.*, 1996). However, the idea that BAL T is truly a mucosal lymphoid tissue is at odds with our finding that most of the BAL T areas in RA patients were not associated with a dome epithelium and in some cases, seemed to be encapsulated by lymphatic endothelium (Rangel-Moreno *et al.*, 2006).

The B cell follicles of RA and SS patients seemed to be participating in local disease as we found plasma cells producing antibodies specific to citrullinated proteins in areas surrounding BAL T. Thus, BAL T areas in RA and SS disease seem to contribute to pathology (Rangel-Moreno *et al.*, 2006). Interestingly, there is a strong association between RA and smoking (Klareskog *et al.*, 2004), which may be explained by the induction of the citrullination process by cigarette smoke (Klareskog *et al.*, 2006).

Moreover, citrullination has been observed in lymphoid areas of the lungs of RA patients (Bongartz *et al.*, 2007), suggesting that autoantigens are located inside BALT areas. Thus, in susceptible individuals, cigarette smoke may actively promote the development of RA, particularly with pulmonary involvement.

A final complication concerning any possible role of BALT in rheumatoid lung disease is that one of the drugs commonly used to treat RA, methotrexate, can itself induce pulmonary inflammation (Kelly and Saravanan, 2008). In fact, methotrexate pneumonitis occurs with an incidence of 1–7%, depending on the study (Saravanan and Kelly, 2004). Moreover, patients with preexisting lung disease were more susceptible to developing methotrexate pneumonitis (Saravanan and Kelly, 2004). Thus, the combination of rheumatoid lung disease with reactivity to methotrexate can exacerbate pulmonary pathology.

10.2. Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive lung disease that involves excessive collagen deposition or fibrosis in the pulmonary interstitium (Selman *et al.*, 2001). IPF is characterized by a diagnosis of usual interstitial pneumonia (UIP), which includes interstitial scarring, interstitial fibrosis in a patchwork pattern, honeycomb changes, and fibroblast foci with minimal associated inflammation (American Thoracic Society, 2002; Selman *et al.*, 2004). Given that there is minimal inflammation associated with this disease, it is not surprising that it is not often associated with the formation or hyperplasia of BALT. In our study of samples from 10 IPF patients, we found small lymphoid aggregates that contained B cells and occasionally T cells, but never contained FDCs, the hallmark feature of B cell follicles (Rangel-Moreno *et al.*, 2006). However, other studies have suggested a more extensive formation of BALT, including the presence of B cell follicles (albeit without examining FDCs), T cell zones, HEVs, DC-LAMP⁺ DCs, and the expression of a variety of chemokines (Marchal-Somme *et al.*, 2006). Studies from this same group showed that DC-Lamp⁺ DCs accumulate in lymphoid areas of fibrotic lungs and coexpress CD83 and CD86 (Marchal-Somme *et al.*, 2007). Given the association of DCs with the formation or at least the maintenance of BALT as discussed in previous sections (GeurtsvanKessel *et al.*, 2009; Halle *et al.*, 2009), and the documented presence of DCs in the T cell zones of BALT (Moyron-Quiroz *et al.*, 2004; Woodland and Randall, 2004), it is not too surprising that DCs are present in BALT areas. Nevertheless, based on the limited data in the literature, the incidence and involvement of BALT in IPF seem minimal, despite the dramatic fibrotic changes in the lungs of these patients.

11. ROLE OF BALT IN PULMONARY MALIGNANCY

11.1. Lung cancer

Given that the formation of BALT is clearly associated with local immune responses to pulmonary pathogens and antigens, one might predict that the development of BALT adjacent to pulmonary malignancies would be beneficial for antitumor immune responses. In fact, such effects have been observed using a pulmonary metastasis model of melanoma in which an LT:antitumor antibody fusion protein was used to simultaneously engage an antigen on the surface of the tumor and the TNFR1 (Gillies *et al.*, 1991). In this situation, LT is thought to be directly cytotoxic to tumor cells and to activate local immune cells and stromal cells, which promoted the accumulation of lymphocytes surrounding the tumors (Reisfeld *et al.*, 1996). Antitumor immunity appeared to require asialo-GM1⁺ cells (probably NK cells) as well as B cells, but not T cells (Reisfeld *et al.*, 1996). Subsequent studies by this same group showed that the LT:antitumor antibody fusion protein induced tertiary lymphoid tissue neogenesis—with accumulations of both CD4 and CD8 T cells, B cells, and HEVs that expressed PNAd (Schrama *et al.*, 2001). The formation of lymphoid tissues was accompanied by changes in the T cell repertoire in local sites, suggesting that clonal expansions of tumor-specific T cells were occurring locally—a finding that was confirmed using ELISPOT and cytotoxicity assays (Schrama *et al.*, 2001). This same group also demonstrated that targeting LT α to the tumor site was effective in *Lta*^{-/-} mice, which completely lack lymph nodes. Thus, they reasoned that the immune response responsible for tumor eradication was generated locally in tertiary lymphoid tissues (Schrama *et al.*, 2008). Together, these results suggest that the local induction of BALT surrounding pulmonary metastases could be beneficial for the induction of antitumor immunity and tumor regression, although it was difficult to distinguish the direct effects of LT signaling on the tumor from the indirect effects of BALT induction on antitumor immunity.

Other studies have also successfully targeted TNF family members, such as LIGHT, directly to tumors and shown an increase in immune cell infiltration to the tumor site and tumor regression (Yu *et al.*, 2004, 2007). Again, the beneficial effects of LIGHT signaling through either LT β R or HVEM could be mediated directly on tumor cells or via environmental influences. In fact, more recent studies have shown that the formation of lymphoid-like stromal elements surrounding tumors may be detrimental to antitumor immune and may in fact, lead to tolerance (Shields *et al.*, 2010). In these studies, the expression of high levels of CCL21 by tumor cells promoted the formation of a lymph node T zone-like environment surrounding the tumors. The formation of this structure correlated with

the recruitment of LT_i-like cells (Shields *et al.*, 2010). Importantly, the lymphoid-like structure surrounding the tumors recruited both DCs and T cells via CCR7-dependent mechanisms and led to the differentiation or recruitment of Tregs that appeared to contribute to immunological tolerance and accelerated tumor outgrowth (Shields *et al.*, 2010). Thus, the development of a lymphoid environment surrounding tumors may lead to antitumor immunity or immune tolerance, depending on some currently unknown factors.

Despite the apparent contradictions in the literature, the ability of BALT to promote immunity to lung tumors is an intriguing idea. Importantly, BALT has been observed to be generated spontaneously in apparent response to the development of pulmonary tumors or the metastasis of other tumor to the lung. For example, we previously observed the presence of BALT in some samples of human pulmonary carcinoma (Rangel-Moreno *et al.*, 2006). However, of the five patient samples that we observed, only one had evidence of BALT despite extensive tumor infiltration of the lung tissue (Rangel-Moreno *et al.*, 2006). These data suggest that the development of pulmonary carcinoma does not always lead to BALT formation and, conversely, that BALT is not a prerequisite for the development of carcinoma (Rangel-Moreno *et al.*, 2006). Intriguingly, a much more comprehensive study showed that the formation of tertiary lymphoid structures (BALT) surrounding nonsmall-cell lung cancer lesions was associated with a better long-term prognosis over 50 months (Dieu-Nosjean *et al.*, 2008). The areas of BALT in these samples were well-formed and consisted of DC-Lamp⁺ DCs, separated B and T cell areas, FDC-containing B cell follicles and, in some cases, well-defined germinal centers (Dieu-Nosjean *et al.*, 2008). However, when these features were correlated with prognostic value, the density of DC-Lamp DCs in the BALT areas was the only feature to correlate significantly with long-term survival. However, the density of DCs did correlate with the infiltration of B cells and T cells and the presence of T-bet⁺ cells, suggesting that the presence of BALT promotes local immune responses to lung tumors. However, there are a number of questions that remain. Was BALT induced independently of tumorigenesis and did it develop prior to tumor formation? If so, then BALT may be responding to some other inflammation-inducing stimuli that helps initiate both BALT and antitumor immunity. Alternatively, was BALT generated in response to the tumor itself and is there something different about the tumors that promoted BALT formation—perhaps high expression of inflammatory cytokines or tumor antigens? These questions need to be answered in order to understand the potential role of BALT in antitumor immunity.

Although the studies above suggest that BALT may contribute to the initiation of antitumor immune responses and promote tumor regression, other studies suggest that the presence of BALT may actually contribute

to the formation of lung tumors or promote the metastasis of tumors to the lung. For example, prostate tumor cells were observed to metastasize to BALT regions in a rat model of pulmonary metastasis, in which mono-dispersed tumor cells were injected intravenously (Geldof and Rao, 1989). Importantly, BALT existed in control rats prior to tumor inoculation and tumor cells rapidly accumulated in BALT areas adjacent to the major artery in BALT (Geldof and Rao, 1989). Thus, these results suggest that alterations in homing molecules and specialized vasculature in BALT areas may recruit tumor cells to the lung and promote metastasis. These data are consistent with other results showing that inflammatory stimuli, such as cigarette smoke, can increase the efficiency of pulmonary metastases (Lu *et al.*, 2007). Although this study showed that the primary effect of cigarette smoke was to impair NK cell function (Lu *et al.*, 2007), other studies have shown that BALT formation is associated with cigarette smoking (Bosken *et al.*, 1992; Escolar Castellon *et al.*, 1992) and is present in patients with severe COPD (Hogg *et al.*, 2004), a pulmonary disease often associated with cigarette smoking.

As mentioned in previous sections, the formation of BALT is often associated with pulmonary inflammation and exposure to a wide variety of inflammatory stimuli. Thus, it is not particularly surprising that the pulmonary administration of Benzo[a]pyrene (B[a]P) leads to the development of BALT hyperplasia in Wistar rats (Silva *et al.*, 2007). However, B[a]P is also associated with tumorigenesis and can induce pulmonary adenocarcinoma (Silva *et al.*, 2007). Thus, inflammatory responses in the lung can simultaneously promote BALT and neoplasia. In fact, given the links between chronic inflammation and the development of cancer (Marx, 2004), it is possible that BALT formation precedes tumorigenesis in this case. Alternatively, BALT formation and adenocarcinoma formation could be independently linked to B[a]P exposure and unrelated to each other. Nevertheless, the link between chronic inflammation and tumorigenesis is intriguing and is discussed further in Section 11.2.

11.2. BALT lymphoma

Pulmonary non-Hodgkin lymphomas are identified as monoclonal lymphoid malignancies that are initially confined to the lungs. Isaacson was the first to identify these low-grade pulmonary lymphomas as a subset of mucosa-associated lymphoid tissue (MALT) lymphomas based on their apparent origins from centrocyte-like or marginal zone-like B cell precursors (Isaacson and Wright, 1983). Given their presence in the lung, they are referred to as BALT lymphomas. These malignancies present diagnostic problems for pathologists due to their heterogeneous appearance and similarity to reactive lymphoid tissue in the lung, including conventional BALT (Koss, 1995, 2004). Most BALT lymphomas are of low

histologic grade and have a relatively benign clinical behavior, typically leading to good clinical prognosis (Stefanovic *et al.*, 2008).

Morphologically, the BALT lymphomas often form dense lymphoid clusters of B cells (Fiche *et al.*, 1995; Koss, 2004; Kurtin *et al.*, 2001). These are often organized into structures reminiscent of B cell follicles in conventional BALT, with most lymphoid cells being positive for CD20 and BCL2 (Koss, 2004; Li *et al.*, 1990) and mixed with nonlymphoid cells that are positive for CD21/CD35 (presumably, FDCs) (Koss, 2004). Some BALT lymphomas even contain histologically defined germinal centers (Koss, 2004; Kurtin *et al.*, 2001; Li *et al.*, 1990). T cells can be found around the outside of peribronchial lymphoid nodules (Koss, 2004), just as they would be around the B cell follicles of conventional BALT. Most BALT lymphomas interact extensively with the bronchial epithelium and form small clusters of lymphoma cells inside polyp-like bulges in the epithelium (Fiche *et al.*, 1995; Kurtin *et al.*, 2001). These interactions are similar to the interactions of B cells in the dome epithelium of typical mucosal lymphoid tissues (Kiyono and Fukuyama, 2004). Thus, many of the histologic features of BALT lymphoma have some correspondence with the architecture of nonmalignant areas of BALT.

The most common type of MALT lymphomas affect the stomach (Isaacson, 1999), where they are often associated with *Helicobacter pylori* infection (Go and Smoot, 2000; Lochhead and El-Omar, 2007). In the case of gastric lymphoma, lymphomagenesis is directly associated with infection as antibiotics can often induce tumor regression (Isaacson, 1999). Thus, chronic inflammation as well as antigen stimulation and T cell help seem to cooperate to trigger tumorigenesis (Isaacson, 1999). Similar events are likely to occur in BALT lymphoma, however, the etiology of the disease is not as clear-cut (Koss, 2004). For example, some BALT lymphomas are associated with underlying autoimmune diseases, such as SS (Imai *et al.*, 2009; Ingegnoli *et al.*, 2008), systemic lupus erythematosus (SLE) (Gayed *et al.*, 2009), and RA (Bende *et al.*, 2005). In contrast, the development of some BALT lymphomas is associated with chronic mycobacterial infections, including infection with *Mycobacterium avium* and *Mycobacterium tuberculosis* (Gaur *et al.*, 2004; Inadome *et al.*, 2001; Klein *et al.*, 2007). This may not be altogether surprising, given the association with pulmonary mycobacterial infection with the development of BALT in both mice and humans (Kahnert *et al.*, 2007; Tsai *et al.*, 2006; Ulrichs *et al.*, 2004). Interestingly, a recent study used an analysis of 16S-RNA to determine the entire bacterial population found in samples from nine BALT lymphomas (Adam *et al.*, 2008). In eight of the nine samples, the authors found RNA from bacteria of the Alcaligenaceae family. These gram-negative bacteria are widespread in the environment and are not typically considered to be pathogenic. Thus, further studies are required to confirm this link and establish any connection between pulmonary

exposure to bacteria of the Alcaligenaceae family and lymphomagenesis. Nevertheless, there is likely to be some connection between chronic inflammation from some source and the development of BALT lymphoma.

12. SUMMARY AND PERSPECTIVES

Based on the accumulating data, it is clear that term "BALT" encompasses a wide range of lymphoid tissues in the lung from small disorganized clusters of lymphocytes and DCs, to highly organized lymphoid tissues with B cell follicles, germinal centers, FDCs, HEVs lymphatics, and a well-developed dome epithelium. All of these tissues seem to be functional to some degree, although there are likely to be functional differences that are not currently appreciated. In any case, we now know that BALT can be a fully functional lymphoid tissue that collects antigens from the pulmonary airways, primes B and T cell responses, and contributes to the clearance of infectious diseases. In addition, the development or expansion of BALT is also associated with a wide variety of chronic inflammatory diseases. However, the role of BALT in these diseases is less well established. Thus, future research should be directed toward understanding how BALT may contribute (both positively and negatively) to chronic lung diseases.

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