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T Cell Accumulation in B Cell Follicles Is Regulated by Dendritic Cells and Is Independent of B Cell Activation

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Abstract

We investigated the mechanism of CD4 T cell accumulation in B cell follicles after immunization. Follicular T cell numbers were correlated with the number of B cells, indicating B cell control of the niche that T cells occupy. Despite this, we found no role for B cells in the follicular migration of T cells. Instead, T cells are induced to migrate into B cell follicles entirely as a result of interaction with dendritic cells (DCs). Migration relies on CD40-dependent maturation of DCs, as it did not occur in CD40-deficient mice but was reconstituted with $CD40^+$ DCs. Restoration was not achieved by the activation of DCs with bacterial activators (e.g., lipopolysaccharide, CpG), but was by the injection of OX40L–huIgG1 fusion protein. Crucially, the up-regulation of OX40L (on antigen-presenting cells) and CXCR-5 (on T cells) are CD40-dependent events and we show that T cells do not migrate to follicles in immunized OX40-deficient mice.

Key words: T lymphocyte migration • B lymphocytes • OX40 • CXCR5 • lymphoid follicles

Introduction

T-dependent humoral immune responses are characterized by the development of germinal centers (GCs)* in B cell follicles of the secondary lymphoid organs, the formation of immunological memory, and long-term production of specific antibodies by bone marrow (BM) plasma cells. Both memory B cells and long-lived plasma cells are generated in GCs. T cells provide help to initiate the GC, maintain B cell proliferation, cause differentiation within the GC, and finally deliver long-term survival signals (via CD40) that allow GC B cells to enter the memory pool. These events require the presence of T cells inside or close to B cell follicles. T cells located in the follicle have long been recognized and are thought to be antigen specific (1). These T cells are heterogeneous in phenotype and apparent function. Those located around the GC edge contain preformed CD40L rapidly expressed after TCR activation (2) and are likely to direct GC B cells into the memory pool. On the other hand, T cells inside the GC of human tonsils display a unique cytokine profile that seems to enhance their capacity to help B cell antibody production (3–5). Many of these T cells can be identified by the markers $CXCR\text{-}5^+$ $CD57^+$ $CCR7^-$ and are proposed as a new, specialized subset of Th cells called "follicular helper T cells" (3–5), which is neither Th1 nor Th2.

Some of the naive T cells first activated in T zones by peptide antigen presented by dendritic cells (DCs; reference 6), as a secondary event engage in cognate interaction with antigen-specific B cells, either in the T zones (7, 8) or after migration to the border of the follicle (9). After this T–B interaction, some of the B cells enter follicles to initiate GCs and a subset of the activated Th cells migrate with them. The activation-induced events that control T cell migration to the B cell follicles are not well understood. Roles for the costimulatory molecule OX40L and the chemokine receptor CXCR-5 have been proposed. CXCR-5 confers responsiveness to B lymphocyte chemokine (BLC; CXCL13), which is produced by follicular stroma cells (10, 11). CXCR-5 is constitutively expressed by recirculating B cells and is required for their migration into B cell follicles (12). It is also induced on T cells after antigenic stimulation (13) and most follicular T cells seem to express CXCR-5. T cells stimulated in the presence of OX40L transfectants up-regulate CXCR-5 transcription (14) and transgenic mice, expressing large amounts of OX40L on DCs, display an increased number of T cells in B cell follicles (15). In addition, human T cells were recently shown to up-regulate CXCR-5 after activa-

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**Abbreviations used in this paper:* BLC, B lymphocyte chemokine; BM, bone marrow; DC, dendritic cell; ELC, Epstein Barr virus–induced molecule 1 ligand chemokine; FDC, follicular DC; GC, germinal center; MIP, macrophage inflammatory protein.

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tion by DCs (16). However, CXCR-5 expression does not strictly correlate with T cell migration in B cell follicles. Upon transfer, purified CXCR-5^+ T cells do not migrate to the B cell follicles (13) and after immune challenge a large proportion of CXCR-5^+ T cells accumulate at the border between the T zone and the follicles, but do not enter (3, 5). These observations show that CXCR-5 expression alone is not sufficient for follicular localization and that ancillary factors are required.

Despite the data discussed above there is no direct evidence that interaction with DCs under normal circumstances in vivo controls migration of T cells to follicles. In fact, the selectivity of the process (only a fraction of T cells enter follicles) suggests that DCs, as the primary APCs that activate all naive T cells, are unlikely to be solely responsible for this migration. Indeed, several observations point to activated B cells as regulators of T cell migration to follicles. First, CXCR-5–expressing T cells accumulate at the follicular border after transfer (13), a place where B and T cells engage in cognate interaction (9). Second, we showed previously that the generation of T cell help for antibody production depends upon antigen presentation by B cells (17). Third, activated B cells are known to produce chemotactic factors acting selectively on primed T cells, e.g., IL-16 (18), macrophage inflammatory protein (MIP)-1α, MIP-1β (19), ABCD-1, ABCD-2 (20), and fractalkine (21). Finally, B cell control could explain the selectivity of the migration. T cells in contact with DCs would only migrate to inflammatory sites whereas those interacting with B cells would be induced to migrate to follicles. The involvement of B cells in this process has not yet been investigated and therefore we set out to analyze the respective roles of DCs and B cells. Our starting point in these studies was our observation that T cell migration to follicles is CD40 dependent as it was absent in CD40-deficient mice. To investigate further we used a number of novel BM chimera models to restrict expression of genes of interest to defined cell populations within lymphoid tissues.

Materials and Methods

Mice and Immunizations. C57BL6, CD40$^{-/-}$ (22), CD40L$^{-/-}$ (23), Aβ$^{-/-}$ (24), MD4-RAG (25), OT-II (26), and μMT (27) mice were bred and maintained in the Science Faculty Animal Facility at the University of Edinburgh under specific pathogen-free conditions. All mice were backcrossed onto the C57Bl/6 background between five (Aβ$^{-/-}$) and seven (CD40$^{-/-}$) generations. OX40-deficient mice (28) were provided by M. Kopf, Swiss Federal Institute of Technology Zürich, Zürich, Switzerland, and immunized in his lab. Immunizations involved injection of 100 μg alum-precipitated antigen (see below) intraperitoneally. 200 μg stimulating anti-CD40 (FGK-45) antibody was injected 6 h after immunization. B7.1–huIgG1 and OX40L–huIgG1 fusion proteins were injected intravenously at a dose of 200 μg daily from days 1 to 5 after immunization. 10 μg LPS, 10 μg CpG (5′-TCCATGACGTTCCTGATGCT-3′), and 10 μg non-CpG (5′-TCCATGAGCTTCCTGATGCT-3′) phosphorothioates oligonucleotides (MWG Biotech) and 2×10^9 chemically killed *Bordetella pertussis* (Calbiochem-Novabiochem) were all injected intraperitoneally at the same time as antigen. Antigens used for immunization were KLH (Calbiochem-Novabiochem), DNP-KLH, or DNP-OVA, prepared as previously described (29).

Antibodies and Fusion Proteins. Monoclonal antibodies to Thy1 (T24) and IA^d (MK-D6) were originally obtained from the American Type Culture Collection (ATCC). Monoclonal antibodies AF6-120.1 (anti-IA^b), HL3 (anti-CD11c), RM134L (anti-OX40L), and biotin-labeled MR9-4 (anti-Vβ5) were all purchased from BD Biosciences. Ly-2 (anti-CD8a) was purchased from Caltag. Sheep anti–mouse IgD was purchased from The Binding Site. Anti-CD40, FGK-45 (30), was provided by A. Rolink, Basel Institute for Immunology, Basel, Switzerland. B7.1–huIgG1 fusion protein was provided by P. Lane, University of Birmingham, Birmingham, United Kingdom, and the OX40L–huIgG1 was provided by Cantab Pharmaceuticals Research Ltd. (31). The antibody to mouse CXCR5 (clone 2G8) was provided by M. Lipp, Max Delbrück Center for Molecular Medicine, Berlin, Germany.

BM Chimeras. Recipient mice were lethally irradiated (1150cGy γ radiation delivered from caesium source) on day 0. On day 1, BM was extracted from the femurs of donor mice and T cells were depleted by negative selection using anti-Thy1(T24)-biotin and Streptavidin microbeads (Miltenyi Biotec). Separation was performed on a MACS magnetic column (Miltenyi Biotec) according to manufacturer's instructions. 5×10^6 BM cells were injected intravenously into the irradiated recipients. The chimeras were used after 8 wk to allow complete reconstitution.

To construct BM chimeras in which gene expression was restricted to defined cell populations, we had to completely replace the host hematopoietic system with donor BM cells. To be certain that our method of constructing chimeras did not allow significant outgrowth or retention of host cells, we tested fully allogeneic chimeras 8 wk after reconstitution. When BM from BALB/c mice (H-2^d) is transferred into lethally irradiated (1150 cGy) C57BL6 mice (H-2^b), >99% of peripheral B cells and DCs are of donor origin as assessed by FACS® staining with IA^d-specific (MK-D6) and IA^b-specific (AF6-120.1) antibodies (unpublished data). In addition, essentially all DCs generated in vitro by culture of BM cells from these chimeras in the presence of GM-CSF are of the donor MHC haplotype (unpublished data).

To assess the differential function of molecules (e.g., CD40 or MHC class II) that are expressed on both B cells and DCs, the following mixed BM chimera system was used, with the result that CD40 was expressed only on DCs and was lacking on B cells. Irradiated (1150 cGy) C57Bl/6 or μMT mice, carrying a gene deletion of the μ heavy chain causing a block in B cell differentiation (27), were reconstituted with BM from μMT mice (80%) and CD40$^{-/-}$ mice (20%). Thus, 80% of the hematopoietic cells (except B cells) in the chimeric mice will be wild-type in gene expression whereas the B cells can only be derived from CD40$^{-/-}$ precursors. Over an 8-wk period, the 20% knockout BM completely repopulated the peripheral lymphoid system with B cells while contributing only 20% to other lineages. In this way, we ensured that the function of DCs was minimally impaired whereas CD40 function in B cells was abolished. To ensure that all the B cells were CD40$^{-/-}$, we stimulated purified B cells from these chimeric mice with anti-CD40 and IL-4 and measured proliferation. This assay, which detected 1% spiked contamination of wild-type B cells in a CD40$^{-/-}$ spleen culture, showed no proliferative response from these chimeras.

DCs and Macrophages. DCs were derived from BM cells according to the procedure developed by Inaba et al. (32). In brief,

BM cells were cultivated in RPMI plus 10% FCS supplemented with GM-CSF (X63-GM-CSF–producing cell line supernatant; reference 17). On the third day, nonadherent cells were washed away and remaining cells were cultivated in RPMI 1.5% mouse serum supplemented with GM-CSF. An additional wash was performed on day 6. On day 7, the culture typically contained >90% pure BM-DCs. DCs were pulsed for 3–6 h with 300 μg/ml DNP-KLH or 100 μg/ml KLH or OVA. 10^6 cells were then injected intravenously. In some experiments, mice received a concurrent intraperitoneal injection of alum-precipitated antigen.

Splenic DCs were purified according to the protocol previously described (33). Contaminating B cells were depleted using anti-CD19–coated Dynabeads (Dynal). This gave a preparation containing 80–90% pure DCs.

Macrophages were derived by culturing BM cells in DMEM plus 25% FCS, supplemented with 20% M-CSF–containing supernatant (from the L929 cell line; ATCC). Nonadherent cells were washed off the plates at days 4 and 6 and the macrophages were harvested from the plates at day 7, pulsed with antigen, and injected as described above for DCs.

OT-II T Cell Transfers. 2.5×10^6 lymph node cells from OT-II TCR transgenic mice (H2-A^b–restricted OVA peptide, 323–339-specific) backcrossed onto a CD40-deficient background representing 1.5×10^6 transgenic T cells were transferred intravenously into nonirradiated recipient mice. After 24 h, the mice were immunized with either 100 μg OVA peptide or 100 μg KLH in CFA. Accumulation of cells in follicles or FACS® analysis was performed 4–6 d after immunization. OT-II TCR transgenic T cells were recognized by their expression of Vβ5.

Immunohistology. Spleens were harvested, embedded in Cryo-M-Bed embedding compound (Bright Instrument Company Ltd.), and frozen at −80°C. 5-μm thick frozen sections were fixed on cold acetone and dried extensively. The sections were stained with T24 (anti–Thy-1) for T cells and anti-IgD for B cells (The Binding Site). Biotinylated anti–sheep/goat immunoglobulin was added 2 h later. Streptavidin–Texas Red (Southern Biotechnology Associates, Inc.) and FITC-conjugated $(Fab')_2$ mouse anti–rat (Jackson ImmunoResearch Laboratories) were added 1 h later.

Quantitative Histology. B cell follicles were delineated in splenic tissue sections by staining with anti-IgD. T cells were identified as $Thy1^+$ cells. Slides were viewed on an Olympus BX50 microscope under reflected light fluorescence. Images were captured using a Hamamatsu digital camera and Openlab image analysis software (Improvision). The number of T cells within the IgD^+ area was counted. For each mouse in a group, 10–15 follicles were counted in this way. The area of the follicles (in arbitrary units) was quantified using the Openlab program. The number of B cells occupying any follicular area could be calculated from this by extrapolation from previous counts of B cells in defined follicular areas. The total follicular area counted in all mice varied by <5%. The number of follicular T cells was expressed per follicular section. In some analyses this was plotted against the number of B cells within the follicular section or the number of T cells in the adjacent T cell zone.

Results

T Cell Accumulation in B Cell Follicles Requires CD40 and Is Proportional to Follicular Size. The dynamics of T cell accumulation in B cell follicles were assessed in normal C57BL6 mice after immunization with DNP-OVA. The number of T cells per follicle progressively increased from around day 4 when it was first noticeable and peaking between days 6 and 12, with on average 63 T cells per follicle (Fig. 1 A). As Fig. 1 shows, there is some variation around the median number of T cells per follicle. To see if this variation was related to the number of responding B cells, space within the follicle, or the number of responding T cells in the adjacent T zone, we measured the area of each follicle counted and the area of the neighboring T zone. It is clear from Fig. 2 that the number of follicular T cells is

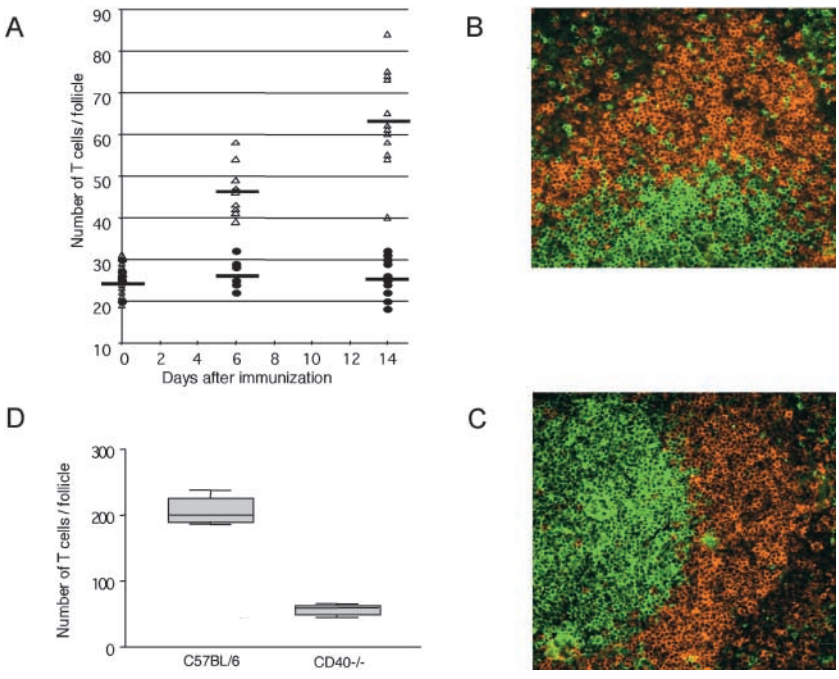

Figure 1. Antigen-driven accumulation of T cells in B cell follicles is dependent on CD40. (A) The number of $Thy1^+$ T cells in follicles plotted against time after immunization with alum-precipitated DNP-OVA in normal C57Bl/6 mice (△) or $CD40^{-/-}$ mice (●). Each point represents a count from a single follicle. The bars show the median values. The data is a compilation from three mice in each group. This result is representative of four such experiments. (B and C) Immunohistological stain showing T cells in B cell follicles in C57Bl/6 (B) and $CD40^{-/-}$ mice (C) 6 d after immunization. Note the relative lack of green $Thy\text{-}1^+$ cells in the IgD^+ follicle of the $CD40^{-/-}$ mouse. (D) The number of OT-II TCR transgenic T cells (identified by Vβ5 staining) in inguinal lymph node follicles in normal (C57Bl/6) and $CD40^{-/-}$ mice 6 d after immunization in the footpad with 100 μg OVA peptide (323–339) plus CFA.

proportional to the size of the follicles but not to the size of the T zone. The presence of GCs in follicles made no difference to this analysis and conclusion. There is a remarkably stable ratio of 4 T cells to 100 B cells in the follicles. This indicates an influence of the B cell compartment on the accumulation of T cells in the follicle.

In contrast to normal C57BL6 mice, the number of follicular T cells in $CD40^{-/-}$ mice was unchanged after immunization (Fig. 1 B). We also observed no migration of T cells to follicles in $CD154^{-/-}$ ($CD40L^{-/-}$) mice after immunization (unpublished data). Therefore, the accumulation of Th cells in B cell follicles that occurs after immunization is CD40 dependent. Follicles in nonimmunized C57BL6 and $CD40^{-/-}$ mice contained an average of 25 T cells. This constitutive, CD40-independent presence of T cells in follicles may represent localization of memory T cells or possibly the (CD1-restricted) NK1.1 CD4 T cells seen in the follicles of MHC class II–deficient mice (24). We confirmed that the migration of T cells to follicles was antigen specific by transferring OT-II H2-A^b–restricted, OVA peptide–specific TCR transgenic T cells into normal (B6) or $CD40^{-/-}$ mice and then immunizing with either OVA or KLH. Fig. 1 D shows that OT-II localize to follicles after OVA immunization in normal mice but to a much lesser extent in $CD40^{-/-}$ mice. We were unable to see any $V\beta5^+$ OT-II T cells in the follicles after transfer when mice (normal or CD40-deficient) were immunized with the irrelevant antigen, KLH (unpublished data).

Expression of CD40 Is Required on Hematopoietic Cells for T Cell Accumulation in B Cell Follicles. To determine the cell type on which CD40 was required, we took the approach of making mixed BM chimeras in which the expression of CD40 could be restricted to defined populations of cells. First, to validate this methodology, we performed C57Bl/6 BM → C57Bl/6 transfers and the resulting chimeras showed normal T cell migration to follicles comparable to nonmanipulated C57Bl/6 mice (Fig. 3 A), whereas $CD40^{-/-}$ BM → $CD40^{-/-}$ chimeras did not. Thus, the process of irradiation and reconstitution did not impair quantitatively or qualitatively the behavior of T cells.

To assess whether hematopoietic and/or non-BM-derived stromal cells were necessary for T cell accumulation in follicles, we made chimeras in which all the hematopoietic cells were replaced with CD40-deficient BM ($CD40^{-/-}$ BM → C57Bl/6). In such mice, radio-resistant stromal cells (e.g., follicular DCs [FDCs]) in lymphoid tissues would be $CD40^+$ whereas all BM-derived lineages would be $CD40^-$. FDCs express CD40 and produce chemokines such as BLCs (CXCL13), which cause the migration of B cells to follicles (10, 12). In these ($CD40^{-/-}$ BM → C57Bl/6) chimeras, T cells did not accumulate in follicles after immunization (Fig. 3 A). In contrast, C57Bl/6 BM → $CD40^{-/-}$ chimeras, where all hematopoietic lineages were $CD40^+$ but stroma was $CD40^-$, exhibited normal T cell migration to follicles. Taken together, these data demonstrated a crucial role for CD40-expressing hematopoietic cells in follicular migration of T cells. The obvious candidates to investigate further were DCs and B cells.

Expression of CD40 on B Cells Is Not Required for T Cell Accumulation in B Cell Follicles. In the light of our previous data on the influence of B cells on T cell differentiation (17) and the observation that follicular T cell number was related to follicular size (Fig. 2), we chose first to investigate the possibility that B cells regulate T cell entry into follicles. To test this hypothesis, we developed mixed BM chimeras in which the CD40 deficiency was restricted to the B cell compartment. Thus, chimeras were generated where all B cells lack CD40 but the majority (80%) of the DCs are $CD40^+$. This was achieved by injecting a mixture of BM from μMT mice (80%) and $CD40^{-/-}$ mice (20%) into lethally irradiated (11.5Gy) C57BL6 mice. The μMT BM gives rise to no B cells and therefore all B cells in these chimeras were derived from $CD40^{-/-}$ hematopoietic stem cells. The other hematopoietic cell types were mostly derived from the μMT BM as it made up 80% of the BM inoculum and were therefore wild type with respect to CD40. There was a complete replacement of the host B cell system in these chimeras and any residual host ($CD40^+$) B cell contamination was significantly <1%. In these chimeras, T cells accumulated normally in B cell follicles after immunization (Fig. 3 B). Therefore, expression of CD40 on B cells is not required for the migration. No IgG response or GC formation was seen in these chimeras (unpublished data).

Antigen Presentation by B Cells Is Not Required for T Cell Accumulation in B Cell Follicles. Although the previous experiment excludes a role for CD40 on B cells, their presen-

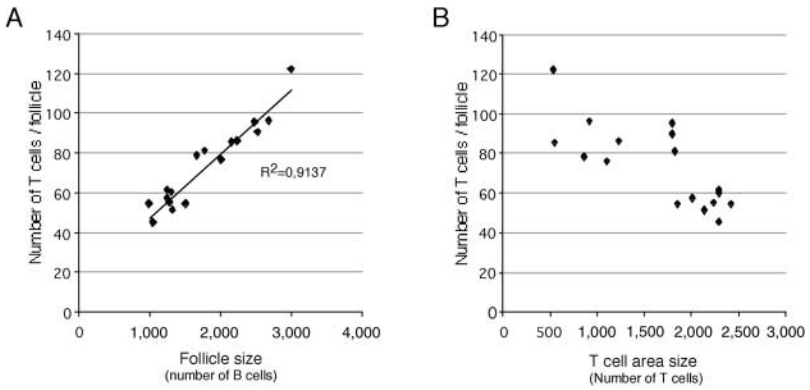

Figure 2. T cell accumulation in B cell follicles is correlated with the follicular size. Spleens from C57Bl6 mice were collected 6 d after immunization with alum-precipitated DNP-KLH intraperitoneally. Each point represents a single follicle. The data are a compilation of counts of T cells in splenic follicles from three mice. The result is representative of two such experiments. (A) The number of Thy-1^+ T cells present in a follicle is proportional to the size of the follicle ($P < 0.001$, using Pearson correlation coefficient). (B) There is no correlation between the number of follicular Thy-1^+ cells and the size of the adjacent T cell area.

tation of MHC class II–peptide complexes during a T–B interaction might still be important. This was tested directly by constructing chimeras in which DCs (and macrophages) express MHC class II but B cells do not. Thus, we injected a mixture of μMT (80%) and $A\beta^{-/-}$ (20%) BM into lethally irradiated μMT recipients. In these chimeras, B cells can capture and process the antigen but they are unable to present it to Th cells (although T cells can be activated by DCs). Fig. 4 A shows that Th cells accumulated in B cell follicles in normal numbers in these mice. Therefore, cognate interaction between B and T cells is not required for T cell accumulation in B cell follicles. Additional experiments showed that follicular localization of T cells occurred in the absence of any B cell activation. Thus, in BM chimeras (μMT + MD4-RAG BM → μMT) in which all the B cells are hen egg lysozyme (HEL)-specific (on $RAG1^{-/-}$ background), immunization with KLH provoked normal T cell migration to follicles (unpublished data).

Expression of CD40 on DCs Is Necessary for T Cell Accumulation in B Cell Follicles. If B cells do not control migration, the next likely candidates are DCs. Indeed, mixed BM chimeras in which CD40 expression was restricted to non-B, nonstromal cells (μMT + $CD40^{-/-}$ BM → $CD40^{-/-}$) exhibited normal follicular migration of T cells (Fig. 3 B), implicating myeloid cells including DCs. Using BM chimeras to restrict the effects of a gene knockout to the DC population was not possible in the absence of a mouse line that specifically lacks DCs. Therefore, we asked directly if expression of CD40 on DCs was sufficient to promote T cell migration to B cell follicles. This was done by injecting CD40-deficient mice with antigen-pulsed DCs generated from $CD40^{+}$ or $CD40^{-}$ BM (by growth in

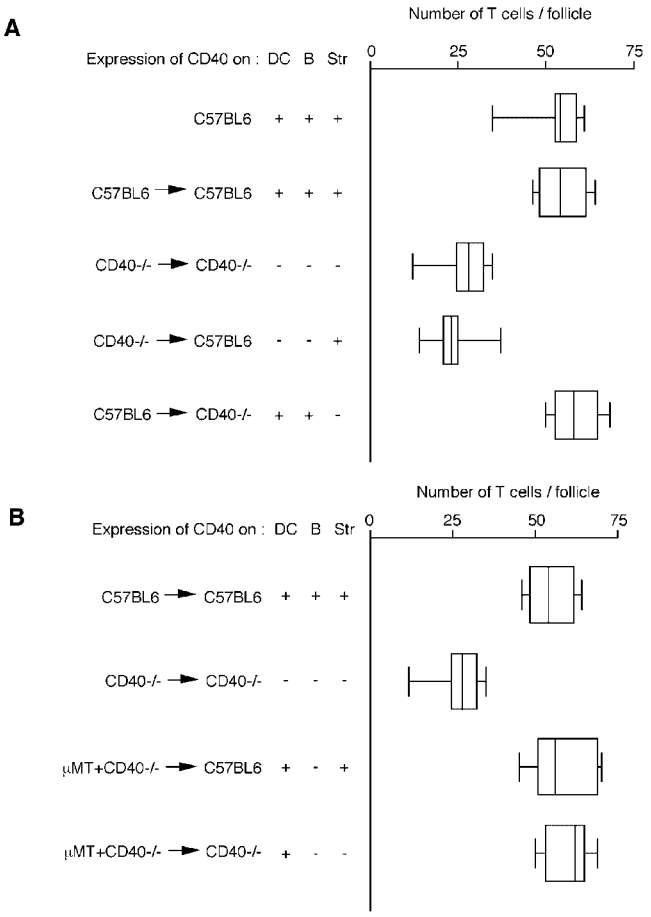

Figure 3. Cell interactions involved inCD40-dependent accumulation of T cells in B cell follicles. Chimeras were immunized with alum-precipitated DNP-KLH intraperitoneally 8 wk after reconstitution with BM cells. Spleens were collected 6 d after immunization. (A) Follicular T cell localization requires CD40 on hematopoietic cells. (B) Follicular T cell localization does not require CD40 on B cells. The data were plotted using a box and whiskers representation. The box extends from the 25th to the 75th percentile, with a horizontal line at the median (50th percentile). Whiskers extend down to the smallest value and up to the largest. The data are a compilation of counts of T cells in splenic follicles from three mice. These results (A and B) are representative of three such experiments. Donor BM is shown to the left of the arrow and the irradiated recipient to the right. CD40 phenotype of DCs, B cells, and radio-resistant stromal cells (Str) in each chimera is shown.

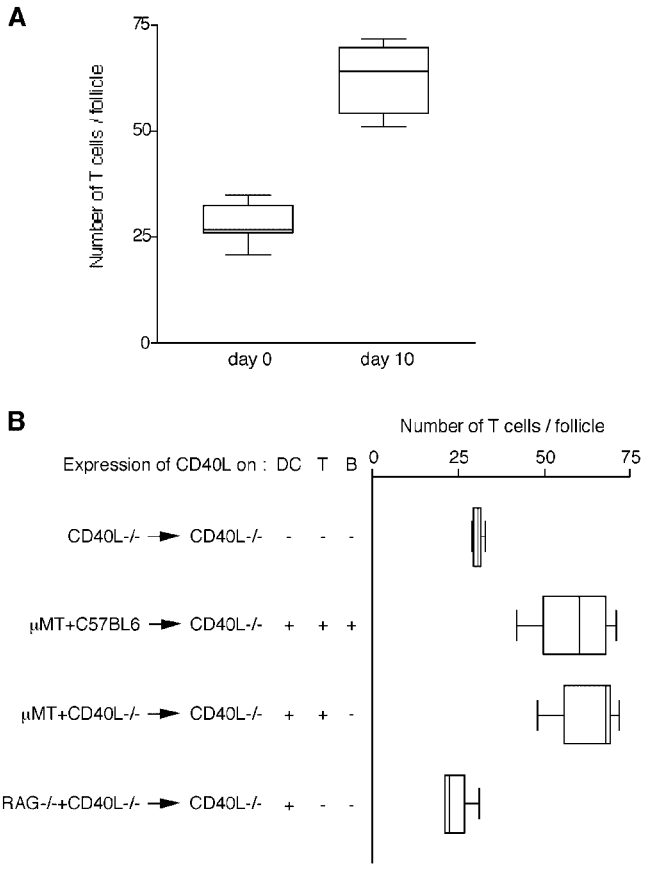

Figure 4. T cell accumulation in lymphoid follicles does not require MHC class II nor CD40L expression by B cells. (A) Follicular T cell localization in chimeras lacking MHC class II on B cells (μMT [80%] + $IA\beta^{-/-}$ [20%] → μMT). (B) Follicular accumulation of T cells requires CD40L expression by T cells but not B cells. The CD40L phenotype of DCs, T cells, and B cells in each chimera is shown. Chimeras were immunized intraperitoneally with alum-precipitated DNP-KLH 8 wk after reconstitution with BM cells. Spleens were collected 6 (B) or 10 d (A) after immunization. The data are a compilation of counts of T cells in splenic follicles from three (A) or four mice (B), analyzed as described in Fig. 3. The result is representative from three such experiments.

GM-CSF cultures). T cell localization in follicles was rapidly restored by $CD40^+$ but not by $CD40^{-/-}$ DCs (Fig. 5). DCs ($CD8\alpha^-$) purified from the spleen were also potent inducers of follicular T cell migration in $CD40^{-/-}$ mice (unpublished data). It is possible that macrophages could also cause T cell accumulation in follicles. To test this, we derived macrophages from cultures of BM cells grown for 7 d in M-CSF. Upon antigen pulsing and transfer to $CD40^{-/-}$ mice, these macrophages were unable to reconstitute follicular T cell migration (Fig. 5 A).

CD40L Is Required on T Cells for Them to Accumulate in the Follicles. The results so far suggest that T cell accumulation in the follicles depends on a CD40-dependent maturation of DCs. To learn more of this maturation, we investigated which cell type delivered CD40L to the DC. T cells (34, 35), B cells (36), and DCs (37) have all been shown to express CD40. Delivery of a signal from the CD40L expressed by B cells (36) might explain the selective nature of the migration. To dissect this, we constructed chimeras where expression of CD40L was restricted to the DC by injecting a mixture of BM from $RAG1^{-/-}$ (80%) and $CD40L^{-/-}$ (20%) mice into $CD40L^{-/-}$ mice (RAG1 + $CD40L^{-/-} \rightarrow CD40L^{-/-}$). In these mice, all lymphocytes are derived from $CD40L^{-/-}$-deficient BM whereas DCs are derived mainly from $RAG^{-/-}$ BM. We also constructed chimeras in which DCs and T cells, but not B cells, expressed CD40L (μMT + $CD40L^{-/-} \rightarrow CD40L^{-/-}$) and chimeras in which DCs, T cells, and B cells expressed CD40L (μMT + C57BL6 $\rightarrow CD40L^{-/-}$). Fig. 4 B shows that T cell migration occurs when both T cells and DCs express CD40L. Because expression of CD40L by DCs alone is not sufficient (Fig. 4 B), we infer that CD40L signals are delivered by T cells to DCs.

T Cell Migration into B Cell Follicles Requires CD40 Activation and Cannot Be Elicited by LPS, CpG DNA, or B. pertussis. We wished to know whether other activators of DCs (38), in particular those derived from microorganisms, could replace the CD40 signal to allow follicular T cell migration in $CD40^{-/-}$ mice. $CD40^{-/-}$ mice were immunized with DNP-KLH together with LPS, CpG DNA, or chemically killed *B. pertussis*. None of these adjuvants would permit T cell accumulation in the follicles at days 5 or 9 (Fig. 6). In contrast, injection of a stimulating anti-CD40 antibody (FGK-45) into $CD40L^{-/-}$ mice restored T cell migration to follicles (Fig. 6).

T Cell Accumulation in B Cell Follicles Is Dependent on OX-40 Signals. OX40 signals have been implicated in the migration of T cells to follicles (14, 15, 39). We investigated whether the CD40-dependent event on DCs was the up-regulation of OX40L. As shown in Fig. 7 A, OX40L was most efficiently induced on DCs by anti-CD40. LPS had no effect and CpG DNA only had a marginal one (Fig. 7 A). In B cells, OX40L was also only up-regulated in the presence of CD40 signals (unpublished data). To see if a lack of OX40L expression could be a major cause of the migration defect in $CD40^{-/-}$ mice, we treated them with OX40L–huIgG1 fusion protein. An agonistic costimulatory role for this fusion protein has previously been shown (31). Fig. 7 B shows that OX40L–huIgG1 restored T cell migration to follicles when injected into $CD40^{-/-}$ mice whereas B7.1–huIgG1 did not. This data, taken in conjunction with the conclusion that CD40-expressing DCs are necessary for follicular T cell migration, implies that up-regulation of OX40L on DCs is the inductive event. We confirmed the role of OX40 in follicular migration of T cells by analyzing their accumulation after the immuniza-

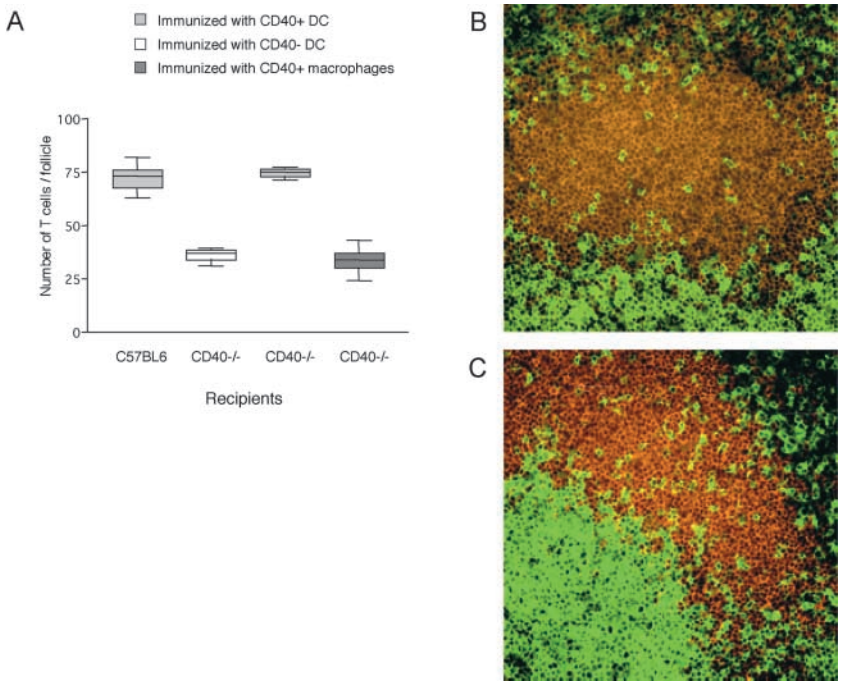

Figure 5. T cells accumulate in B cell follicles of $CD40^{-/-}$ mice immunized with $CD40^+$ DCs. (A) $CD40^{-/-}$ and C57Bl6 mice were immunized by intravenous injection of 10^6 $CD40^+$ (light gray boxes) or CD40-, BM-derived DCs (white box) or macrophages (dark gray box) pulsed with DNP-KLH. Spleens were collected 4 d after immunization. The data are a compilation of counts of T cells in splenic follicles from three mice, analyzed as described in Fig. 3. (B and C) Immunohistological stain showing T cells in B cell follicles in $CD40^{-/-}$ mice immunized with $CD40^{-/-}$ (B) and $CD40^+$ DCs (C). Note the relative lack of green Thy-1^+ cells in the IgD^+ follicle of the $CD40^{-/-}$ mouse immunized with $CD40^{-/-}$ DCs. This experiment was repeated three times with similar results.

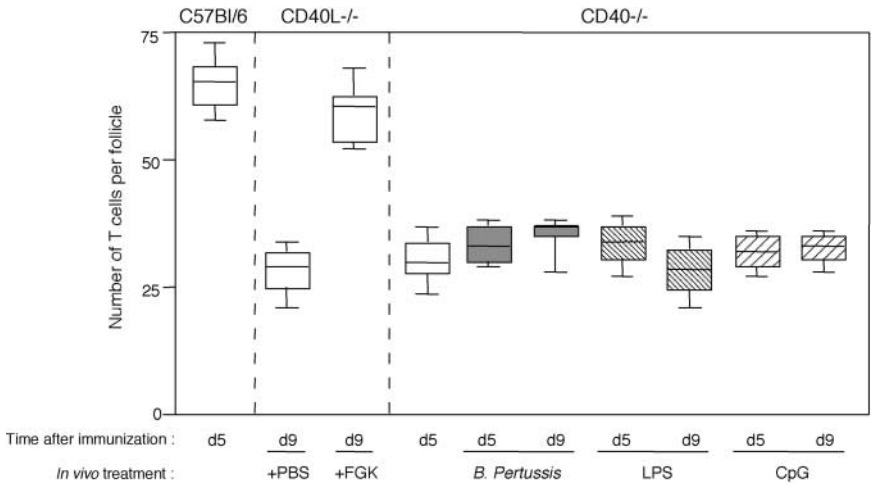

Figure 6. T cell accumulation in B cell follicles requires a CD40 signal, which cannot be substituted by microorganism-derived activators of DCs. $CD40^{-/-}$ mice were immunized intraperitoneally with alum-precipitated DNP-KLH together with one of the following products: chemically killed *B. pertussis*, LPS, or CpG, all at the doses indicated in Materials and Methods. $CD40L^{-/-}$ mice were injected once with 200 μg FGK-45 intravenously 6 h after immunization with alum-precipitated DNP-KLH. The data are a compilation of counts of T cells in splenic follicles from three mice, analyzed as described in Fig. 3. This result is representative of two similar experiments.

tion of OX40 knockout mice. As shown in Fig. 7 C, the OX40-deficient mice show little antigen-driven accumulation of T cells in follicles.

CXCR5 Induction on Naive T Cells Is Antigen Specific and Impaired in CD40-deficient Mice. To see if CXCR5 induction on T cells in vivo is an antigen-driven, CD40-dependent event, we transferred 2.5×10^6 OT-II T cells (from H2-A^b–restricted, OVA peptide–specific TCR transgenic mice, backcrossed to a CD40-deficient background) into normal and CD40 knockout mice and immunized them with OVA or KLH (in CFA). We could see up-regulation of CXCR5 on the majority (66%) of Vβ5 (OT-II β chain)-expressing T cells in OVA-immunized normal mice, but relatively few (19%) in KLH-immunized mice (Fig. 7 D). The up-regulation of CXCR5 expression on antigen-activated T cells in $CD40^{-/-}$ mice was impaired despite blasting, proliferation, and up-regulation of CD44 (unpublished data).

Discussion

The migration of T cells into B cell follicles is a selective process as only a fraction of the T cells activated in the first days after immunization have the capacity to localize here. T cells are required in follicles to provide help for the development of high affinity antibodies and memory B cells. However, the transfer of information between T and B cells is not unidirectional as B cells also influence T cell differentiation by eliciting Th2 responses advantageous to the B cells (17, 40–42). For these reasons it seemed likely that B cells would regulate the process of migration and entry into follicles. However, the evidence presented here shows

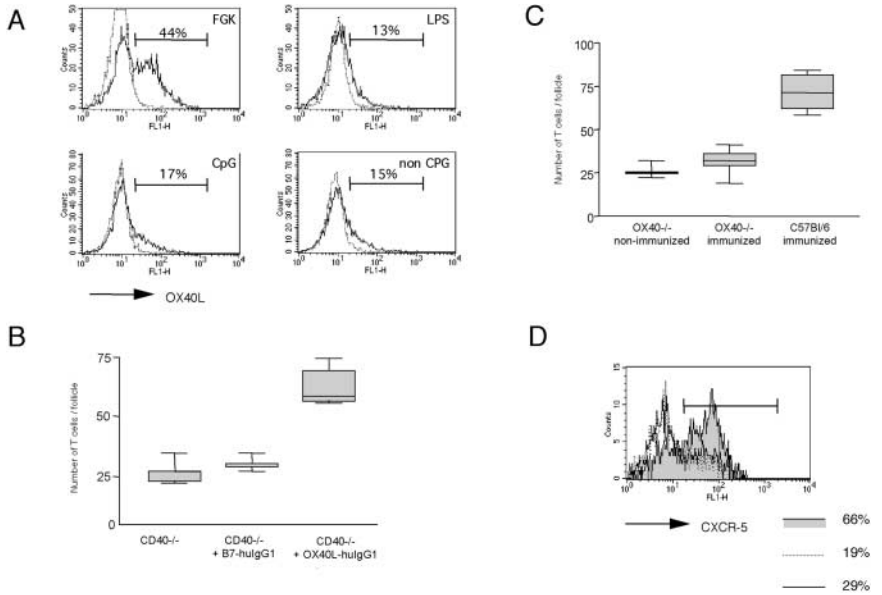

Figure 7. The role of OX40L on DCs and B cells in follicular migration of T cells. (A) Expression of OX40L on purified splenic DCs stimulated for 48 h with FGK-45 (anti-CD40), LPS, CpG DNA, and non-CpG DNA (as indicated). Data were collected and analyzed on a FACScalibur® using CELLQuest™ software. Dead cells are excluded using 7AAD. DCs were gated on $CD11c^+$ cells. The dotted line shows the staining profile of isotype-matched controls. (B) T cell migration to follicles is reconstituted in $CD40^{-/-}$ mice by injection of OX40L–huIgG1. Mice were immunized intraperitoneally with alum-precipitated DNP-KLH and then injected daily intravenously from days 1 to 5 with 200 μg B7–huIgG1 or OX40L–huIgG1. Spleens were collected at day 6. This result is representative of two similar experiments. (C) T cell migration to follicles is impaired in OX40-deficient mice. Spleens were taken from knockout and control mice (five of each) 8 d after immunization. The data are a compilation of counts of T cells in splenic follicles from three to five mice and analyzed as described in Fig. 3. (D) Up-regulation of CXCR5 on T cells is antigen specific and CD40 dependent. FACS® plots of CXCR-5 expression on OVA-specific OT-II ($V\beta5^+$) $CD4^+$ T cells after transfer into normal (filled profile) and $CD40^{-/-}$ mice (solid line) after immunization with OVA (323–339) peptide or normal mice immunized with KLH. Data is representative of two experiments.

that B cells do not direct T cell migration in any immediate or obvious way. Rather, T cell migration to follicles is controlled by DCs.

Our initial observation was that T cells do not accumulate in the follicles of CD40-deficient mice. Restoration of CD40 expression on DCs is sufficient to reinstate T cell localization. Furthermore, a selective absence of CD40 on B cells (in BM chimeras) had no effect on follicular migration of T cells. Interestingly, T cell activation in these chimeras (in which CD40 on DCs was normal) is unimpaired. Proliferative responses and cytokine production are similar to T cells from control chimeras when isolated 10 d after immunization and restimulated in vitro (unpublished data). Thus, B cells that lack CD40 are not inherently tolerogenic as reported previously for allogeneic responses (43, 44). Cognate interaction between B and T cells, thought to occur at the margins of the follicles, is also dispensable as T cells migrated to follicles in chimeras in which B cells expressed no MHC class II (DCs in these mice were MHC class II^+). Therefore, B–T cell contact involving antigen presentation and CD40 ligation is unnecessary for T cell migration to follicles. Another possible means of B cell action is through the release of chemokines or cytokines after BCR cross-linking with antigen. These could initiate T cell migration directly or induce the up-regulation of receptors that would allow T cells to respond to a chemotactic gradient originating from the follicular stromal cells (e.g., BLC; references 10 and 11). However, in chimeras (μMT + MD4-RAG BM $\rightarrow$ μMT) where there is no possibility of B cell activation via the BCR, T cells still migrated to follicles. Thus, the chemokines made by activated B cells, ABCD-1, ABCD-2 (20), fractalkine (21), IL-16 (18), MIP-1α, and MIP-1β (19) do not seem to be required to attract T cells to follicles. However, they might be important at a later stage to bring follicular T cells into GCs.

The observation that activated B cells do not attract T cells to lymphoid follicles suggests to us that the migration of T cells to the follicles is not solely for the purpose of helping B cells, but also for reasons related to normal T cell differentiation pathways. For instance, the interactions that occur in the follicle may optimize clonal expansion of T cells. There is evidence that the follicle is a major site of CD4 T cell proliferation (45, 46). It is also intriguing that Fas-expressing activated B cells can protect activated T cells from activation-induced cell death (47). In vivo this is likely to occur in the B cell follicle. Survival of cells after activation-induced cell death is likely to be related to the formation of the memory pool and the action of B cells as Fas decoys might be the basis of their role in the development or maintenance of CD4 T cell memory that we (48) and others (49) have shown. It may not be coincidence that markers that distinguish various memory T cell subsets, e.g., the chemokine receptors CCR7 and CXCR-5 (3–5, 50–52), are important for the positioning of T cells in or out of the lymphoid follicles, suggesting a link between passage through the follicle and differentiation into these subsets.

The strong correlation of the number of T cells accumulating in B cell follicles with the size of the follicle might be related to the B cell control of events within the follicle as discussed above. At the peak of the response, we could detect 4 T cells per 100 B cells in the follicle, whether the B cells were responding to antigen or not (e.g., in μMT + MD4-RAG BM $\rightarrow$ μMT chimeras). This indicates that the process of accumulation in the follicle is limited by factors present in follicles and is unrelated to the scale of T cell activation in the T zone. Such factors might be constitutively delivered by B cells, follicular stroma cells such as FDCs, or DCs present within the follicle. They could act by regulating T cell proliferation, survival, entry, or exit from the follicle.

Our data demonstrate that DCs control the initial migration of T cells into follicles. T cell migration was restored in $CD40^{-/-}$ mice when they were immunized with $CD40^+$ DCs. In addition, because treatment of $CD40L^{-/-}$ mice with a stimulating anti-CD40 antibody allowed T cell migration to occur, the important step is the maturation of the DCs induced via CD40. It is important to note that T cell migration could not be reinstated in CD40-deficient mice by immunizing with products derived from microorganisms, which are known activators of DCs (e.g., LPS and CpG). This result emphasizes the importance of CD40 in the development of helper function. In the absence of CD40-dependent maturation, DCs prime $CD4^+$ T cells as demonstrated by the normal clonal expansion and T-dependent IgM response in $CD40^{-/-}$ mice (40), but these primed T cells fail to develop effector functions. They do not accumulate in B cell follicles and do not develop helper function to support GC formation or an IgG response by adoptively transferred wild-type B cells (40 and unpublished data). However, both GCs and IgG are restored when wild-type splenocytes (including DCs and B cells) are transferred into $CD40^{-/-}$ mice (unpublished data). Similarly, the generation of CTLs also requires a CD40-dependent maturation of DCs that cannot be achieved by LPS (53).

The CD40-dependent maturation of DCs required for T cells to accumulate in B cell follicles seems to consist of the acquisition of specific costimulatory capacity, as it can be overcome by the injection of OX40L–huIgG1 (but not B7.1–huIgG1) into $CD40^{-/-}$ mice. The crucial role of OX-40 is confirmed, as OX40-deficient mice show essentially the same defect in follicular T cell accumulation as $CD40^{-/-}$ mice. As we also show that up-regulation of CXCR-5 on activated T cells is impaired in $CD40^{-/-}$ mice, we surmise that CD40 signals to DCs induce OX40L, which in turn stimulates CXCR-5 expression by T cells. Our results are in agreement with those of Flynn et al. (14) and Brocker et al. (15) who have shown that stimulation of T cells via OX40L resulted in CXCR-5 expression in vitro (14) and increased accumulation of T cells into the follicles in vivo (15). The expression of OX40L on the DC, in turn ligating OX40 on the T cell, profoundly changes its migratory behavior. Interestingly, neither LPS nor CpG could induce OX40L expression on purified

splenic DCs or B cells. Being strictly dependent upon CD40 stimulation, OX40L expression by DCs appears to be a specific signature of the cognate interaction with T cells and is not associated with "innate" activation. Our results support a sequential model of T cell activation where DCs first prime T cells and induce CD40L up-regulation, which in turn stimulates the DC via CD40. This induces the up-regulation of "late" costimulatory molecules such as OX40L that regulate T cell differentiation (14, 54), migratory properties via CXCR-5 (14, 15), and survival by promoting Bcl-xL and Bcl-2 expression (55). Many of the other effects of CD40 ligation on DCs, such as up-regulation of IL-12p70 (56), antigen loading onto MHC class II (57), expression of MHC class II (58), costimulatory molecules CD80 (B7-1) and CD86 (B7-2; references 59 and 60), and enhanced DC survival (58, 61) can also be achieved by signals such as LPS and CpG.

The impairment of T cell responses in OX40- and OX40L-deficient mice (28, 62, 63, 64) may well relate to secondary effects absent due to the suboptimal B–T cell interaction in follicles that we demonstrate here. OX40 has been shown to be necessary for long-term CD4 T cell survival and memory (55) and it remains to be seen if this a wholly direct effect or if it is a correlate of impaired migration to follicles and the subsequent events in that site. Despite minimal T cell migration to follicles in $OX40^{-/-}$ mice have a normal GC response and normal T-dependent antibody responses, which may mean that T–B cell interactions in follicles are not crucial for these responses. Certainly, we believe that the induction of a costimulatory molecule, other than OX40, on DCs or B cells by CD40 ligation is important for these.

Our results do not explain the selectivity of the migration process. Not all activated T cells enter B cell follicles (65). We assume that CXCR-5 is involved in this migration, although there is no definitive proof of this. In human tonsils, CXCR-5 was found on ~90% of memory type CD4 T cells (5), however, not all of these inhabit follicles. Levels of CCR7 expression might be an important parameter as movement of activated T cells to the follicles seems to require CCR7 down-regulation (66). Maintenance of CCR7 expression may override CXCR-5–mediated BLC chemoattraction and retain activated T cells within the T cell area, a notion supported by the observation that $CXCR\text{-}5^+$ $CD57^+$ T cells, which are only located in GCs, have completely lost CCR7 expression and Epstein Barr Virus–induced molecule 1 ligand chemokine (ELC)/CCL19 responsiveness (5). In contrast, $CXCR\text{-}5^+$ $CD57^-$ T cells, found in the follicles and at the border between the T zone and the follicle, still respond to ELC as a population and some of them express CCR7 (5). Similarly, B cell follicular exclusion of antigen-stimulated (9, 67) or anergic B cells (67, 68) is not associated with a decreased BLC responsiveness (69). The follicular exclusion of these cells correlates with an increased responsiveness to T zone chemokines ELC and secondary lymphoid chemokine (SLC)/CCL21 (69, 70). It seems likely, based on the recent work of Reif et al. (71) that T cells, in the same way as B cells, are responding to opposing gradients of SLC/ELC generated by T zone DCs on the one hand and BLC from follicular stroma on the other. CCR7 persistence on some activated T cells could be due to priming by different DC subsets or signal strength (72). It is conceivable that the basis of selective follicular migration lies in the heterogeneity and position of DC subsets, programming T cell differentiation (and migration) in a step-wise fashion.

In conclusion, the following intriguing issues await resolution: (a) the basis of selective regulation of follicular migration, whether differential responsiveness to gradients of two or more chemokines or the involvement of different subsets of DCs, (b) the control of migration and differentiation of T cells within the follicle (e.g., into GCs), and (c) the identity of the factors controlling the follicular T cell pool size. Although we have gone some way to answer the question of how T cells migrate to follicles, it is important that we not forget to ask why. Providing help for B cells is only part of the story and delving further may reveal the influences of B cells on T cell differentiation and memory generation.

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