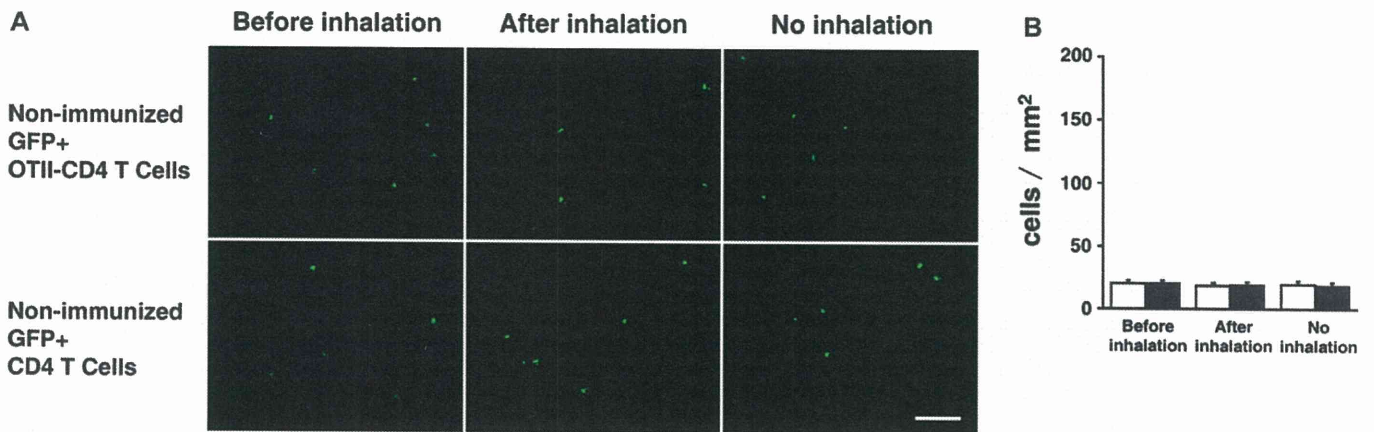

FIG E2. Fluorescence imaging of naive OT II–CD4 T and CD4 T cells in the lung. **A,** Images of naive OT II–CD4 T and CD4 T cells. Unprimed GFP$^+$ OT II–CD4 T and CD4 T cells were monitored in the lung before ovalbumin inhalation and 24 hours after ovalbumin inhalation by using the OV100 Small Animal Mouse Imaging System. *Bar,* 150 μm. **B,** Summary of the accumulation of fluorescent cells of *A.* Data are from 15 fields from 3 mice with SD. *Open bar,* OT II–CD4 T cells; *closed bar,* CD4 T cells.

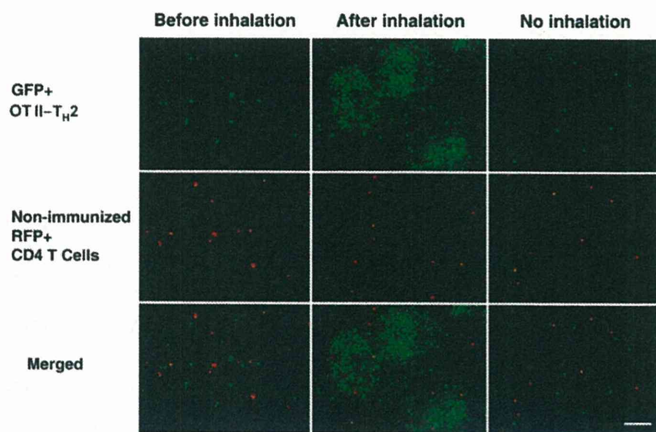

FIG E3. Color -coded fluorescence imaging of OT II–T_H2 cells and CD4 T cells in the lung. Primed GFP^+ OT II–T_H2 cells and RFP^+ CD4 T cells from nonprimed RFP Tg mice were monitored before ovalbumin inhalation and 24 hours after ovalbumin inhalation by using the OV100 Small Animal Mouse Imaging System. *Bar,* 150 μm. The results are representative of 3 experiments.

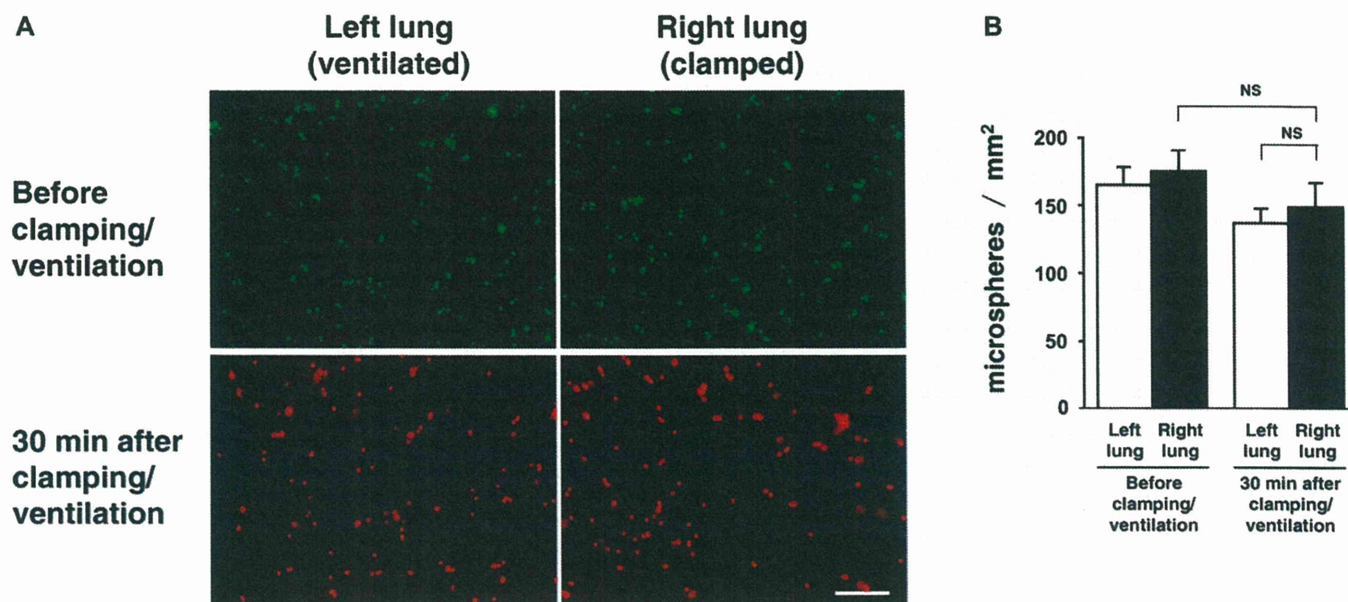

FIG E4. Measurement of blood flow in the clamped right lung and the ventilated left lung. **A,** Assessment of blood flow using fluorescent microspheres. Yellow-green fluorescent microspheres (10 μm, Invitrogen) were infused before ventilation and red fluorescent microspheres (10 μm, Invitrogen) infused 30 minutes after ventilation. The excised lungs were assessed by using the OV100 Small Animal Mouse Imaging System. *Bar,* 150 μm. **B,** Summary of the number of fluorescent microspheres. Data are from 9 fields from 3 mice with SD. *Open bar,* left lung; *closed bar,* right lung *NS,* not significant.

VIDEO E1-E6. Dynamic real time cellular imaging of ovalbumin-specific GFP^+ OT II–T_H2 cell migration into the lung after ovalbumin inhalation in living mice. Ovalbumin-specific GFP^+ T_H2 cell migration and accumulation in the lung before ovalbumin inhalation (Videos E1 and E2 [high magnification]) and after ovalbumin inhalation: 6 hours (Video E3), 12 hours (Videos E4 and E5 [high magnification]), and 21 hours (Video E6). Individual cells were imaged every 5 seconds. Videos are at ×225 real-time.

Organization of immunological memory by bone marrow stroma

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Abstract | Immunological memory is a hallmark of the adaptive immune system. Plasma cells and memory B and T cells collectively provide protective immunity and effective secondary immune responses to invading pathogens. Here, we discuss how mesenchymal stromal cells regulate immunological memory by organizing defined numbers of dedicated survival niches for plasma cells and memory T cells in the bone marrow and also, to a lesser extent, in secondary lymphoid organs. An understanding of the biology of mesenchymal stromal cells and their interaction with cells of the immune system is key to fully understanding immunological memory.

The stroma is the supporting structure of an organ; it consists of stromal cells and the extracellular matrix that they produce. Stromal cells were initially defined as non-haematopoietic cells that were adherent in cell culture[1], but the term is now generally used to describe non-haematopoietic cells that form a matrix. Although the cellular basis of stroma is poorly understood, recent evidence suggests that stromal cells have an important role in controlling immune responses by influencing the generation of lymphocytes, the induction of central tolerance, the response to antigen and the maintenance of immunological memory. Therefore, understanding the dialogue between stromal cells and lymphocytes becomes an essential facet of understanding the organization of the immune system. The role of stromal cells, in particular follicular dendritic cells, fibroblastic reticular cells and endothelial cells, in the initiation and maintenance of immune responses in secondary lymphoid organs (SLOs) — that is, the spleen and lymph nodes — have been recently reviewed[2]. Here, we discuss the immunological memory-regulating functions of stromal cells in the bone marrow and SLOs.

Immunological memory is a hallmark of the adaptive immune system and is highly complex. It is thought to be restricted mostly, if not exclusively, to T cell-dependent immune responses, in which memory lymphocytes are generated, by mechanisms that are currently poorly understood[3–6]. Memory B cells express high-affinity antibodies, often of IgG, IgE or IgA isotype, that have been generated by somatic hypermutation and class-switch recombination. Antigen-experienced CD4$^+$ T helper (T_H) cells are transcriptionally and epigenetically imprinted to re-express specific cytokines and chemokines following re-exposure to the antigen. According to the cytokines that these T cells produce, they can be classified as T_H1, T_H2 or T_H17 cells, which can be effector and/or memory CD4$^+$ T cells[7,8]. For information on the phenotype of effector and memory cell types, see TABLE 1.

The relationship between effector and memory lymphocytes is still debated[9], as are the mechanisms that maintain the memory phenotype and determine the size, stability and adaptation of the memory cell pool to new specificities. Much confusion in this debate is due to diverse interpretations of the term 'memory'. Here, we define memory as 'the maintenance of information in the absence of the original instruction' — that is, memory cells are cells that are maintained in the absence of functional antigen. This definition differentiates true memory cells from long-lived effector lymphocytes that develop during long-lasting and persistent immune responses[10]. Memory B and T cells provide a 'reactive' memory response: they react quickly to antigenic challenge by proliferating and expressing their imprinted effector functions[3].

Memory B cells can differentiate into non-dividing antibody-secreting plasma cells in antigen-recall responses. Plasma cells can provide a 'protective' memory response by secreting high levels of antibodies that neutralize a pathogenic antigen[11]. Plasma cells can be short lived or long lived[12–16], and long-lived plasma cells constitute an independent compartment of immunological memory[5,15–17]: the 'memory plasma cell'. Memory plasma cells are no longer responsive to antigen and are resting in terms of proliferation. Whether a plasma cell is short lived or whether it

Stromal cells
Cells of non-lymphoid origin that form the framework of each organ. These cells can support adhesion, proliferation and survival of distinct cell subsets.

Central tolerance
Tolerance to self that is created at the level of the central lymphoid organs. Developing T cells (in the thymus), and B cells (in the bone marrow) that strongly recognize self antigen face deletion or marked suppression.

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Table 1 | **Comparison of effector and memory cell phenotypes**

Cell type	Markers	
	Effector cell	**Memory cell**
CD8^{+} T cell	CD44^{hi} IL-7Rα^{low} IL-2Rβ^{low} KLRG1^{hi} LY6C$^{low/-}$	CD44^{hi} IL-7Rα^{hi} IL-2Rβ^{hi} KLRG1^{low} LY6C^{hi}
CD4^{+} T cell	CD44^{hi} IL-7Rα^{low} IL-2Rβ^{low} CD49b^{+} LY6C$^{low/-}$	CD44^{hi} IL-7Rα^{hi} IL-2Rβ^{hi} CD49b^{+} LY6C^{hi}
B cell*	B220^{+} CD138^{-} GL7^{+} sIg^{+} LY6C$^{low/-}$	B220^{+} CD138^{-} GL7^{-} sIg^{+} LY6C$^{low/-}$
Plasma cell‡	B220$^{+/-}$ CD138^{hi} GL7^{-} sIg$^{low/-}$ LY6C^{hi}	B220^{-} CD138^{hi} GL7^{-} sIg$^{low/-}$ LY6C^{hi}

IL, interleukin; KLRG1, killer cell lectin-like receptor subfamily G, member 1; R, receptor; sIg, surface immunoglobulin. *The effector B cell is a germinal centre B cell. ‡The effector plasma cell is a plasmablast.

becomes a long-lived memory plasma cell is determined by poorly defined molecular competence and by survival signals from its environment[5,18]. It was originally suggested that, after the generation of plasma cells in the spleen or lymph nodes, these cells translocate to the bone marrow[19], where they individually dock with stromal cells that express CXC-chemokine ligand 12 (CXCL12; also known as SDF1) and vascular cell-adhesion molecule 1 (VCAM1; also known as CD106), which is a ligand of α4β1 integrin[20]. It is thought that these stromal cells form dedicated survival niches for plasma cells and determine the size of the protective memory response[5].

Recent evidence suggests that other memory cell types are also maintained by stromal cells of the bone marrow. Activated effector CD4^{+} T_H cells translocate to the bone marrow at the end of an immune response, dock with stromal cells expressing interleukin-7 (IL-7) and VCAM1, and are maintained there as resting memory CD4^{+} T cells[21]. Currently, the situation is less clear for memory CD8^{+} T cells and for memory B cells, but it is likely that stromal cells have a key role in their maintenance[22]. Here, we discuss the stability and plasticity of immunological memory and focus on the emerging role of dedicated stromal cells in organizing the location, differentiation, numbers and persistence of memory cells.

Follicular dendritic cell
A cell type that is normally found only in the germinal centres of lymphoid tissue. It presents antigen to selected B cells and provides survival signals required for affinity maturation.

Memory B cell
An antigen-experienced B cell that expresses high-affinity antibodies and quickly differentiates into a plasma cell in antigen recall responses.

Plasma cell
A terminally differentiated quiescent B cell that develops from a plasmablast and is characterized by an ability to secrete large amounts of antibody.

The organization of bone marrow stroma

Bone marrow stroma includes mesenchymal stromal cells, endothelial cells, osteoblasts and adipocytes[23–28]. Understanding the cellular basis of bone marrow stroma in more detail — in particular, its role in the maintenance of immunological memory — is hampered by the fact that little is currently known about the heterogeneity of stromal cells in the bone marrow. Information on the functional diversity of stromal cells *in vivo* is scarce (see below), and data from the analysis of these cells *in vitro* has to be taken with care, as *ex vivo* stromal cells undergo substantial phenotypical, and probably also functional, changes when cultured *in vitro*[29,30].

Mesenchymal stromal cells. Mesenchymal stromal cells are so named because of their mesenchymal origin[31]. Owing to their morphology, they have also been named 'reticular' stromal cells, as they show extensive, elongated cellular processes[26]. Mesenchymal stromal cells generate an extracellular matrix, mainly composed of collagen III[32], and form a cellular network by themselves — hence the term 'reticular'[33] (FIG. 1). The physical structure of the stroma and most mesenchymal stromal cells of the bone marrow are radiation resistant, indicating that these cells are not proliferating[34]. Bone marrow mesenchymal stromal cells express VCAM1 (REFS 35,36), as do endothelial cells of the bone marrow[37]. Of note, endothelial cells also express platelet endothelial cell adhesion molecule 1 (PECAM1; also known as CD31)[38]. *In vitro*, cultured mesenchymal stromal cells have been shown to express fibronectin[39], proteoglycan[40], collagen I, collagen III[41,42], laminin-β1 (REF. 43), CD34 (REF. 44), STRO1 (REF. 45), CD10 (also known as neprilysin)[46] and vimentin[47]. Fibronectin, collagen and laminin are ligands of receptors of the integrin family, which are expressed by haematopoietic cells. Proteoglycans are ligands of the receptors CD44 (expressed by memory T cells), syndecan (expressed by memory plasma cells) and neuropilin[48]. *In vivo*, mesenchymal stromal cells show heterogeneous expression of these surface and secreted matrix molecules, but this heterogeneity has not been elucidated further[41,42].

A distinct subpopulation of mesenchymal stromal cells is characterized by the expression of CXCL12. These cells have been reported to be in contact with pre-pro-B cells, multipotent haematopoietic progenitors and memory plasma cells[20]. Approximately 17% of all VCAM1^{+} cells express CXCL12, and all CXCL12^{hi} stromal cells in the bone marrow also express VCAM1, but not PECAM1, classifying them as mesenchymal stromal cells.

Recently, it has been shown that mesenchymal stromal cells that express platelet-derived growth factor receptor-α (PDGFRα) but not stem cell antigen 1 (SCA1; also known as LY6A2–LY6E1) produce 10 times more CXCL12 than their SCA1^{+} counterparts[28]. PDGFRα^{+}SCA1^{+} mesenchymal stromal cells include multipotent progenitor cells (also termed mesenchymal 'stem' cells), which can differentiate into osteoblasts, chondrocytes or adipocytes[28,49–53]. PDGFRα^{+}SCA1^{-} mesenchymal stromal cells do not have this developmental potential. Their high expression of CXCL12 and their developmental stability suggest that they might be the mesenchymal stromal cells that provide a survival niche for memory plasma cells although, at present, no data are available to support this claim.

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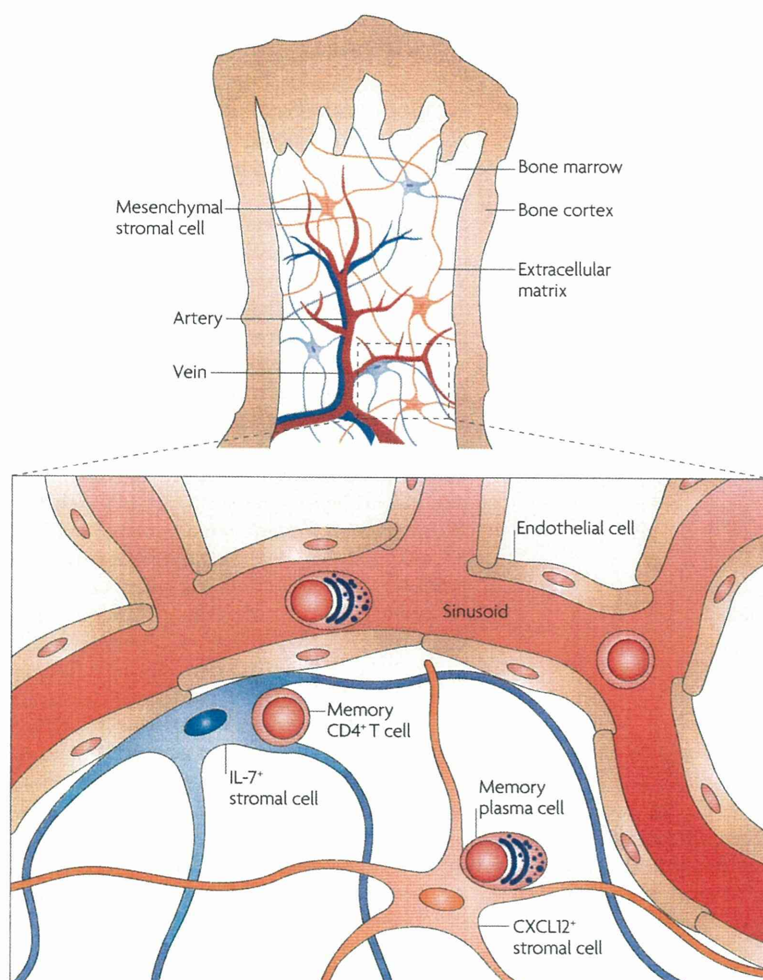

Figure 1 | **Overall organization of bone marrow stroma.** Bone marrow is the soft tissue that fills the cavities of the bone. The tissue is formed by mesenchymal stromal cells and the extracellular matrix they secrete. Haematopoietic progenitors and mesenchymal stem cells reside and move on this matrix. Memory CD4+ T cells and memory plasma cells also reside in the bone marrow; memory CD4+ T cells are maintained on interleukin-7 (IL-7)+ stromal cells and memory plasma cells are maintained on CXC-chemokine ligand 12 (CXCL12)+ stromal cells.

Sinusoid
A specialized blood vessel in haematopoietic tissues through which venous circulation occurs. It has thin walls formed by a discontinuous, irregularly shaped endothelium that allows cells to pass in and out of circulation.

Germinal centre
A lymphoid structure that arises within follicles after immunization with, or exposure to, a T cell-dependent antigen. It is specialized for facilitating the development of high-affinity, long-lived plasma cells and memory B cells.

Another fraction of ~46% of VCAM1+ bone marrow cells expresses IL-7. These cells are in contact with pro-B cells and memory CD4+ T cells[20,21] and do not co-express CXCL12 (REF. 20). Of the IL-7-expressing stromal cells, ~50% also express the endothelial cell marker PECAM1. However, memory CD4+ T cells only dock with PECAM1− stromal cells (K.T., unpublished observations). Whether PECAM1+VCAM1+IL-7+ stromal cells are truly endothelial cells, as their marker expression suggests, remains to be proved.

In general, bone marrow stromal cells have been shown to have immunomodulatory properties and they can suppress allogenic and mitogenic T cell proliferation[54–57], induce T cell anergy[58] or apoptosis[59] and modulate cytokine production by T cells[60]. However, it remains to be shown whether IL-7-expressing stromal cells of the bone marrow can also modulate the function of T cells beyond maintaining memory CD4+ T cells.

Thus to date, several subpopulations of mesenchymal stromal cells have been identified *in situ*: CXCL12+VCAM1+ cells, IL-7+VCAM1+PECAM1− cells, IL-7+VCAM1+PECAM1+ cells and CXCL12−IL-7−VCAM1+ cells (FIG. 2), as well as SCA1+ and SCA1− variants of these cells. It is conceivable that considerably more heterogeneity among mesenchymal stromal cells in the bone marrow will be identified if more markers are used for their analysis.

Vascular endothelial cells. Vascular endothelial cells form the lining of sinusoids, arterioles and venules in the bone marrow. Because bone marrow is not connected to the lymphatic system, immune cells travel to and from the bone marrow through the blood[61], and sinusoidal endothelial cells organize their entry and exit. Endothelial cells of both the arterioles and sinusoids express PECAM1, VCAM1 and vascular endothelial growth factor receptor 1 (VEGFR1; also known as FLT1) and VEGFR2 (also known as FLK1)[62,63]. Arteriolar endothelial cells selectively express SCA1, whereas sinusoidal endothelial cells express VEGFR3 (REF. 64). Sinusoidal endothelial cells provide niches for haematopoietic progenitor cells[64,65]. They have also been suggested to have a role in the regulation of peripheral B cell numbers by positioning immature B cells in sinusoids and regulating their exit[66,67]. Defective retention of B cells in the sinusoids of mice deficient in cannabinoid receptor 2 results in decreased numbers of B cells in the periphery[67].

It is obvious that endothelial cells must be involved in recruiting memory lymphocytes or their immediate precursors from the blood into the bone marrow; however, the molecular mechanisms involved are currently unknown. Furthermore, it is not clear whether some or all endothelial cells also provide survival niches for memory lymphocytes. Memory plasma cells and memory CD4+ T cells are maintained in close proximity to bone marrow blood vessels (A.H., unpublished observations) (FIG. 1), but no CXCL12-expressing VCAM1+PECAM1+ cells have been observed in the bone marrow. This suggests it is unlikely that endothelial cells (which express VCAM1 and PECAM1) are an integral part of the memory plasma cell survival niche. Memory CD4+ T cell maintenance is different, as approximately half of the IL-7-expressing bone marrow stromal cells express both VCAM1 and PECAM1, and thus could be endothelial cells (see above and FIG. 1).

Bone marrow and SLO stromal cells

A prominent feature of secondary and tertiary lymphoid organ stroma is the presence of follicular dendritic cells, which provide the network that organizes the B cell follicles of germinal centres[2]. They express the complement receptors CR1 and CR2, the adhesion molecules VCAM1, intercellular adhesion molecule 1 (ICAM1) and mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), which is a ligand of α4β7 integrin[68–71]. In germinal centres, follicular dendritic cells present antigen-containing immunocomplexes, bound by low-affinity Fc receptors for IgG (FcγRIIb) and complement receptors,

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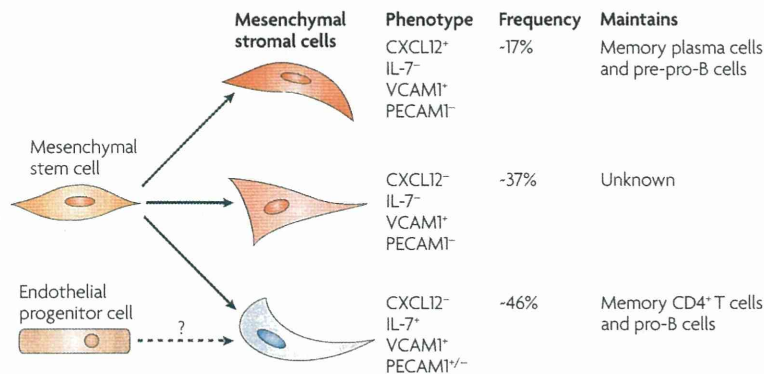

Figure 2 | **Development of bone marrow stromal cells and their role in B cell ontogeny and memory maintenance.** Mesenchymal stem cells differentiate into diverse subpopulations of mesenchymal stromal cells. The CXC-chemokine ligand 12 (CXCL12)-expressing population constitutes ~17% of vascular cell-adhesion molecule 1 (VCAM1)$^+$ bone marrow stromal cells. This subpopulation maintains pre-pro-B cells and memory plasma cells. The interleukin-7 (IL-7)-expressing population constitutes ~46% of VCAM1$^+$ bone marrow stromal cells, and approximately half of this IL-7-expressing population also express platelet endothelial cell adhesion molecule 1 (PECAM1). This IL-7-expressing population maintains pro-B cells and memory CD4$^+$ T cells. Approximately 5% of all the cells in the bone marrow express VCAM1.

High endothelial venule
A specialized venule that occurs in secondary lymphoid organs, except the spleen. High endothelial venules allow continuous transmigration of lymphocytes as a consequence of the constitutive expression of adhesion molecules and chemokines their luminal surface.

Plasmablast
A dividing antibody-secreting cell of the B cell lineage that has migratory potential. These cells can further mature into plasma cells, which do not divide.

to B cells[72] and are the main producers of the B cell-zone chemokine CXCL13 (REF. 73). The progenitors of follicular dendritic cells are not currently known, but it is assumed that they are related to bone marrow mesenchymal stromal cells[74,75]. Follicular dendritic cells have been shown to present antigen over long time periods[76,77] and thus have been implicated in the maintenance of effector lymphocytes by persistent antigen, a process that is often confused with immunological memory.

Fibroblastic reticular cells produce the collagenous reticular fibres that constitute a supportive lattice in SLOs[78]. These cells have an important role in the initiation of immune responses by transporting soluble antigens from afferent lymph to the T cell zone of lymph nodes[79] and are the main producers of IL-7 in SLOs, qualifying them as potential organizers of the CD4$^+$ memory T cell survival niche (see below). They also secrete CC-chemokine ligand 19 (CCL19) and CCL21, which are important for positioning and motility of T cells[80–83]. A network composed of fibroblastic reticular cells and extracellular matrixes in the lymph nodes provides a place for the interaction of T cells with antigen-presenting cells and constitutes a conduit system for the delivery of soluble molecules such as cytokines from lymph to the high endothelial venules[84,85].

The conduit system of lymph nodes is distinct from that of the bone marrow. Immune cell-containing lymph enters the lymph node and percolates through the subcapsular sinus. In addition, active immigration of lymphocytes from the blood occurs through high endothelial venules[86]. Reticular fibroblastic cells organize the conduit system connecting lymph and high endothelial venules in the lymph nodes. By contrast, bone marrow has no lymph vessels or high endothelial venules but contains sinusoids. The cells that line the sinusoids are thought to have a role in regulating the diameter of the sinusoids and thus the distribution and velocity of blood, as described for sinusoids of the liver[87]. In the bone marrow, sinusoidal-lining cells may contribute to the control of cell entry and exit by regulating the distribution and velocity of blood.

The different cellular composition of stroma in SLOs and the bone marrow may reflect the essential roles of stroma in organizing the initial phase of immune responses and organizing immunological memory, respectively. However, there must be some overlap in these functions, as memory cells can be maintained in low numbers in SLOs[88], and immune niches have been described in the bone marrow[66]. The conduit networks formed by endothelial cells are prominent features of stroma of SLOs and provide topographical orientation and coordination for the complex cellular interplay of immune responses. By contrast, bone marrow serves as a protective environment for the development of lymphocytes and their maintenance as memory cells[22,89]. Therefore, bone marrow stroma should be organized to optimize the recruitment of memory cell precursors and provide stable niches for the survival of memory cells.

Organization of immunological memory by stroma

Conversion into memory cells. In the initial phase of an immune response, referred to here as an immune reaction, antigen-specific lymphocytes expand and differentiate in response to antigen. In the memory phase of an immune response, antigen-specific memory lymphocytes are maintained in an antigen-independent manner. Follicular dendritic cells have an essential role in the selection of high-affinity B cells during germinal centre reactions in SLOs[88], and they determine the length of an immune reaction owing to their ability to store and present antigen–antibody complexes over extended time periods[76,77]. In the transition of an immune reaction into the formation of immune memory, when antigen-dependent signalling ceases and activated lymphocytes switch to antigen-independent signals for survival as memory lymphocytes, mesenchymal stromal cells seem to have a central role. The transition of effector T_H cells into memory cells requires IL-7, but not antigen[90–92]. Although it is currently not clear where in the body memory T_H cell differentiation occurs, this step can occur on IL-7-expressing mesenchymal stromal cells *ex vivo*[93]. Recently it was shown that, *in vivo*, most precursors of memory CD4$^+$ T cells home to the bone marrow, dock with IL-7-expressing stromal cells and differentiate into memory CD4$^+$ T cells, as shown by their loss of proliferation, expression of a distinct gene signature and ability to provide rapid and efficient helper function[21]. Taken together, these results suggest that the *in vivo* conversion of the precursors of memory T_H cells and/or effector T_H cells into memory CD4$^+$ T cells probably occurs in the bone marrow, organized by IL-7-expressing stromal cells.

Similar to memory T helper cells, the precursors of memory plasma cells, plasmablasts, migrate to the bone marrow[94,95], where they dock with CXCL12-expressing stromal cells[20] and differentiate into memory plasma cells. The transition from plasmablast to plasma cell

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includes termination of proliferation and loss of migratory potential[5], but it is unclear to what extent signals from stromal cells regulate this transition. For memory CD8+ T cells and memory B cells the situation is even less clear, as the stromal cells required for their maintenance have not been identified. It is possible that the instruction for differentiation of activated lymphocytes into memory lymphocytes, rather than being induced by stromal memory niche cells, is already occurring in the antigen-driven immune reaction, generating precursors of memory cells competent to reach a survival niche and respond to the survival signals. Stromal cells providing such survival signals would then allow the final differentiation into memory lymphocytes but would not induce it.

Guidance of lymphocytes. Stroma provides guidance for lymphocytes in SLOs and the bone marrow. Lymphocytes travel along the network formed by the extracellular stroma matrix using adhesion molecules and chemokine receptors for orientation. The relocation of antigen-specific effector CD4+ T cells to the bone marrow is thought to require the expression of α2 integrin by the T cells[21]. α2 integrin preferentially binds to collagen I, the main type of collagen found in the bone marrow stroma but rarely elsewhere[21]. *Ex vivo* plasma cells strongly adhere to VCAM1 and ICAM1, which are expressed by stromal cells, but it remains unclear how relevant this adhesion is to the homing of plasmablasts to the bone marrow.

It is still not clear which chemokines attract T_H cells to the bone marrow. Plasmablasts are probably attracted to the bone marrow by CXCL12, which is highly expressed by mesenchymal stroma cells that provide a niche for plasmablast survival[20,94,95]. Thus, although our molecular understanding of how the precursors of memory T_H cells and plasma cells home to the bone marrow is incomplete, it seems that stromal cells may guide these precursor cells to their respective niches where they can survive.

Little is known about the niches for memory CD8+ T cells, and memory B cells are thought to home to particular sites in the spleen and not to the bone marrow[96–98]. Although attraction and adherence is still undefined on the molecular level, it would be surprising if the guidance of precursors of memory CD8+ T cells and B cells did not follow the same basic principle of migration under the supervision of stromal cells.

Maintenance of memory lymphocytes. In the memory phase of an immune response, stromal cells organize the maintenance of memory lymphocytes, at least of memory plasma cells and memory T_H cells. The survival of plasma cells *in vivo* depends on signals from the microenvironment, as plasma cells die within a few hours when isolated from the bone marrow[5,99,100]. They require signalling through the receptor B cell maturation antigen (BCMA) for survival, which is provided by the ligands B cell-activating factor (BAFF; also known as TNFSF13B) or a proliferation-inducing ligand (APRIL; also known as TNFSF13)[101]. Numerous other signals support the survival of plasma cells, including CXCL12, which also attracts the cells to the niches[5,95,102]. In their bone marrow stromal niches, plasma cells survive without detectable proliferation[15]. Thus, plasma cells are maintained in the bone marrow as resting cells in survival niches that are organized by dedicated stromal cells expressing CXCL12.

The survival of memory CD4+ T_H cells seems to be more complex. It is thought that, once activated, T_H cells can be maintained as proliferating cells by antigen and cytokines for long time periods in SLOs and blood, a process termed 'homeostatic proliferation'[93,103,104], but whether antigen-dependent signalling is essential for homeostatic proliferation is still controversial; that is, it is not known to what extent antigen-dependent signalling reflects memory as opposed to a persistent immune response[22,90,105]. Recent studies have provided evidence for an alternative antigen-independent mechanism for the maintenance of memory T_H cells as resting cells by tracking antigen-specific CD4+ T cells through an immune reaction into the memory phase[21,106]. During various immune responses in mice, activated CD4+ T_H cells were shown to translocate to the bone marrow following the termination of the immune reaction, where they docked with IL-7-expressing stromal cells[21]. No MHC class II-expressing cells, such as macrophages, dendritic cells or B cells, were detected near memory T_H cells in the bone marrow, suggesting that antigen is not involved in memory T_H cell maintenance. The memory T_H cells were dormant in terms of proliferation and transcriptional activity[21]. Interestingly, these memory T_H cells expressed LY6C, which we have proposed to be a marker of 'true' memory T_H cells[21]. More than 80% of all mouse LY6C^{hi}CD4+ T cells are located in the bone marrow, whereas approximately 15% are found in the spleen[21]. Thus, most *bona fide* memory T_H cells are maintained as resting cells in the bone marrow by dedicated stromal cells expressing IL-7. It seems that effector T_H cells can also be maintained *in vivo* for some time, either by homeostatic proliferation or as resting cells, outside of the bone marrow[9]. Whether stromal cells are involved in the maintenance of effector T_H cells remains to be shown.

It has been shown that memory B cells do not proliferate for at least 12 weeks after their generation[107], indicating that they can be maintained as resting cells in SLOs. For memory B cells specific for a particular antigen, it has been shown that ~20 times more of these cells reside in the spleen than in the blood. Thus, it has been suggested that memory B cells may be maintained in the spleen and not in the bone marrow[96–98], although the bone marrow was not examined in these studies. It is currently not clear whether dedicated stromal cells are required to maintain memory B cells in the spleen.

It is our opinion that, to date, the conversion of effector CD8+ T cells into memory CD8+ T cells and the maintenance of these cells has not been analysed during a clearly defined memory phase of an immune response. Instead, the continued activation and proliferation of CD8+ T cells and their disputed dependency on antigen, as observed in some studies, suggest that the cells analysed were actually part of an extended immune response

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that was driven by persistent antigen[108,109]. For most of these studies, virus infection models had been used in which viral antigen persisted for a long time[76,77]. It will be interesting to follow the fate of effector CD8+ T cells through a defined and limited immune reaction into a clearly defined memory phase of an immune response.

Limiting the numbers of memory lymphocytes. The concept that memory lymphocytes are maintained as resting cells in survival niches organized by dedicated stromal cells, mainly in the bone marrow but also in other organs[5,21,22,110], implies an intriguing mechanism to define the size, complexity and plasticity of immunological memory. Such a mechanism would require that a memory niche can host only a defined number of memory cells. Indeed, for plasma cells and memory CD4+ T_H cells, one memory lymphocyte docks with only one stromal cell[20,21]. The molecular reason for this restriction is not known, but it limits the number of memory cells maintained to the number of stromal cells that provide the dedicated survival niche. The frequency of CXCL12-expressing stromal cells is ~1% of all bone marrow cells in mice[20] and the physiological frequency of plasma cells among bone marrow cells of adult humans and mice is also ~1%[111,112]. Thus, CXCL12-expressing stromal cells put plasma cell memory (protective memory) in the context of the volume of the bone marrow (that is, body size), which is a physiological necessity to prevent hypergammaglobulinaemia and plasmacytosis[113,114]. The frequency of IL-7-expressing stromal cells is ~2.5% of all bone marrow cells. Accumulating with age, memory CD4+ T cells make up ~1% of bone marrow cells in 64-week-old mice (K.T. and A.R., unpublished observations). *A priori*, for resting memory CD4+ T_H cells, a strict numerical limitation seems less necessary than for memory plasma cells, but we may have to revise this view when we learn more about reactivation of this memory cell population.

Stroma therapy

To date, therapeutic strategies for chronic inflammatory diseases primarily target cells and molecules of the haematopoietic system, in particular innate and adaptive immune cells[115,116]. Pro-inflammatory immune cells include memory CD4+ T cells and memory plasma cells[5,18,117], and these memory cells are of high therapeutic interest, as they are resistant to conventional therapy, including immunosuppression[116,118]. It is possible that these memory cells are the reason why we currently cannot cure chronic inflammatory diseases.

In view of the fundamental dependency of memory cells on guidance and survival signals provided by dedicated stromal cells, new therapeutic strategies could target the communication between stromal cells and memory and effector cells to achieve a selective depletion of those cells that drive chronic inflammation. One such target would be memory plasma cells that secrete autoantibodies or allergen-specific IgE. Because plasma cells depend on BCMA signals for their survival in the bone marrow, inhibition of the BCMA ligands APRIL and BAFF depletes memory plasma cells from the bone marrow but spares memory B cells[101]. Such plasma cell depletion is not selective for autoreactive versus protective memory plasma cells, but — with a broadening of our understanding of the factors necessary for plasma cell survival in inflamed organs — new, more selective therapeutic options might be discovered[102]. The same applies for memory and long-lived effector T_H cells: a molecular understanding of the role of stromal cells for their survival might identify new therapeutic options for the selective depletion of pathogenic memory or effector T_H cells.

Concluding remarks

Stromal cells regulate the development, migration, activation and selection of lymphocytes and also the maintenance of immunological memory. Bone marrow stromal cells provide dedicated and distinct survival niches for memory plasma cells and memory CD4+ T cells. They control the numbers of memory cells that can survive by providing limiting numbers of survival niches, and thus also regulate the stability and plasticity of immunological memory. The stability of an immunological memory cell population for a given antigen depends on persistence of the stromal niches that maintain the memory cells; the inclusion of new specificities requires competition for the limited numbers of niches between 'new' and 'old' memory cells[5]. It is now apparent that an understanding of immunological memory requires an understanding of the dialogue between the dedicated stromal cells and the memory cells themselves. There are several questions that remain unanswered: what is the phenotype of the stromal cells that organize memory B and CD8+ T cells? How do stromal cells organize memory niches; do they form the niche themselves or do they organize niches composed of several cell types? How do dedicated stromal cells develop, and how is the number of niches determined? Answers to these and other questions (BOX 1) will help to shape our understanding of stromal cells as regulators of immunity and immunological memory.

Box 1 | Questions for future research

- Are memory CD8+ T cells and memory B cells located in the bone marrow and the spleen, respectively, and, if so, what is the microanatomical location of these cells, and why are memory B cells the only cells to preferentially reside in the spleen?
- What is the tissue distribution, localization and physiological role of memory natural killer cells?
- How heterogeneous are bone marrow stromal cells, and how does heterogeneity develop?
- Do cells other than stromal cells participate in the formation of memory niches?
- What are the molecular signals involved in attracting memory cells to the niches, maintaining them there and keeping them alive?
- Is there crosstalk between memory cells in the bone marrow?
- What are the mechanisms of memory cell reactivation *in vivo*?

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Competing interests statement
The authors declare no competing financial interests.

DATABASES
UniProtKB: http://www.uniprot.org
CR1 | CR2 | CXCL12 | ICAM1 | IL-7 | LY6C | MADCAM1 | PDGFRα | PECAM1 | SCA1 | VCAM1 | VEGFR1 | VEGFR2 | VEGFR3

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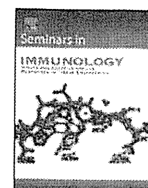

Review

The TCR-mediated signaling pathways that control the direction of helper T cell differentiation

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ABSTRACT

In the periphery, upon antigen recognition by αβTCR, naïve CD4 T cells undergo functional differentiation and acquire the ability to produce a specific set of cytokines. At least four Th cell subsets, i.e., Th1, Th2, Th17 and iTreg cells have so far been identified and the differentiation of each subset is driven by distinct cytokine sets. Antigen recognition by TCR and the activation of the TCR-mediated signaling pathways that follows, however, are most critical for initiating Th cell differentiation. This review focuses on the TCR signal strength and the TCR-mediated signaling pathways that control the differentiation into these four Th cell subsets.

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1. Introduction

After antigen recognition by TCR, naïve CD4 T cells undergo clonal expansion and differentiate into functionally polarized helper T (Th) cell subsets. At least four distinct Th subsets, i.e., Th1, Th2, Th17 and iTreg cells have been identified [1–5]. The direction of functional Th cell differentiation depends on the cytokines present in the priming environment. Naïve CD4 T cells stimulated with antigens differentiate into Th1 cells in the presence of IL-12 and IFNγ [6,7], whereas IL-4 plays an important role for Th2 cell differentiation [8,9]. The activation of downstream signaling molecules of these cytokine receptors, such as STAT4/STAT1 [10–13] or STAT6 [14–16] are required for differentiation into either Th1 or Th2 cells, respectively. Recently, TGFβ was shown to co-operate with IL-6 to induce Th17 cell differentiation [17–19], while TGFβ plus IL-2 induced the generation of iTreg cells [20,21]. Several master transcription factors for differentiation into Th1, Th2, Th17, iTreg cells have been identified; T cell-specific T-box transcription factor (T-bet) [22], GATA3 [23,24], retinoid-related orphan receptor gamma t (RORγt) [25] and Foxp3 [26], respectively.

In addition to appropriate cytokines, antigen recognition by TCR and events triggered by this interaction is most critical for achieving the functional differentiation of all these Th subsets. More specifically, various TCR-mediated signal transduction pathways are activated in naïve CD4 T cells after antigen recognition [27–29]. This review focuses on: (i) the TCR signal strength and the outcome of Th1 and Th2 cell differentiation, and (ii) the role of the TCR-mediated signal transduction pathways or signaling molecules that control the direction of Th cell differentiation.

2. The effect of antigen dosage and signal strength on the differentiation into Th1 and Th2 cell subsets

In addition to cytokines such as IL-12, IFNγ and IL-4, the generation of Th1 and Th2 cells is also controlled by the antigen dosage and overall TCR-mediated signal strength induced in naïve CD4 T cells. More than a decade ago, Bottomly and co-workers [30,31] used moth cytochrome *c* peptide and antigen-specific αβTCR Tg CD4 T cells, and demonstrated that low antigen doses induced early IL-4 in naïve CD4 T cells and thus preferentially induced Th2 cell generation. Paul and co-workers [32] also reported that the early IL-4 production from naïve CD4 T cells was induced by low antigen doses which appeared to be GATA3-dependent. Independently, O'Garra and co-workers [33] used DO11.10 αβTCR Tg CD4 T cells and a broad range of antigenic peptide, and demonstrated that Th2 cell development was observed at very high doses and very low doses of antigenic peptides, whereas midrange peptide doses directed the development of Th1 cells. Burstein and Abbas [34] reported that high-dose aqueous protein antigens induced tolerance in Th1 cells, but not in Th2 cells, and thus it appears that high-dose antigens favored enhanced Th2 responses. We used highly purified naïve CD4 T cells and immobilized anti-TCR mAb, and reported a stronger stimulation with higher anti-TCR mAb concentrations in the presence of IL-4 *in vitro* induced an increased Th2 cell differentiation but with a decreased Th1 cell differentiation [35,36]. Similar results were observed even when DO11.10 αβTCR Tg CD4 T cells and

Abbreviations: APC, antigen presenting cells; dnCN, dominant-negative calcineurin; dnRas, dominant-negative Ras; TCR, T cell receptor; Th, helper T; Tg, transgenic; Treg, T regulatory.

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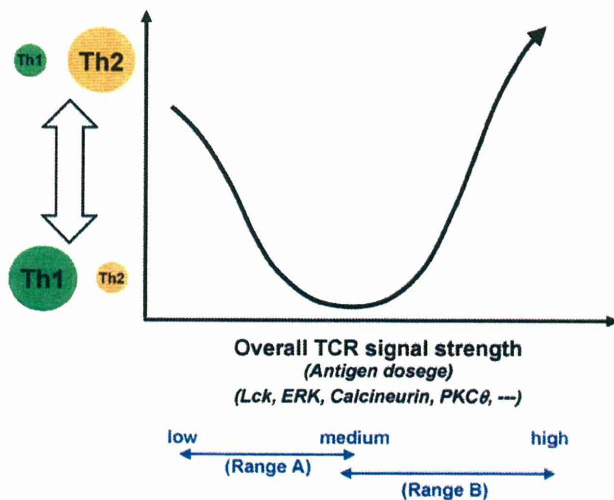

Fig. 1. A bi-phasic Th2 cell differentiation model. After antigen recognition by TCR, naïve CD4 T cells usually undergo differentiation into both Th1 and Th2 cells. The direction of the differentiation into Th1 or Th2 cells is controlled by the overall TCR signal strength. Some reports indicate that low-level antigenic stimulation induced Th2 rather than Th1 cell differentiation (Range A) [30,32,41,42]. However, other experimental results support the notion that low-level TCR stimulation induced Th1 cell differentiation and high-level stimulation induced increased a Th2 cell generation (Range B) [33–35,37,43,44]. These two sets of conclusions seem to be contradictory, but the range of TCR signal strength could be different between these two sets of experiments, although the experimental system used in each study is different, we cannot directly compare them with each other. In this bi-phasic Th2 cell differentiation model, naïve CD4 T cells differentiate into Th2 cells preferentially when naïve CD4 T cells were stimulated with a very low-level TCR signal strength (low dose antigen) and also a very high-level TCR signal strength (high-dose antigen). The levels of activation of major signal transduction pathways, including the ERK/MAPK, calcium/calcineurin, and PKC/NF-κB pathways, may reflect the TCR strength. The ranges of the experiments addressing each signal transduction pathway may be classified as belonging to the Range B, because the decreased signaling activity of these pathways resulted in impaired Th2 cell differentiation.

the specific antigenic peptide were used [35–37]. Several reviews on co-receptors have suggested that stronger activation signals favor Th2 cell differentiation [38–40]. Based on these experimental results, we would like to propose a bi-phasic Th2 cell differentiation model (Fig. 1). A weak stimulation of TCR, such as stimulation with a low dose antigenic peptide, may induce early IL-4 in naïve CD4 T cells, and thereby initiates Th2 cell differentiation in some experimental systems [30,32,41,42]. Under these conditions, although a preferential Th2 cell generation could be observed, the absolute number of effector Th2 cells generated may not be so large, because the extent of T cell expansion is considered to be limited. Relatively higher doses of antigens induced Th1 cells in these experimental settings, where the range of stimulation corresponds to Range A (low-medium) in Fig. 1 [30,32,41,42]. Regarding the signal strength reported in the other studies, they generally correspond to Range B (medium to high) [33–35,37,43,44], where mild infection, low antigen concentration or low dose anti-TCR mAb would favor the induction of Th1 cell differentiation, whereas high-dose stimulations preferentially induced Th2 cells. One characteristic example was observed in experiments using parasites, in which a low dose challenge induced Th1 responses [45,46].

A likely explanation as to why stimulation with high-dose antigen favors Th2 cell differentiation, particularly in the *in vivo* experimental systems is that high-dose antigen may be efficiently presented by APCs, resulting in repeated stimulation of the antigen-specific naïve CD4 T cells and also early developing Th2 cells. Therefore, in addition to early IL-4 production from naïve CD4 T cells, a relatively high amount of IL-4 produced by early developing Th2 cells after stimulation with the same antigen may thus induce the developing Th2 cells themselves to accelerate both polarization and proliferation. This IL-4 may also induce other surrounding naïve CD4 T cells to undergo Th2 cell differentiation. These autocrine and paracrine IL-4 circuits may function efficiently if high-dose antigen is provided. In contrast, although IFNγ influences the magnitude of Th1 cell differentiation, IL-12 plays an important role in the initiation of Th1 cells [47]. Since IL-12 is not produced by either naïve CD4 or developing Th1 cells, no such enhanced circuit would occur during Th1 cell differentiation. Therefore, Th2 cells would dominate over Th1 cells at the high-doses of antigen. Interestingly, if naïve CD4 T cells are cultured in the presence of both IL-4 and IL-12, they differentiate preferentially into Th2 cells (T.N. and M.Y., unpublished observation) [48].

A high concentration of antigenic peptides is required for the production of Th2 cytokines in effector Th2 cells. The TCR and CD4 are efficiently recruited to lipid rafts in Th1 cells, but not in Th2 cells offering an explanation for the decreased ability of Th2 cells to respond to low-affinity peptide stimulation [43]. The functional significance of the expression levels of CD4 was demonstrated by the experiment with the restoration of high-level expression of CD4 in Th2 cells using a retrovirus gene transfer system [44]. Therefore, particularly in the case of *in vivo* experimental systems, Th2 responses appear to be induced more efficiently if a large amount of antigens is available.

3. Effect of the affinity of antigens and signal strength on the generation of Treg (regulatory T) cells

More recently, low-affinity antigenic peptides have been shown to induce a significant expansion of $CD4^{+}Foxp3^{+}$ Treg cells, whereas high-affinity antigenic peptides favored the expansion of $Foxp3^{neg}$ Th cells [49]. The inverse correlation of Foxp3 expression and Akt/mammalian target of rapamycin (mTOR) signaling has been reported. Similarly, the premature termination of TCR signaling and the inhibition of the PI3K/Akt/mTOR pathway conferred Foxp3 expression and Treg-like gene expression profiles [50]. Continued TCR signaling and constitutive activation of the PI3K/Akt/mTOR pathway antagonize the expression of Foxp3. In addition, the inhibition of calcineurin by FK506 resulted in the inhibition of the proliferation of conventional T cells, but FK506 less efficiently inhibited the proliferation of Treg cells [51]. Therefore, the activation levels of calcineurin appear to control the proliferation of Treg cells and their numbers.

4. Role of the TCR-mediated signal transduction pathways in Th cell differentiation

After antigen recognition by TCR, the TCR/CD3 complex initiates the activation of various signal transduction pathways. The most proximal biochemical event is the tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of four components of the TCR/CD3 complex, i.e., CD3γ, CD3δ, CD3ε and TCR-ζ [27–29]. The src family tyrosine kinases including Lck play an important role in the tyrosine phosphorylation of ITAMs. Then, the Zap70 syk family kinase is recruited to the phosphorylated ITAMs through the SH2 domains of ZAP70. The recruited ZAP70 is then phosphorylated and activated by surrounding Lck tyrosine kinase, thus leading to the phosphorylation of LAT molecules. Following these proximal events, the activation of various distinct signaling pathways is initiated, including (i) the Ras/ERK MAPK cascade, (ii) the Ca/calcineurin/NF-AT pathway, and (iii) the PKC/NF-κB pathway (Fig. 2).

We summarize the experimental results that have addressed the role of each signaling pathway or signaling molecules in the Th cell differentiation and Th responses noted below. Interestingly,

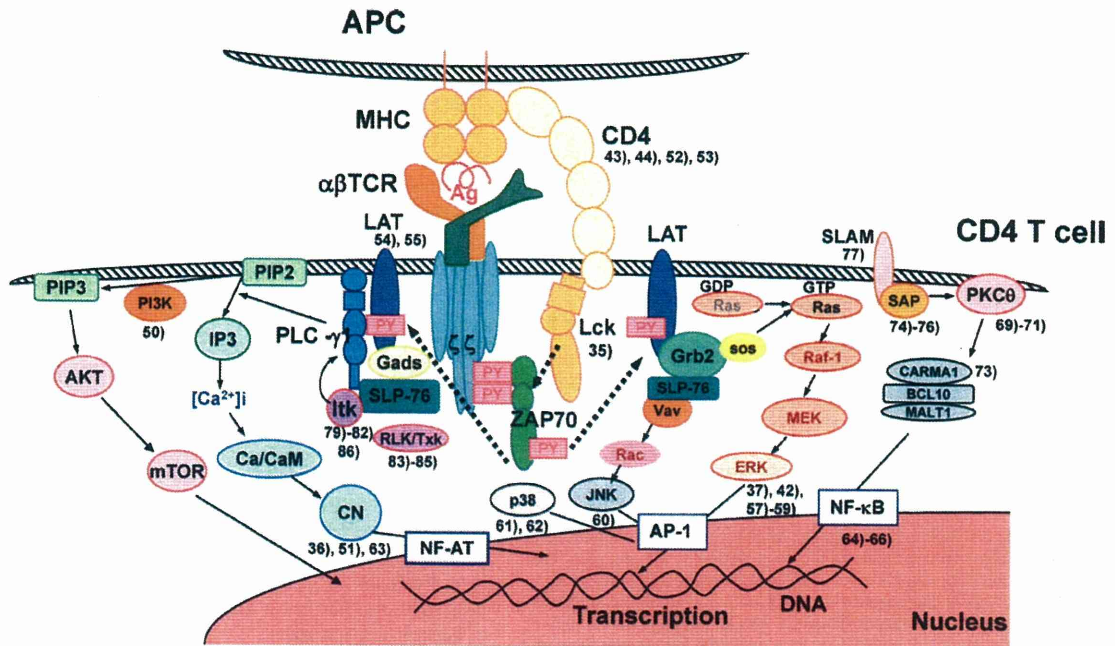

Fig. 2. Major signal transduction pathways downstream of TCR and their roles in Th cell differentiation. The references addressing each molecule in Th cell differentiation are indicated. See text in detail.

the loss-of-function of various signaling molecules tended to lead to the defects in Th2 cell differentiation while leaving Th1 cell differentiation intact although some exceptions have been reported. Therefore, the most of these loss-of-function experiments addressing the role of each signaling pathway may fit within Range B as shown in Fig. 1.

4.1. Lck

We reported that strong Lck kinase activity is required for the efficient generation of Th2 cells [35]. Dominant-negative Lck Tg mice showed impaired Th2 cell differentiation while leaving Th1 cell differentiation intact. It is known that as much as 50% of Lck protein is constitutively associated with the CD4 cytoplasmic domain. Reiner and co-workers [52] demonstrated a critical role of the cytoplasmic domain of CD4 for Th2 cell differentiation. The blockade of CD4/MHC class II interaction by peptides resulted in the inhibition of Th2 responses more efficiently than of Th1 responses [53]. In polarized Th2 cells, the expression levels of CD4 are lower in comparison to Th1 cells, and a decreased CD4 expression resulted in suboptimal TCR-induced phosphorylation and reduced Ca^{2+} signaling [44]. These results are also consistent with the notion that weak TCR stimulation may not efficiently activate Lck and thus favor Th1 cell differentiation, whereas strong stimulation may sufficiently activate Lck to induce Th2 cell differentiation.

4.2. LAT

Gene manipulated mice with mutations of critical tyrosine residues (Y136 and Y132) of LAT showed a severe defect in the development of T cells, and the few remaining T cells caused a spontaneous Th2-like allergic inflammation [54,55]. In MHC class II-deficient mice, a similar spontaneous allergic phenotype accompanied with a severe defect in the CD4 T cell development was observed. The remaining T cells in these animals did not show normal antigen-specific Th2 cell responses [56]. Therefore, the Th2 phenotype observed in these CD4 T cell deficient mice appears to be unrelated to the strength of the activation of the specific signaling pathways, but more likely due to the secondary effects of the lymphopenic environment, such as abnormal homeostatic proliferation.

4.3. The MAPK cascade

In mammals, there are three distinct MAPK pathways, ERK, JNK and p38MAPK. We previously reported that the activation of the ERK/MAPK cascade was required for Th2 cell differentiation [37]. The inhibition of this cascade by an inhibitor or dominant-negative Ras resulted in impaired Th2 cell differentiation with an increased Th1 cell generation even in the presence of a sufficient amount of exogenous IL-4 (Fig. 3). These results indicate that the inhibition of the activation of the ERK/MAPK cascade induced a shift from Th2 to Th1 fate. This shift has been confirmed *in vivo* using a Th2-driven murine model of asthma [57]. In addition, we more recently found that ERK controls the level of GATA3 protein post-transcriptionally in Th2 cells through the inhibition of the ubiquitin–proteasome degradation pathway [58]. Interestingly, the activation of the ERK induced increased ribosomal components in Th2 cells to accommodate the production of Th2 cytokines [59]. In contrast, Jorritsma et al. [42] reported that the sustained activation of ERK induced by a high-affinity peptide in naïve CD4 T cells led to Th1 cell differentiation, which can be switched to Th2 differentiation when the activation of ERK was reduced. They suggest that the strong activation of ERK resulted in the down-regulation of IL-4 expression by altering the composition of AP1 complex in naïve CD4 T cells. Thus, the activation of ERK appears to be an important regulator of Th2 cell differentiation and functions at multiple levels. Th1 cell differentiation and Th1 cytokine production are dependent on other MAPK pathways, including JNK and p38, respectively [60,61]. p38 MAPK has also been shown to promote IL-5 expression through the phosphorylation of GATA3 protein [62]. Thus, p38 MAPK appears to be involved in both the Th1 and Th2 responses.

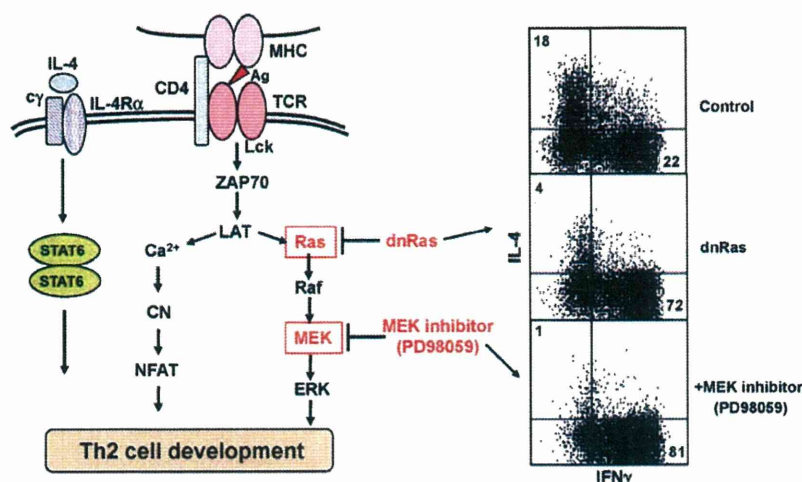

Fig. 3. Decreased activation of Ras and MEK in the ERK/MAKP cascade resulted in decreased Th2 cell differentiation and increased Th1 cell differentiation. Naïve CD4 T cells from normal C57BL/6 mice (control) and dominant-negative Ras Tg mice under the control of Lck distal promoter (dnRas) were stimulated with immobilized anti-TCR mAb in the presence of IL-4, and 5 days later, the cultured cells were harvested and subjected to the cytoplasmic staining with anti-IL-4 and anti-IFNγ. A specific inhibitor for MEK (PD98059) was added to the control C57BL/6 CD4 T cell culture (+MEK inhibitor). The percentages of the cells present in each quadrant are indicated [37].

4.4. The Ca/calcineurin/NF-AT pathway

Ca^{2+}/calcineurin signaling has been reported to play a crucial role in Th cell differentiation. We reported that the inhibition of the calcineurin activity by a specific inhibitor FK506 or by the overexpression of dominant-negative calcineurin Aα chains resulted in the impaired proliferative responses of both Th1 and Th2 cells, although, the inhibition of Th2 cell differentiation was more prominent than Th1 cells [36]. Therefore, when the activity of calcineurin was inhibited, the generation of Th2 cells was affected more profoundly in comparison to Th1 cells (Fig. 4). In this analysis, we identified that the TCR-induced activation of calcineurin modified the IL-4R signaling complex. Efficient calcineurin activation upregulated Jak3 expression and induced both the physical and functional associations of STAT5 with IL-4 receptors. The inhibition of IL-4-induced STAT5 activation resulted in a diminished IL-4-induced proliferation of Th2 cells. This study highlights the crosstalk that exists between the TCR-mediated signaling pathway and the cytokine mediated signaling pathway during Th cell differentiation. In addition, Scheinman and Avni [63] reported that the TCR-mediated activation of NF-AT1 is involved in the transcriptional regulation of the GATA3 expression in both naïve and differentiated Th2 cells. Thus, GATA3 is a common target of the IL-4- and TCR-mediated signaling pathways.

4.5. The PKC/NF-κB pathway

NF-κB appears to be a critical transcription factor that regulates Th2 cell differentiation. NF-κB p50 subunit-deficient mice were unable to induce OVA-induced airway inflammation [64]. The lack of inflammation was not due to defects in T cell priming, T cell proliferation, or the overexpression of important cell adhesion molecules, but rather it was due to the impaired induction of GATA3. We reported that the CD28-mediated activation of NF-κB was required for efficient Th2 cell differentiation [65] and the chromatin remodeling of the IL-5 gene locus [66].

PKCθ is a key component of the T cell immunological synapse whose activity is required for the activation of AP-1, NF-AT and NF-κB [67,68]. Th2 cell-mediated immune responses were markedly impaired in PKCθ-deficient mice, whereas the Th1 immune responses developed normally [69,70]. More recently, the role of PKCθ in Th17 cell-dependent immune responses was reported [71]. CARMA1 (caspase recruitment domain-containing MAGUK protein 1) is a scaffolding protein, which is thought to link PKCθ to NF-κB [72]. This molecule is also required for the induction of the Th2 responses *in vivo*. CARMA1-dificient mice exhibit impaired Th2 cell-mediated allergic airway inflammation [73]. Signaling lymphocyte activation molecules (SLAM) associated protein (SAP), was shown to activate PKCθ and promote Th2 cell differentiation. SAP-deficient T cells showed impaired TCR-induced GATA3 activation and subsequent Th2 cell differentiation, and increased Th1 cell generation [74–76]. SLAM-deficient CD4 T cells exhibited a similar defect in Th2 cell differentiation [77]. SAP promotes the recruitment of PKCθ to the contact site, which is enhanced by the engagement of SLAM. The TCR-induced phosphorylation of Bcl10 and the NF-κB1/p50 nuclear translocation were both decreased in SAP-deficient CD4 T cells. Thus, SLAM, SAP, PKCθ, Bcl10 and CARMA1 are all required for the induction of Th2 cell differentiation through the activation NF-κB and resulting positive regulation of GATA3 expression.

4.6. TEC family tyrosine kinases

ITK is a member of the TEC family of non-receptor tyrosine kinase and plays a key role in the activation of PLCγ1 (Fig. 2). Therefore ITK-deficient T cells show a defect in the activation of the MAPK signaling pathway, the calcium influx and NF-AT [78]. ITK-deficient mice exhibited defects in the Th2 responses [79–81]. Using IL-4 reporter mice, ITK was found to be dispensable for early IL-4 expression during Th2 cell differentiation, however, it was critical for the IL-4 production in Th2 effector cells [82]. Another TEC family kinase, RLK/Txk, is preferentially expressed in Th1 cells in comparison to Th2 cells [83]. Although RLK mRNA is rapidly down-regulated after TCR stimulation, it is re-expressed in Th1 cells but not in Th2 cells [81]. In human T cells, the overexpression of RLK resulted in the increased production of IFNγ without affecting either the IL-2 or IL-4 production [84]. RLK has been reported to localize in the nucleus after receiving TCR-mediated stimulation, and it may control the transcription of the IFNγ gene [85]. It, therefore, remains to be elucidated whether the activation of RLK promotes Th1 cell differentiation, in addition to enhancing the production of IFNγ in Th1 cells. More recently, ITK was shown to be required for IL-17A production but not for IL-17F production [86]. ITK-dependent activation of NF-AT regulates IL-17A transcription in Th17 cells. The

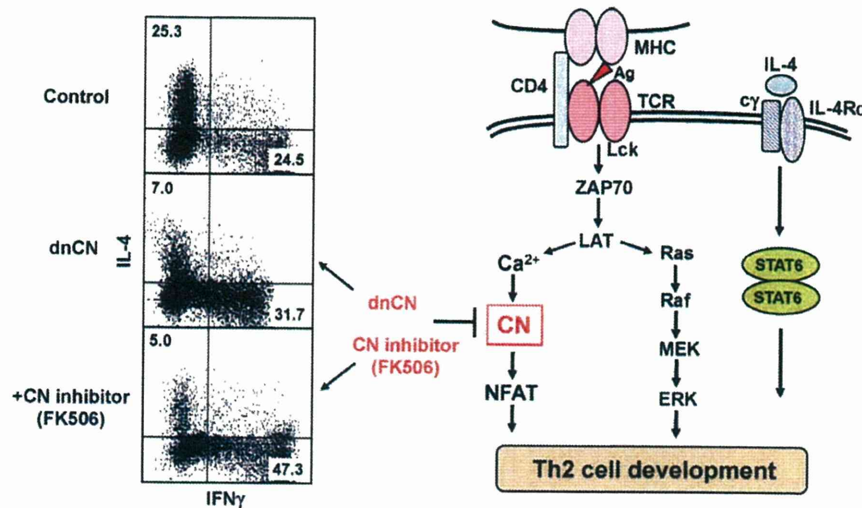

Fig. 4. Decreased activation of calcineurin resulted in decreased Th2 cell differentiation. Naïve CD4 T cells from normal C57BL/6 mice (control) and dominant-negative calcineurin Tg mice (dnCN) were stimulated with immobilized anti-TCR mAb in the presence of IL-4, and 5 days later, the cultured cells were harvested and then were subjected to the cytoplasmic staining with anti-IL-4 and anti-IFNγ. A specific inhibitor for calcineurin (FK506) was added to the control C57BL/6 CD4 T cell culture (+CN inhibitor). The percentages of the cells present in each quadrant are indicated [36].

expression of RORγt, which is the master regulator of Th17 cells, did not decrease in ITK-deficient Th17 cells. Taken together, the TEC family tyrosine kinases play important roles in various processes including the induction of the Th1, Th2 and Th17 responses (Fig. 4).

5. Concluding remarks

At least two distinct processes are required for the induction of each Th response, namely the TCR-mediated Th differentiation process and the TCR-induced Th cytokine expression process. The former represents the chromatin remodeling of the Th cytokine gene loci, while the latter involves the activation of specific transcription factors to induce the transcription of appropriate cytokines. Both of them are consequences of the TCR-mediated signaling events. Therefore, some signaling molecule downstream of TCR may only play a role in the former process, while others may be involved in only the latter process, but collectively they are required for the Th responses. In evaluating the experimental results, particularly those observed in the *in vivo* analysis, it will be necessary to clearly elucidate whether a given interesting molecule is involved in either process or both.

Essential for understanding CD4 T cell-mediated immunity will be a complete definition of the role of each signaling molecule in Th cell differentiation. This is becoming quite important from a clinical point of view because various inhibitors have been used clinically, and many other inhibitors can be expected to be developed in the near future. Since the direction of the generation of Th cell subsets is dependent on the overall signal strength, critical awareness is necessary for any inhibitors that modify the CD4 T cell-dependent immune responses in human patients. The effects of the inhibitors may depend on the dose of inhibitors, the duration of the intake, and also on the individual patients themselves.

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Application of NKT Cells in Immunotherapy

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Abstract: Invariant natural killer T (iNKT) cells are a conserved T cell sublineage and an important component of the innate immune system. The invariant T cell antigen receptor (TCR) α chain on iNKT cells interacts with glycolipid presented *via* CD1d on antigen-presenting cells (APCs), resulting in the production of a variety of cytokines, and thus bridging the innate and adaptive immune systems. In this review, we discuss two strategies of immune modulation that target iNKT cells using either liposomal α-galactosylceramide (α-GalCer) or α-GalCer-loaded APCs. Liposomal α-GalCer generates regulatory iNKT cells, which serve to induce regulatory T cells (Treg) and can be used to diminish immune responses as is seen in autoimmunity and allergic diseases. In contrast, α-GalCer-pulsed APCs generate stimulatory iNKT cells capable of releasing pro-inflammatory cytokines and leading to adaptive immune responses that can be used for treating malignancies. Here, we summarize the modalities used to manipulate the dual nature of iNKT cell function and their tremendous potential in treating both allergic and malignant disease.

Keywords: NKT cells, immunotherapy.

INTRODUCTION

Natural killer T (NKT) cells were originally defined as lymphocytes that had T-cell receptors (TCRs) and natural killer (NK) cell markers such as CD161 (NKR-P1) on their cell surface, and a capacity to show biological activities of both NK and T cells. However, a number of distinct types of NKT cells have been described. Invariant NKT (iNKT) cells have a single invariant TCRα chain encoding Vα14-Jα18 in mouse and Vα24-Jα18 in human and are restricted by CD1d, a non-classical major histocompatibility (MHC) molecule. Unlike classical MHC molecules which present peptides to T cells, CD1d binds lipids and presents them to iNKT cells [1, 2]. Another subclass of CD1d-restricted iNKT cells, type II NKT cells, are identified by particular TCRα chains, e.g. Vα3.2 and Vα8 [3]. And finally, some types of NKT cells having no CD1d-restriction had been reported, though these cells have not been fully characterized [4-6].

TCR on iNKT cells are composed of a single α-chain and a restricted number of β-chains, such as Vβ8, Vβ7, and Vβ2. Once assembled, the TCR of iNKT cells recognizes CD1d-loaded glycolipids [7, 8]. KRN7000 is the first synthetic ligand for iNKT cells, usually termed α-galactosylceramide (α-GalCer is used hereafter) [9-12]. Originally α-GalCer was discovered while screening compounds for anti-tumor effects [13], and Taniguchi *et al.* determined it as an iNKT cell ligand [14]. α-GalCer can be pulsed onto APC such as dendritic cells (DCs) and used to activate iNKT cells. Once activated, iNKT cells produce various cytokines such as IL-2, IL-4, IL-10, IL-13, IL-17, IL-21 and IFN-γ [15, 16].

Because of the unique ability of iNKT cells to bridge the innate and adaptive immune system, these cells have been proven to be valuable targets for clinical applications. The role of iNKT cells in tumor surveillance has been widely investigated since the original studies of anti-tumor effects of α-GalCer [17, 18]. iNKT cells are activated by α-GalCer-pulsed DCs. This iNKT cell activation causes DC maturation and IL-12 production, which in turn causes iNKT cells to produce IFN-γ for the activation of NK cells and anti-tumor cytotoxic T cells (CTLs). Hence, the delivery of α-GalCer to DCs and the efficient production of IL-12 from DCs are critical for the application to a tumor immunotherapy using iNKT cells.

IL-12 production by DCs skews the differentiation of naïve helper T cells toward the inflammatory type I (Th1), not the allergic type 2 (Th2). For this reason, increased IL-12 secretion by DCs could attenuate Th2-dominant allergic diseases. iNKT cells activated by α-GalCer produce IL-4 as well as IFN-γ, so it was initially unclear whether *in vivo* treatment with α-GalCer would activate or suppress allergic responses. However, Cui *et al.* showed that Th2 differentiation and IgE antibody formation were suppressed in α-GalCer-treated wild type mice, but not in iNKT-deficient or IFN-γ-deficient mice, suggesting that Th1 type iNKT cells are most likely responsible for these findings [19].

In addition to Th1 cytokine production, Th2 cytokine production by iNKT cells could be clinically useful for its

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