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Early simultaneous production of intranodal CD4 Th2 effectors and recirculating rapidly responding central-memory-like CD4 T cells

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This study characterizes the diversity of CD4 Th cells produced during a Th2 response *in vivo*. Kinetics of effector and memory cell differentiation by mouse OVA-specific CD4 T cells was followed during primary responses to alum-precipitated OVA. The complexity of the CD4 T response was assessed in nodes draining and distant from the site of immunization for phenotype, location and function. By 3 days IL-4-producing effector CD4 T cells developed in the draining node and a proportion of the responding cells had migrated to B-cell follicles, while yet others had left the draining node. Some of these early migrant cells were recirculating cells with a central memory phenotype. These had divided four or more times in the draining node before migrating to distant nodes not exposed to antigen. We questioned the responsiveness of these early central-memory-like cells on secondary antigen challenge at sites distant to the primary immunization. They re-entered cell cycle and migrated to B-cell follicles, much more rapidly than naïve CD4 T cells and could still be induced to produce IL-4. Their production and survival were independent of the starting frequency of antigen-specific CD4 T cells. Thus intranodal effector cells and recirculating, rapidly responding central-memory-like cells emerged simultaneously from the third day of a primary Th2 response.

Key words: Alum · Cell trafficking · Effector and memory CD4 T cells · Vaccination

Introduction

There is evidence to support at least two models of memory T-cell generation [1]. These are the linear differentiation pathway, where naïve cells become effectors that subsequently convert into memory cells, and the divergent differentiation pathway, where naïve cells simultaneously give rise to effector and memory cells. In the linear differentiation pathway the classical view is that Ag encounter leads to rapid Ag-specific T-cell proliferation when differentiation programs are triggered that modify their properties giving rise to cytokine-producing effector Th subsets. This extensive expansion is followed by a contraction phase when the vast majority of Ag-specific T cells undergo apoptosis leaving a minority that become a long-lived memory population. The contraction phase is usually closely associated with Ag exhaustion. Although the linear differentiation model is widely accepted for both CD8 [2, 3] and CD4 [4] T cells, other data support the divergent differentiation model [5, 6]. It has also been reported that asymmetric cell division of a newly activated T cell can give rise to two daughter cells that, respectively, form effector and memory lineages [7]. The relative importance of these two pathways of memory cell formation, which need not be mutually exclusive, may differ in responses to different Ag. Thus, depending on *in vitro* or *in vivo* model systems that are used,

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the Ag form and its administration, IFN-γ- or IL-4-producing CD4 T cells, as well as non-cytokine producing CD4 T cells have all been shown to differentiate into memory cells [4, 8–11]. These disparate results maintain the debate on the origin of memory T cells and whether they emerged before Ag exhaustion, or are derived from effector T cells, or arise by both pathways.

These issues are further complicated by the memory T-cell classification into two categories [12, 13]: (i) central memory T cells, which lack immediate effector functions but rapidly proliferate in secondary responses and are found principally in the T zones of secondary lymphoid tissues, and (ii) effector memory T cells, which are characterized by immediate functions, slow proliferation rate and are found in the blood, peripheral tissues and spleen.

We have investigated *in vivo* the emergence of diversity within CD4 Th2 cell subsets in response to alum-precipitated Ag. Aluminum potassium (alum) is a widely used adjuvant worldwide in human and animal vaccines [14, 15]. It promotes Th2 responses with the induction of Th2 cytokines in CD4 T cells [16] and strong antibody production associated with elevated titers of the Th2-dependent IgG1 and IgE antibodies [17]. We previously reported that several weeks after immunization with alum-precipitated Ag recirculating memory CD4 T cells are found in lymphoid organs not exposed to the Ag and mount accelerated secondary responses [18]. In the present study we questioned the precise timing of appearance and responsiveness of recirculating memory CD4 T cells in Ag-free lymphoid organs.

These studies were carried out using OVA-specific CD4 T cells from transgenic (OT-II) mice. The response to alum-precipitated OVA (alumOVA) injected in footpads is confined to the draining popliteal LN. The first CD4 T cells responding to alum-precipitated Ag start to leave the draining LN (popliteal LN draining the site of footpad immunization) for other secondary lymphoid organs as early as the third day after immunization and already show phenotypic features associated with central memory cells. Importantly, these early central-memory-like cells are immediately responsive to further Ag stimulation and produce effectors more rapidly than naïve cells. In addition to early memory formation there is production of effector helpers that produce Th2 cytokines [16] and direct B cells to undergo Ig class switching and to form plasmablasts and germinal centers [17–19]. Finally, the effect of the initial T-cell precursor frequency has been considered, for evidence has emerged that this can influence central and effector memory commitment [20], and memory T-cell survival [21, 22].

Results

To monitor T-cell diversification, CFSE-labeled CD45.1$^+$ OT-II cells were transferred into congenic WT CD45.2$^+$ recipients and the chimeras were immunized 1 day later in rear footpads with alumOVA. OT-II cells were analyzed, at various times after immunization, for proliferation, phenotype and location in the draining popliteal LN and distant Ag-free brachial LN.

Early migration of primed OT-II cells to distant LN during primary response to alumOVA

CFSE dilution shows that OT-II cells in the draining popliteal LN start to divide between 24 and 48 h after immunization (Fig. 1). Proliferation is then rapid, with the responding OT-II cells completing up to seven divisions by 72 h. In contrast, only naïve

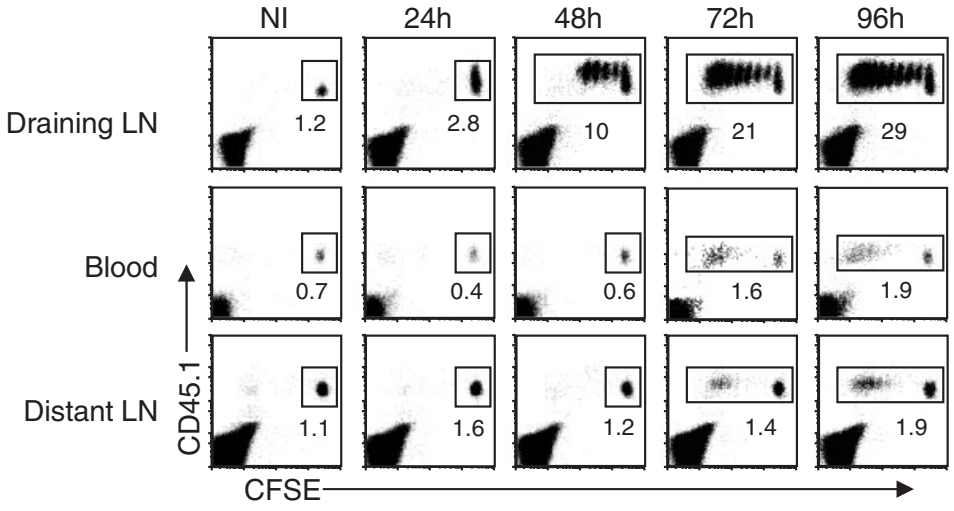

Figure 1. Early OT-II cell emigration from responding LN to distant non-responding LN. CFSE-labeled CD45.1$^+$ CD4$^+$ T cells from naïve OT-II mice were transferred into naïve CD45.2 congenic WT mice. These chimeras were immunized with alumOVA in both footpads. Popliteal LN draining the site of immunization and brachial distant LN not exposed to Ag and blood were harvested at the indicated times after immunization. Cells from these tissues were analyzed for the presence of OT-II cells by flow cytometry. Dot plots are gated on CD4$^+$ cells. The numbers in the dot plots indicate the percentage of OT-II cells within the total CD4 population. The data are representative of a total of ten mice in three independent experiments each with three or four mice.

CFSE^{high} OT-II cells were detected in the blood or distant (brachial) LN 48 h after immunization, indicating that under the immunization conditions used, the Ag is confined to the LN draining the site of injection. As expected, when precipitated in alum the Ag is slowly and locally released from the alum-depot at the injection site [14, 15]. This delays Ag exhaustion and prolongs the T-cell response, compared with the same amount of free Ag (data not shown). Strikingly, by 72 h some CFSE^{low} OT-II cells have left the draining LN and entered both the blood and Ag-free distant LN (Ag-free brachial LN outside the drainage area of the site of immunization). These OT-II cells are likely emigrants from the responding LN, for they have completed at least four divisions. If they had been activated in the distant LN, some OT-II cells that had divided fewer than four times would have been observed there from 48 h and onwards and these were not present. The primed immigrant OT-II cells were present in distant nodes in increased numbers at 96 h. As the distant LN contains both naïve CFSE^{high} and primed immigrant CFSE^{low} OT-II cells the phenotype and responsiveness of these two cell types can be studied in the same natural environment.

Phenotypic diversity of OT-II cells in the draining LN induced by alumOVA

Phenotypic changes occurring within the OT-II population in the draining and distant LN of chimeras before and at intervals after immunization are shown in Fig. 2. In all lymphoid organs before immunization the OT-II cells are CFSE^{high} and present a homogeneous phenotype (CD69$^-$, CD25$^-$, CD44$^-$ and CD62L$^+$) characteristic of naïve CD4 T cells (Fig. 2A). By 16 h post-immunization almost all of the OT-II cells in the draining LN have been recruited into the response, for they have become CD69$^+$, CD25$^+$ and CD62L$^-$. These phenotypic changes have already occurred in some OT-II cells within 4 h of immunization (Fig. 2C). After this initial activation the OT-II cells enter a prolonged lag-phase before completing their first division at around 30 h (data not shown; see the two divisions observed at 36 h in Fig. 2A). By 48 h the OT-II cells have divided up to four times (Fig. 2A), indicating a doubling time of 6 h, consistent with previous reports [23–25]. With each division the level of CD69 expression by OT-II cells falls and CD62L increases. The extent of these changes varies so that by 72 h OT-II cells that have undergone seven divisions are heterogeneous in relation to CD69, CD62L and CD25 expression. On the other hand, the CFSE^{low} OT-II cells show uniform strong CD44 expression. Thus, the responding OT-II cells in the draining popliteal LN show homogenous phenotypic changes before cell division, followed by diversification during proliferation.

The phenotype of primed OT-II cells that migrate to distant LN

As indicated in Fig. 1 there is no proliferation of OT-II cells in the distant Ag-free LN. The OT-II cells in these LN at 36 h remain CFSE^{high} CD62L$^+$, CD25$^-$ and CD44$^-$ (Fig. 2A) and contrast markedly to the activated phenotype of the OT-II cells in the draining LN. Although a small proportion shows a modest increase in CD69 expression at this stage, this low expression level is never associated with proliferation (Fig. 2A compare 36 h draining and distant LN). On average 81%$\pm$11 of the CFSE^{low} OT-II cells that have migrated to distant LN are in the quadrant of small sized cells compared with 38%$\pm$13 of the primed OT-II cells within the responding popliteal LN ($p<0.001$). The CFSE^{low} immigrant OT-II cells that arrive 72 h after immunization are small lymphocytes that have a homogeneous post-activation phenotype, being CD69$^-$, CD62L$^+$, CD25$^-$, CD127/IL-7R$^+$, like naïve recirculating OT-II cells, while showing memory features – CD44$^+$, CD45RB$^-$, CD11a$^+$ (Fig. 2A and B). Finally, to test if residual memory OT-II cells might be responsible for the emergence of these CFSE^{low} immigrant OT-II cells, chimeras were created where small naïve CD4$^+$CD62L$^+$CD44$^-$ OT-II cells from naïve OT-II LN were transferred into naïve recipient mice. A high level of purity of the donor cells was achieved by FACS-sorting them twice (Fig. 2D). From 3 days after immunization early recirculating CFSE^{low} OT-II cells started to colonize the distant Ag-free LN. Thus these early recirculating central-memory-like cells can be derived from highly purified naïve OT-II cells (Fig. 2D). In summary these experiments show that by 3 d post-immunization, some naïve CD4 OT-II cells that have proliferated in the LN draining the site of immunization have migrated to distant non-responding LN. These early migrants have a phenotype associated with recirculating central memory CD4 T cells.

The location of OT-II cells in the responding and distant LN reflects their functions

The location of CD4 T cells provides insight into their function. Classically, naïve CD4 T cells occupy the central T zones where they screen the surface of dendritic cells, while primed CD4 T cells in the outer T zone make cognate interaction with B cells that have captured Ag, and follicular CD4 T cells are key to the differentiation and survival of germinal center B cells [26]. After transfer into non-immunized (NI) mice naïve OT-II cells recirculate between the T zones of LN (Fig. 3A). By 24 h after alumOVA the draining LN has enlarged and the OT-II cell numbers are increased. By 48 h OT-II cell numbers have increased markedly. They are distributed throughout the enlarged T zone and there is now a focus of responding cells in the outer T zone adjacent to the follicles. By 72 h many OT-II cells have migrated into the follicles and the number in the T zone has further increased. The timing of migration into follicles is in keeping with previous reports [27]. The follicular location correlates with strong CXCR5 expression by a proportion of the OT-II cells (Fig. 2B).

IL-4 mRNA was strongly up-regulated (Fig. 3B). We and others have reported that responding CD4 T cells are the main producer of IL-4 in response to alum-precipitated protein [16, 28, 29]. Data shown later also demonstrate that IL-4 mRNA is

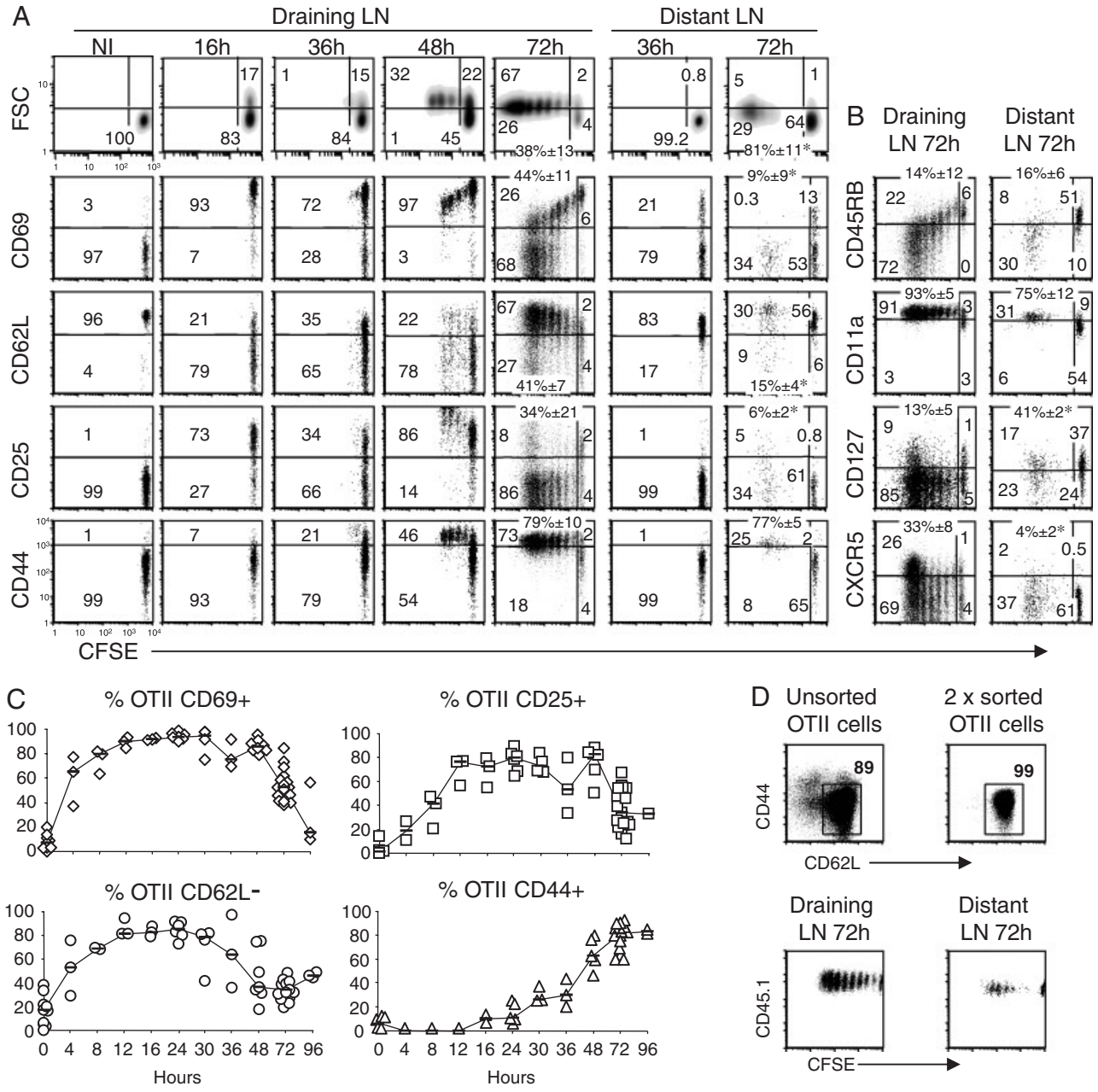

Figure 2. Phenotype and proliferation of OT-II cells responding to alumOVA and the memory phenotype of primed OT-II cells that have migrated to distant Ag-free brachial LN. Chimeras were constructed and immunized as indicated in Fig. 1. (A and B) Representative flow cytometry dot plots comparing the phenotype of OT-II cells gated on $CD4^+$ $CD45.1^+$ cells in the popliteal draining LN and brachial distant LN at various times post-immunization. (A) Forward scatter (FSC), CD69, CD62L, CD25 or CD44 *versus* CFSE (ten mice in six independent experiments). (B) CD45RB, CD11a, CD127 (IL-7Rα) or CXCR5 *versus* CFSE. The average of the $CFSE^{low}$ OT-II cell values ± SD at 72 h that express or do not express a marker are respectively given at the top or bottom of the dot plots (4 mice in 3 independent experiments). *$p<0.005$: statistical differences between draining LN *versus* distant LN groups are indicated in the graph and were assessed by the two-tailed *t*-test. (C) Time course of CD69, CD44 and CD25 up-regulation and CD62L down-regulation in draining LN between 4 and 96 h after immunization. The lines are drawn through the median values at each time point. Each symbol represents value from an independent experiment where cells are pooled from the two draining LN of one mouse. (D) Small naïve $CD62L^+CD44^-CD45.1^+$ OT-II cells were double FACS-sorted (top panel), labeled with CFSE and then transferred into naïve CD45.2 congenic WT mice. These chimeras were immunized with alumOVA in both footpads and 72 h later LN draining or distant to the site of immunization were harvested and analyzed for the presence of early central-memory-like cells (bottom panel). The data are representative of nine mice in total in two independent experiments each with four or five mice.

translated into protein and that about 2% of IL-4-secreting OT-II cells are generated by 72 h after immunization. In addition, a gene expression profiling study shows that molecules strongly associated with follicular B helper functions (Bcl6, CD200, CXCR5, ICOS, IL-21, SAP, PD-1) were highly expressed by alumOVA-responding OT-II cells by 3 days after immuniz-

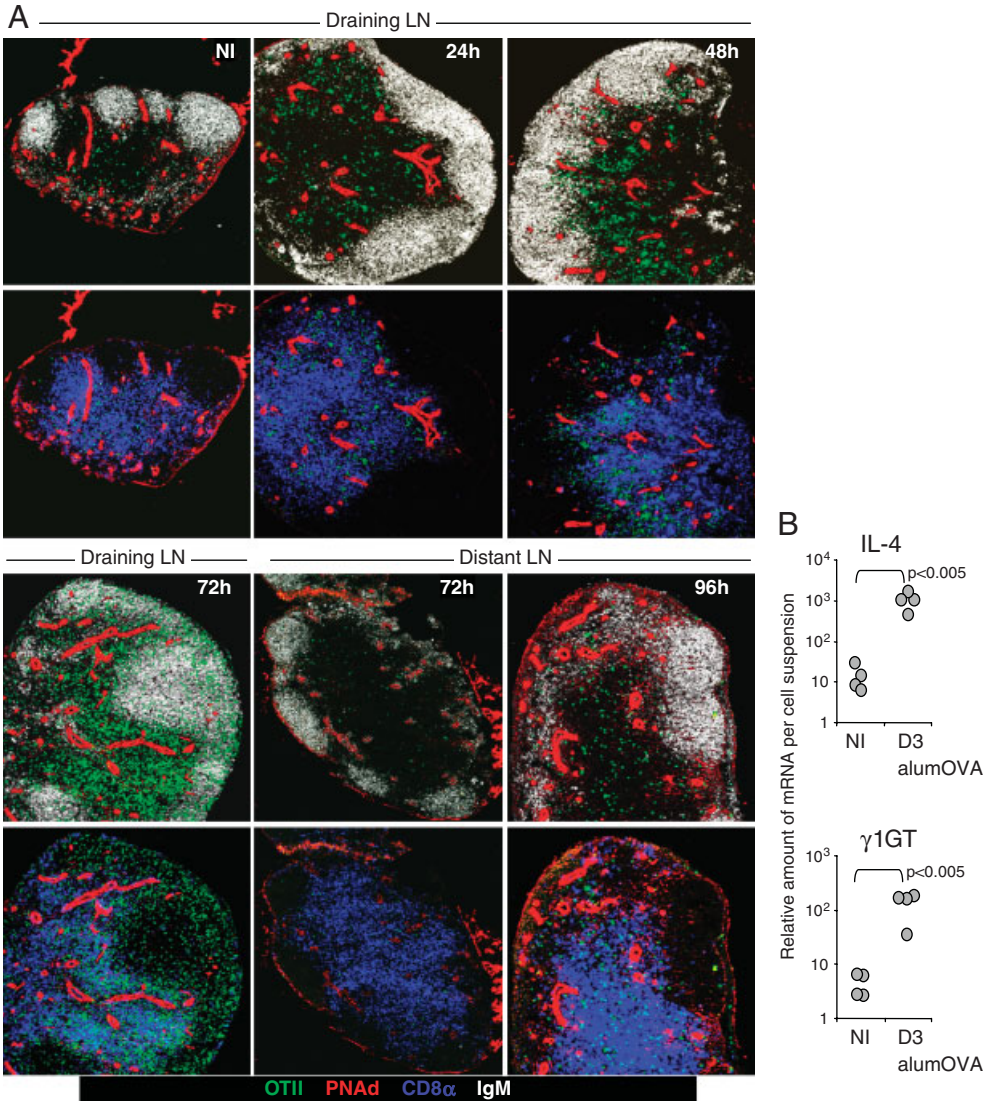

Figure 3. Visualization of OT-II cells in the popliteal draining and brachial distant LN. (A) Mice received CD45.1$^+$ OT-II cells. Some remained NI while others were immunized with alumOVA in both footpads. Chimeras were sacrificed at various days post-immunization. Immunofluorescence histology was used to localize OT-II cells (green) within popliteal draining and brachial distant LN. LN tissue sections were stained to show CD8α (blue) to identify the T-cell zone, IgM (white) to locate B-cell follicles, and PNAd (red) to show high endothelial venules (HEV). Original magnification ×10. The data are representative of three independent experiments. (B) cDNA was prepared from popliteal draining LN total cell suspensions 3 days after immunization with alumOVA and the graphs show IL-4 (top panel) and IgG1 GT (γ1 GT, bottom panel) mRNA levels, assessed by real time RT-PCR. Each symbol represents total cells pooled from two popliteal LN *per* mouse. The data are representative of eight mice in total from three independent experiments.

ation [16]. Evidence that cognate T-cell interaction with B cells has occurred by this time is provided by the presence of processed γ1 germline transcripts (GT) (Fig. 3B) [19]. The induction of these Th2 features is strictly associated with the presence of alum, for as expected free OVA fails to induce IL-4 mRNA or γ1 GT (data not shown). OT-II cells are responsible for the induction of γ1 GT, for in the absence of OT-II cell transfer the level of these transcripts 3 days after immunization is much reduced (data not shown). The low response seen in the absence of OT-II cells is likely to be attributable to endogenous OVA-specific CD4 T cells primed in the response. Thus, at 72 h, effector and helper OT-II cell functions were also induced.

CFSE^{low} primed OT-II cells that have migrated to brachial Ag-free distant LN are not positive for IL-4 mRNA (data not shown). Importantly they are confined to the central T zones, which is the site where recirculating naïve and memory CD4 T cells interact with antigen-presenting dendritic cells (Fig. 3A). This location is consistent with the lack of CXCR5 expression by these CFSE^{low} OT-II immigrants (Fig. 2B). Based on phenotype and location, these primed cells that migrate to distant LN from the third day after immunization have the characteristics of recirculating central memory CD4 T cells. Importantly, these early central-memory-like CD4 T cells develop simultaneously with a range of effector cells.

The proliferation status of primed CFSE^{low} OT-II cells in draining and distant LN

To investigate the activation status of OT-II cells, their proliferation in responding and distant LN was assessed further, both by Ki67 expression, which reflects cell cycle progression, and by BrdU incorporation, which identifies cells in S phase of the cell cycle. From 72 and 96 h the rapidly proliferating OT-II cells in the draining LN are characteristically Ki67$^+$ (Fig. 4A, top panel). In the distant LN, at 72 h after immunization, approximately two third of the immigrant CFSE^{low} OT-II cells were still Ki67$^+$, but this proportion fell to around 10% at 96 and 120 h (Fig. 4A, bottom panel and graph). Ki67 expression remains in OT-II cells for at least 24 h after they have stopped proliferating. Therefore this low residual proportion of Ki67$^+$ OT-II cells in the distant LN at 96 h suggests accumulation of newly generated primed recirculating OT-II cells over time. Migrant OT-II cells exit from cell cycle was further confirmed by BrdU staining. No OT-II cells in the draining LN had started to synthesize DNA by 24 h, but nearly 80% of the OT-II cells in the draining LN had taken up BrdU between 42 and 48 h; two third were BrdU$^+$ at 72 h and half at 96 h. This is consistent with CD4 T cells requiring sustained stimulation to maintain cell cycle progression during the expansion phase [30–32]. In the distant LN, some 25% of the CFSE^{low} cells were labeled by BrdU in the 6 h before 72 h and 15% in the 6 h before 96 h post-immunization. The BrdU in CFSE^{low} emigrant OT-II cells is mainly taken up before they leave the draining LN; for in chimeras given a 2 h BrdU pulse ending at 72 h fewer than 1% of CFSE^{low} OT-II cells in the distant LN were labeled.

Early migration of primed OT-II cells to distant LN is independent of initial OT-II cell frequency

Previous studies using adoptive transfer of transgenic T cells found that the initial number of transferred cells affects (i) quantitative and qualitative aspects of the T-cell differentiation [33, 34], (ii) central and effector memory commitment [20], (iii) survival of T memory cells [21, 22], (iv) proliferation, phenotype and acquisition of effector functions [33], (v) expansion, peak and contraction phases of the T-cell immune response [34] as well as (vi) CD62L expression on recirculating T cells [35]. These findings made it necessary to assess whether the appearance of early primed emigrant OT-II cells occurs only in the presence of high numbers of Ag-specific naïve CD4 T cells. To address this possibility, we compared responses at 3 or 7 days post-immunization in groups of chimeras constructed with 10^7, 10^6 or 10^5 naïve OT-II cells. Proliferation, measured by CFSE dilution, and exit from cell cycle, assessed by lack of BrdU incorporation, were determined in primed CFSE^{low} emigrant OT-II cells (Fig. 5).

Precursor frequency did not affect the kinetics of CFSE^{low} OT-II cell migration from the responding node since from 3 d and onwards these cells started to appear in distant nodes irrespective of precursor frequency (Fig. 5A). Even when 10^5 OT-II were transferred, some BrdU$^-$CFSE^{low} cells were found in distant LN from 3 d.

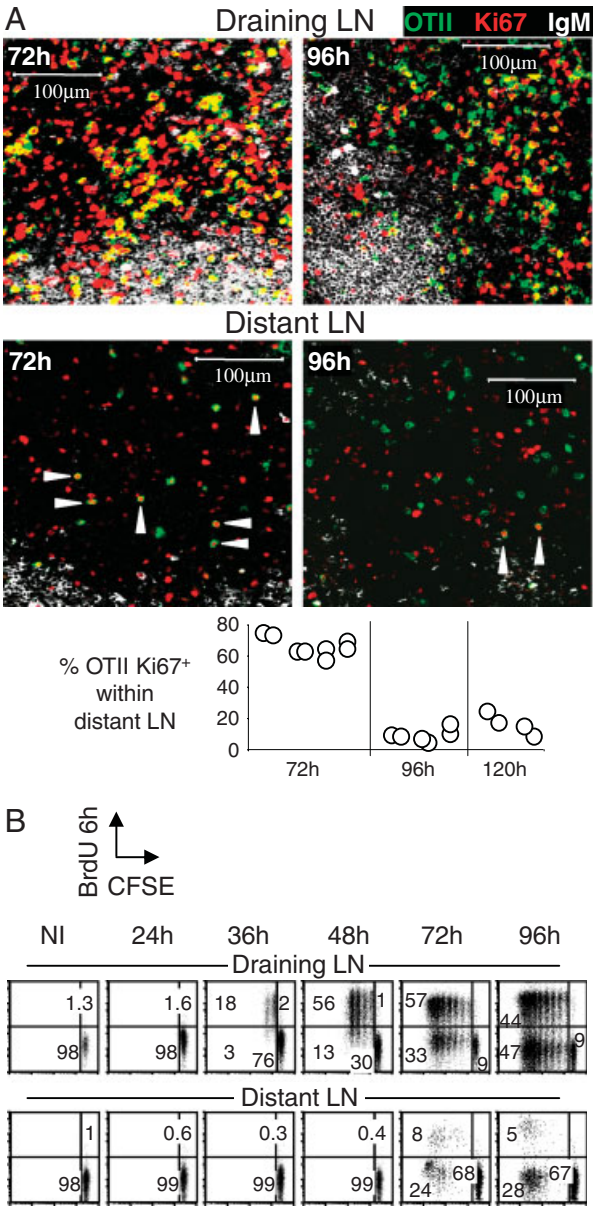

Figure 4. The proliferation status of responding OT-II cells in the draining popliteal LN compared with non-proliferating naïve and primed emigrant OT-II cells in the distant Ag-free LN. Chimeras were constructed and immunized as indicated in Fig. 1. (A) Immunofluorescence histology showing OT-II cells (green) and Ki67 expression (red) within popliteal draining (top row) and brachial distant (bottom row) LN at 72 and 96 h post-immunization. The sections show mainly T zone with B-cell follicles at the margins (identified with white IgM cells). White arrowheads in the sections of distant nodes point to CD45.1$^+$Ki67$^+$ OT-II cells. Original magnification $\times$40. Plots indicate the percentage of Ki67$^+$OT-II cells within distant brachial LN at various time points post-immunization. Each symbol represents the count from an individual tissue section and touching symbols are from two sections derived from one LN from one individual mouse. Data are representative of three independent experiments. (B) Mice received CD45.1$^+$OT-II cells freshly CFSE-labeled and were immunized 1 day later. Various time after immunization chimeras were injected with BrdU. Six hours after BrdU injection, mice were sacrificed and cell suspensions from the draining popliteal LN (top row) and distant brachial LN (bottom row) LN were stained for BrdU, CD4 and CD45.1. Dot plots are gated on OT-II cells (CD4$^+$CD45.1$^+$) and show BrdU incorporation by OT-II cells related to CFSE dilution. The data are representative of one mouse *per* time point in three independent experiments.

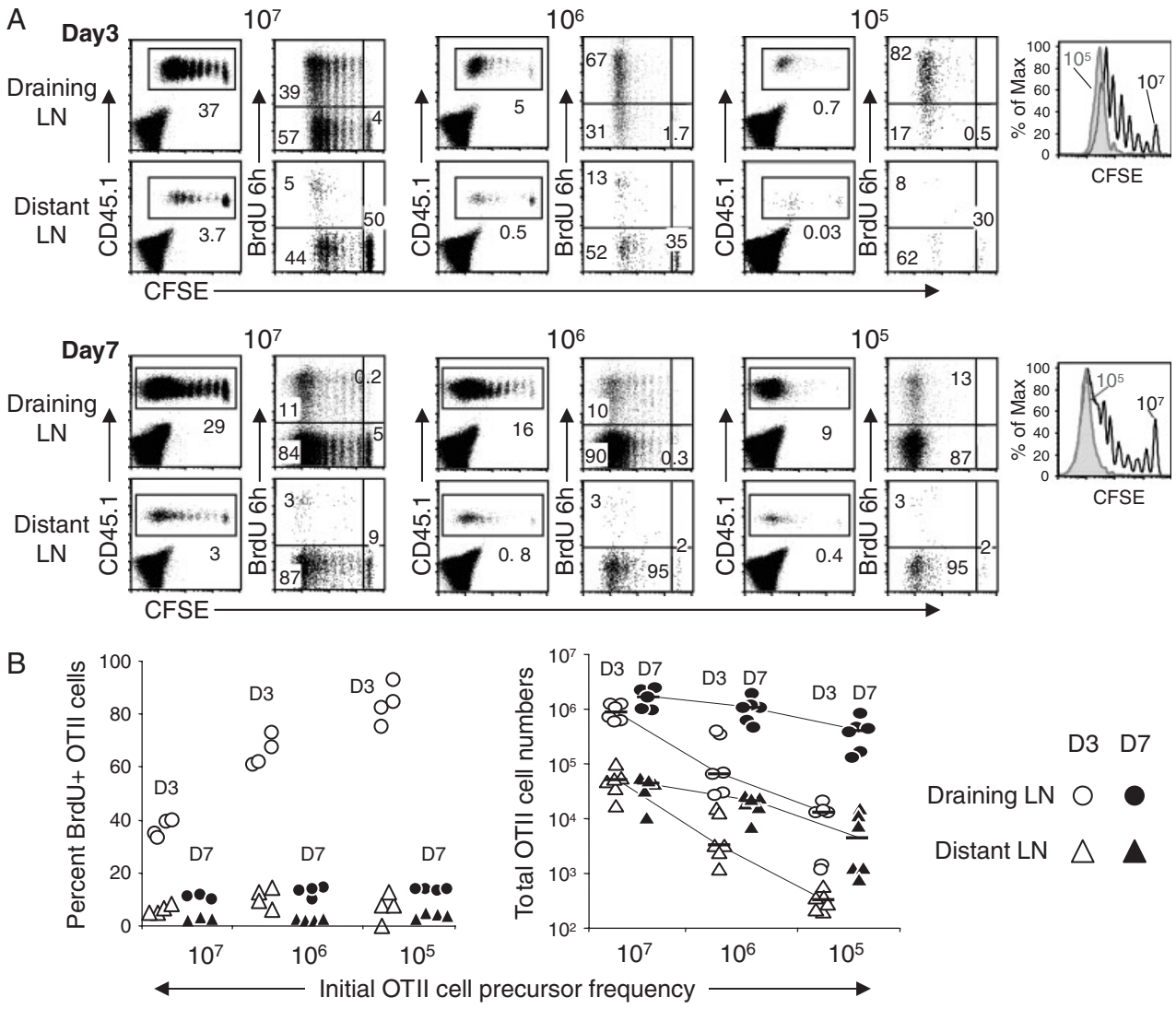

Figure 5. Development of primed recirculating OT-II cells is not dependent upon initial T-cell precursor frequency. Mice received 10^7, 10^6 or 10^5 CFSE-labeled CD45.1$^+$ OT-II cells as indicated and were immunized with alumOVA in footpads. BrdU incorporation was used to assess the proliferation status of the OT-II cells. Chimeras received BrdU either on day 3 (top two rows) or day 7 (bottom two rows) post-immunization and were sacrificed 6 h later. (A) Cell suspensions from draining popliteal LN and distant brachial LN were stained for BrdU, CD4 and CD45.1. For each initial dose of OT-II cells dot plots are either gated on CD4 cells (left column) or on OT-II cells (right column). Histograms are representative of OT-II cell proliferation in draining LN (10^7 empty black line, 10^5 filled gray line). (B) Plots indicate either the percentage of BrdU positive cells within the OT-II population (left) or the total numbers of OT-II cells (right). The lines intersect through median values. Each symbol represents the results for cells pooled from two LN of one mouse. Data derived from two independent experiments ($n = 4$ *per* point, left panel; $n = 6$ *per* point, right panel).

There is published evidence that high precursor frequency is associated with responding cells leaving cell cycle earlier [21, 36]. In the present experiments the maximum extent of proliferation, as detected by CFSE dilution, was only moderately decreased as the precursor number increases (Fig. 5, histograms). Nevertheless, while by 3 d in the recipients of 10^5 cells a median of 84% OT-II cells had incorporated BrdU$^+$ (Fig. 5B), in recipients of 10^7 cells only a median of 38% of the responding OT-II cells were BrdU$^+$ and some of these had stopped dividing after only one or two divisions (Fig. 5A, top row, and Fig. 5B, left panel). This may explain why a few cells migrated to distant LN after 1–3 divisions in the chimeras constructed with 10^7 OT-II cells (Fig. 5A, rows 2 and 4) and may also account for the relatively small difference between groups in the numbers of OT-II cells at 7 d in the draining LN (Fig. 5B, right panel). By 7 d, >90% of the OT-II cells have stopped proliferating in the draining LN irrespective of the initial cell frequency (Fig. 5A, row 3, and Fig. 5B, left panel). These data support the previous reports that high frequency imposes constraints on CD4 T-cell proliferation and favors exit from cell cycle [21, 36].

Central memory cells persist irrespective of initial OT-II cell frequency

Given that high initial precursor frequency may be detrimental to the survival of memory T cells [21, 22] we assessed late

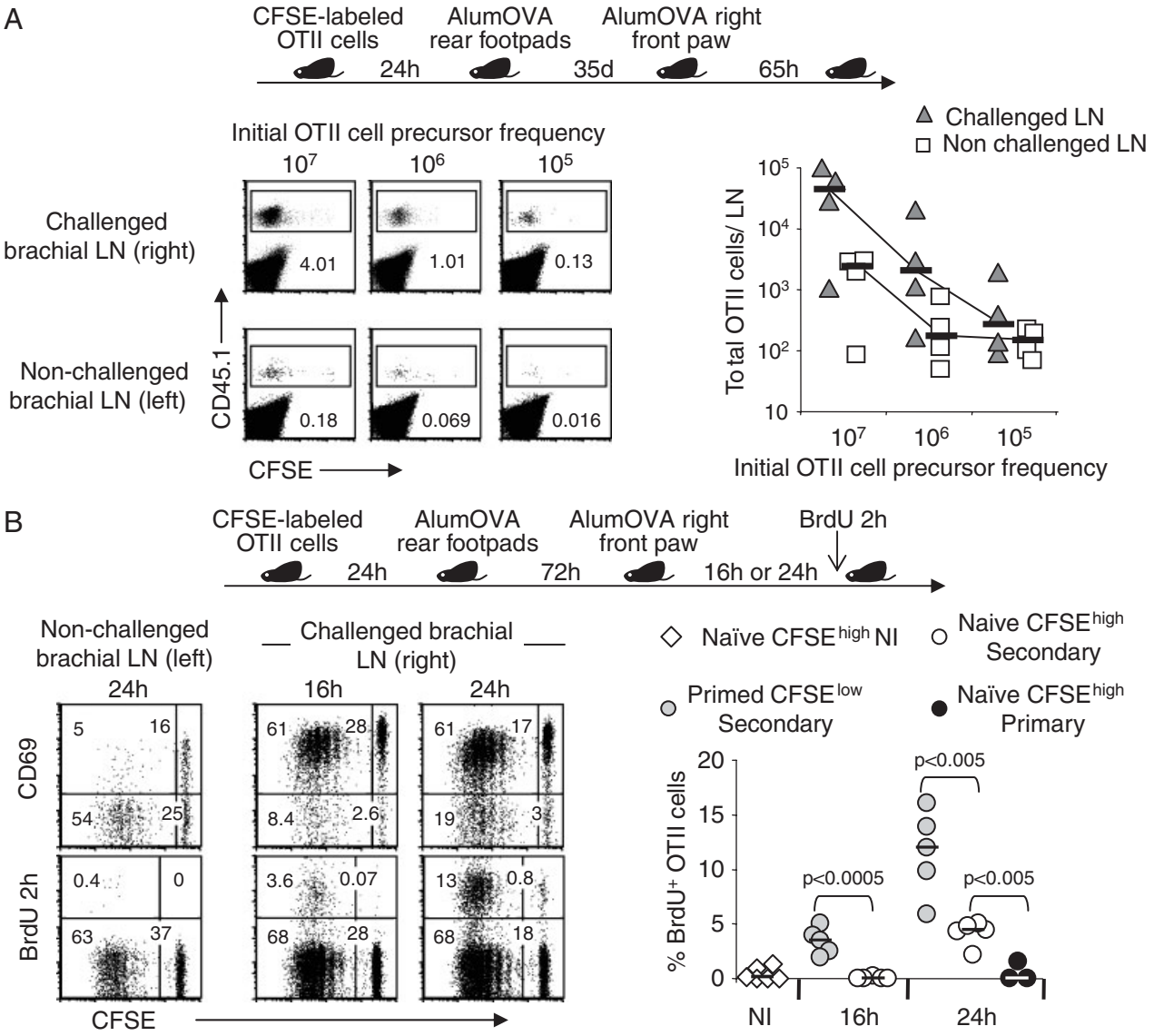

Figure 6. The responsiveness of early and late primed recirculating OT-II memory cells. Top panels show the summary of experimental procedure. (A) Mice received 10^7, 10^6 or 10^5 CFSE-labeled CD45.1$^+$ OT-II cells and were immunized 24 h later with alumOVA in the rear footpads to generate memory cells. At day 35 the chimeras were challenged in the right front paw with alumOVA and LN were harvested after 65 h. Cell suspensions were stained for CD4 and CD45.1. Dot plots were gated on CD4$^+$ cells. The graph shows total numbers of OT-II cells recovered from challenged right brachial LN (closed triangles) to those in the non-challenged left brachial LN (open squares). Data are from four mice from two independent experiments. (B) Chimeras were subjected to the protocol shown in (B). CD69 expression or 2 h BrdU *versus* CFSE. Dot plots are gated on OT-II cells (CD4$^+$CD45.1$^+$). The graph shows the percentages of BrdU$^+$ cells among: (i) naïve OTII cells from NI mice (white diamonds), (ii) naïve OTII cells responding in a secondary environment (white circles), (iii) primed recirculating OTII cells responding to secondary immunization in brachial LN (gray circles) and (iv) naïve OTII cells responding to primary immunization in popliteal LN (black circles). Data are derived from two independent experiments ($n = 5$).

secondary responses of mice that had received 10^7, 10^6 or 10^5 naïve OT-II cells (Fig. 6A, scheme). Chimeras were immunized with alumOVA into rear footpads the following day. They were challenged 35 d later with alumOVA in the front right paw, a site distant to the first immunization. After a further 65 h the challenged right and non-challenged left brachial LN were harvested. Mice from all groups, irrespective of the number of OT-II cells transferred, showed larger numbers of CFSE^{low} cells in the right (challenged) brachial LN compared with those in the left (non-challenged) brachial LN (Fig. 6A, dot plots and graph). Although memory cells persist after all levels of initial transfer, there is a *pro rata* reduction in the number of CFSE^{low} cells recovered from the mice that have received fewer initial OT-II cells. This finding means that the catch-up of cell number seen at 7 d into the primary response in the mice that originally received low numbers of OT-II (Fig. 5B) has not been sustained. Thus, in the current system, memory resulting from large cell transfers persists.

Primed emigrant OT-II cells in distant LN respond faster than naïve OT-II cells to alumOVA

Memory cells are characterized by their rapid response to secondary challenge. On the other hand, some data suggest that recently primed T cells may be refractory to further stimulation, which might be due to TCR unresponsiveness or down-regulation after an initial stimulation [37]. As the primed immigrant OT-II cells are $CFSE^{low}$, while naïve OT-II cells in the same node are $CFSE^{high}$, the responsiveness of these two populations can be compared in the same natural microenvironment. The responsiveness of these $CFSE^{low}$ early central-memory-like cells was assessed, as soon as they leave the draining LN, in chimeras primed with alumOVA in the rear footpads and challenged 3 d later with alumOVA in the front right paw, a site distant to the first immunization (Fig. 6B, scheme). The same immunization protocol (alumOVA) was kept for primary and secondary challenges in order to avoid any differences in Ag delivery and inflammatory response induced by the adjuvant. BrdU was given 2 h before the brachial LN was harvested for analysis at either 16 or 24 h after the challenge. Both $CFSE^{high}$ and $CFSE^{low}$ cells up-regulated CD69 by 16 h in the challenged, but not in the non-challenged LN (Fig. 6B, dot plots). In contrast, $CFSE^{low}$ OT-II cells in the challenged right brachial node entered S phase earlier than naïve $CFSE^{high}$ OT-II cells; indeed 4% of the $CFSE^{low}$ cells had incorporated BrdU at 16 h and 12% at 24 h (Fig. 6B, dot plots and graphs). None of the $CFSE^{high}$ naïve OT-II cells had entered S phase in the challenged brachial LN by 16 h, but surprisingly a median of 5% of the $CFSE^{high}$ cells in these LN were $BrdU^+$ at 24 h. The accelerated naïve cell response possibly reflects a bystander effect of the responding primed $CFSE^{low}$ cells as suggested elsewhere [38]. This requires further study. As expected, neither $CFSE^{low}$ nor $CFSE^{high}$ OT-II cells in the non-challenged left brachial LN had taken up BrdU after 24 h. In conclusion, primed OT-II cells that have migrated to a distant LN by 3 d after primary immunization are not refractory and immediately respond faster in that distant node than naïve OT-II cells in a primary response, which enter only S phase 30 h after immunization (Fig. 4B).

Naïve and early central-memory-like OT-II cells equally generate IL-4-producing OT-II cells

Given that the early central memory cells were rapidly responsive we questioned whether IL-4 production would be induced in a higher proportion of these cells than in responding naïve cells. To address this, mice that have received $CD45.1^+$ OT-II cells were immunized with alumOVA in both footpads. Half the chimeras were sacrificed 3 days post-immunization and popliteal LN were harvested (Fig. 7, scheme). The other half were challenged at day 4 after the primary in the right front paw with alumOVA and right brachial LN were harvested 72 h after this second challenge. In both situations, LN cell suspensions were harvested and OT-II cells were re-stimulated *ex vivo* and analyzed for cytokine secretion (Fig. 7). This shows that the proportion of IL-4-producing OT-II cells is similar, when they are generated from the starting naïve OT-II cell population only, to when they are induced in LN that contained early central-memory-like OT-II cells. Thus the capacity to induce an IL-4 response is retained, but is not augmented in the early central-memory-like OT-II cells. Further assessment of the complete profile of cytokine expression during responses of these cells will be assessed in future studies.

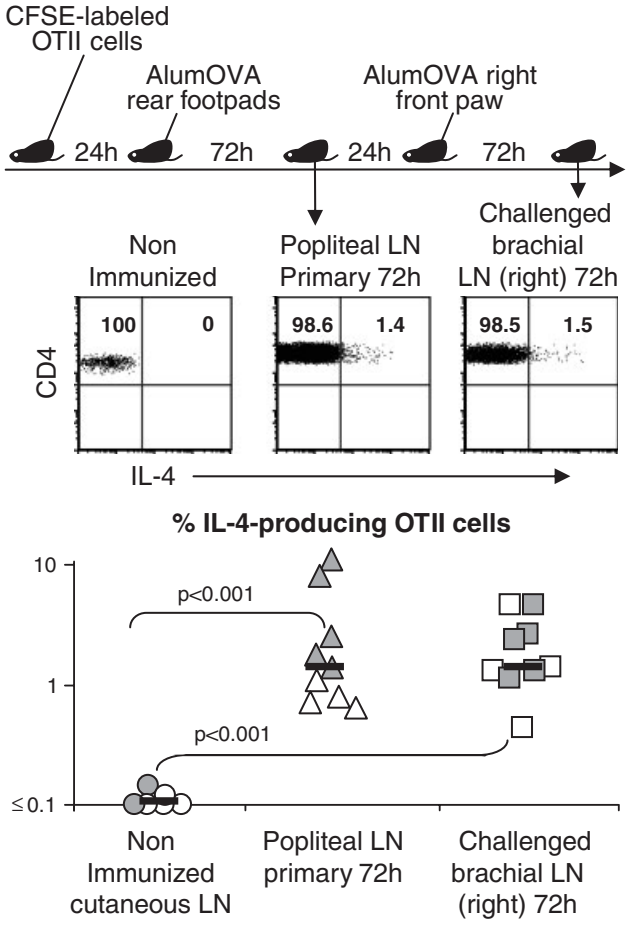

Figure 7. Naïve OT-II cells and early central-memory-like OT-II cells induced to produce IL-4 by alumOVA. Top panel shows summary of experimental procedure. Mice received $CD45.1^+$ OT-II cells and were immunized with alumOVA in both footpads. Half the chimeras were sacrificed 3 days post-immunization and popliteal LN were harvested. The other half were challenged at day 4 after primary immunization in the right front paw with alumOVA and right brachial LN were harvested 72 h after the challenge. LN cell suspensions were incubated *in vitro* for 5 h with 323–339 OVA-peptide to re-stimulate the OT-II cells, and analyzed by intracellular FACS staining for IL-4 secretion. Dot plots are gated on $CD45.1^+CD4^+$ OT-II cells. The graph shows the proportion of responding OT-II cells producing IL-4. Each symbol represents the percentage of OT-II cells producing IL-4 in one mouse popliteal or brachial LN. Naïve OT-II cells derived from pooled brachial, inguinal and popliteal LN from NI mice do not produce IL-4 on stimulation as described above. Bars represent the median. Data show pooled results of a total of nine mice in two experiments, with values for one experiment shown with open symbols and those of the other with filled symbols. The data represented with the filled symbols were derived from chimeras constructed with double-FACS-sorted naïve $CD4^+CD62L^+CD44^-$ OT-II cells as shown in Fig. 2D. Statistical differences between groups are indicated in the graph and were assessed by the two-tailed Mann–Whitney non-parametric test.

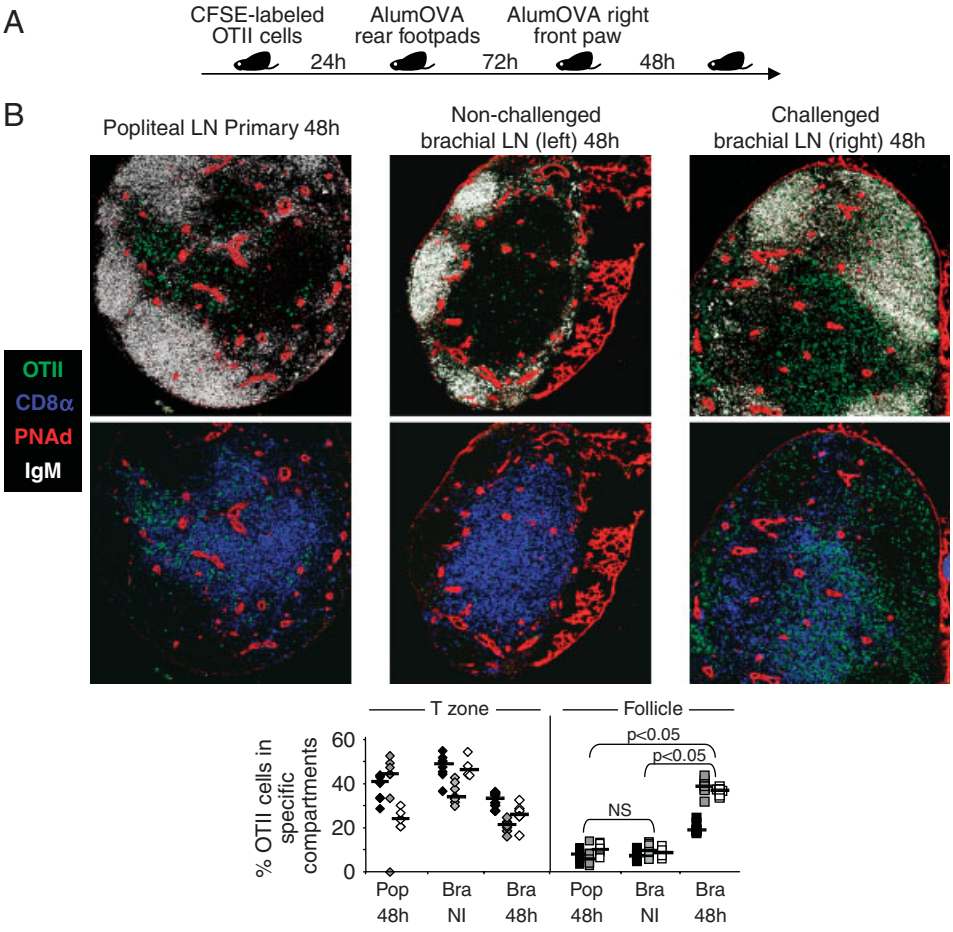

Figure 8. Accelerated entry into follicles of primed emigrant OT-II compared with naïve cells. Sections from popliteal LN 48 h after primary immunization, as well as the left and right brachial LN from chimeras subjected to the protocol described in (A) were examined by immunofluorescence microscopy. (B) OT-II cells are indicated by green color, T zone identified by CD8α staining is indicated by blue color and B-cell follicles identified by IgM staining are indicated by white color. Original magnification × 10. Graphs represent the percentage of OT-II cells in T-cell zone (left panel) and B-cell follicles (right panel) *per* section from popliteal LN 48 h after primary immunization (Pop 48 h) (see Fig. 3), brachial LN 120 h after primary immunization (Bra NI) and brachial LN 48 h after secondary immunization (Bra 48 h). Each color in the gray scale indicates an independent LN. Each symbol represents the percentage of OT-II cells located in a specific zone. Four to eight sections were counted *per* LN. The data are derived from three mice in three independent experiments. Statistical differences between groups are indicated in the graph and were assessed by the two-tailed *t*-test. NS: non significant.

Early central-memory-like OT-II cells colonize follicles more rapidly than naïve OT-II cells

The studies depicted in Fig. 3 show that a proportion of alumOVA-responding OT-II cells migrate into follicles from the third day post-immunization. Chimeras were generated as described in Fig. 8A and they were challenged in the front right paw 3 d after primary immunization in the footpads. The rate of dispersal of the $CFSE^{low}$ OT-II cells in the right brachial LN was accelerated (Fig. 8B). About 20–40% of the primed recirculating OT-II cells in the right brachial node have entered the follicles by 48 h in response to secondary challenge (Fig. 8B). Thus the early central-memory-like cells are capable of accelerated production of follicular helper T cells that are critical for germinal center formation and the production of high-affinity antibody.

Discussion

Diversity becomes apparent in naïve OT-II cells responding to alum-precipitated Ag during the third day of primary immunization. Effector functions appear at this stage including production of the Th2-cytokine mRNA – IL-4 (see also [16]), cognate interactions with B cells – resulting in γ1 GT, and migration of activated T cells into B-cell follicles. At the same time, other OT-II cells leave the responding node and enter distant Ag-free nodes with characteristics of recirculating central memory T cells. Although memory CD8 T cells in some situations can form within a few days after primary immunization [5, 6, 35, 39–41], evidence for simultaneous development of effector and memory CD4 T cells during Th2 immune responses is limited. The early emergence of such primed emigrant cells has been reported for both CD4 and CD8 T cells in Th1 responses to infections, or immunization in the

presence of alum, or intratracheal injection of DC [35, 42–46], but the phenotype and responsiveness of these early emigrants had not been characterized within distant Ag-free nodes.

The primed OT-II cells that migrate to distant Ag-free LN have the features of central memory cells. They have lost the phenotype of naïve cells, for they lack CD45RB while expressing CD44 and CD11a. They express CD62L [35] and in most cases re-express IL-7Rα [47] and are non-proliferating Ki67 cells. These cells also lack the early activation markers CD69 and CD25. Their localization in the central T zone of distant nodes places them with recirculating naïve and memory CD4 T cells in a site where they can respond to antigen presented on dendritic cells. It also correlates with their loss of CXCR5. Finally, when challenged with alumOVA these cells have a shorter activation lag-time and acquire effector localization more quickly than naïve CD4 T cells. On the basis of these features we refer to these cells as early central memory cells.

We questioned how initial frequency affects the extent of naïve CD4 T-cell proliferation and the generation and survival of early recirculating memory cells. *In vivo*, T cells at high frequency compete for access to Ag or survival factors that support expansion [25, 48–52]. Consequently, when clonotypic CD4 or CD8 T cells are present in high numbers, their ability to proliferate is limited [21, 33, 36, 50, 53]. Contrary to previous reports [21, 33], we found that the extent of proliferation was only modestly affected by the initial frequency of naïve OT-II cells. This may reflect the amount, form and route of administration of the Ag used. The alum-precipitated Ag persists longer than intravenously injected peptide with LPS [21] or provides different antigenic stimulus than in the case of a skin graft model [33]. However, 3 d post-immunization in the draining LN the proportion of cells maintained in cell cycle decreases as the precursor number increases. Consequently, by 7 d the median number of early central memory cells in the distant LN of recipients of 10^5 OT-II cells was only tenfold lower than the number in the recipients of 10^7 OT-II cells. Despite this, the relative proportions of central memory cells after 5 wk more closely reflects the relative numbers of OT-II cells transferred. This is perhaps due to a change from early central memory cells to a more stable population of late central memory cells. Therefore the relationship between early and late central memory cells still remains to be questioned. Nevertheless, early central CD8 memory T cells, produced on the third day after immunization with Ag-loaded DC, have been shown to contain precursors of stable memory cells [35]. Finally, although at 3 d post-immunization most OT-II cells in the responding node were in cell cycle in the chimeras constructed with 10^5 OT-II cells early central memory cells were generated. Redistribution of CD8 T cells has also been shown to occur regardless of the number of adoptively transferred CD8 T cells [35]. This reinforces the concept that there may be a normal mechanism for producing early central CD4 and CD8 memory T cells whose significance is not yet clearly appreciated. What advantage would be the generation of central memory cells before Ag disappearance or T-cell contraction phase? If a primary exposure to Ag is localized the response is also local. Should containment, through phagocytosis, Ab neutralization or mechanical barriers fail, the early dispersal of rapidly responsive central memory CD4 T cells that have been produced in parallel with effectors will allow faster responses at distant sites in the body.

Identifying the mechanisms regulating memory formation will help design strategies to modulate the generation and functions of memory T cells in vaccines. It remains to be determined whether there might be extrinsic signals (or lack of signal) from the Ag-presenting DC or other cells in the LN that trigger the commitment into a rapidly responding population of recirculating memory cells. Vaccination with peptide-coated DC generates CD8 T cells with the phenotype and function of memory cells within 4–6 days during a primary response [40]. Conversely, co-injection of peptide-coated DC with CpG, through IFN-γ action on T cells, favors effector cell formation. Withdrawing infection-induced-inflammation-signals during T-cell priming accelerates memory generation [54], indicating that inducers of inflammation may control the rate of memory development. This is of interest in light of the results obtained with alum. Notably, immunization with alum does not induce strong level of IFN-γ, and this can be increased by co-injection with *Bordetella pertussis* toxin [17], or IL-12 [55]. Therefore, it remains to be tested whether co-injection of alumOVA with molecules influencing the extent of inflammation, such as TLR agonists or cytokines, would impair the generation of early central memory CD4 T cells.

Materials and methods

Mice

WT C57BL/6J mice were from HO Harlan OLAC (Bicester, UK). OT-II mice, which are transgenic for TCR specific for 323–339 OVA-peptide in the context of H-2 I-A^b (Charles River, Wilmington, MA), were crossed to CD45.1^+ C57BL/6 congenic mice (The Jackson Laboratory, Bar Harbor, Maine). All animals were maintained under standard animal house conditions in accordance with local and Home Office regulations.

T-cell purification methods, including FACS-cell sorting, and adoptive transfer

CD4 T cells from the LN of OT-II mice were purified using anti-CD4 MACS microbeads (Miltenyi Biotec, Bisley, UK). Of note, no differences in OT-II cell activation or proliferation have been observed when these were negatively purified [56] or positively selected with the anti-CD4 MACS microbeads. Where indicated, small naïve $CD4^+CD62L^+CD44^-$ OT-II cells were sorted by flow cytometry (MoFlo, DakoCytomation, UK). To ensure high purity, and the exclusion of memory ($CD4^+CD62L^-CD44^+$) cells from the naïve $CD4^+CD62L^+CD44^-$ OT-II cell population, samples were sorted twice. Sorted cell purity was assessed on a MoFlo. Before transfer CD45.1^+ OT-II T cells were labeled with CFSE (Cambridge Bioscience, Cambridge, UK) and were injected i.v. at 5×10^6 cells *per* congenic CD45.2^+ recipient mouse, unless otherwise stated. Mice were immunized the following day.

Immunization, antigens and BrdU

Immunizations were with 10 μg of OVA (Imject® Ovalbumin, Perbio Science UK) precipitated with aluminum potassium sulfate (A7167 Sigma) as described previously [16]. Injections were given subcutaneously into the plantar surface of the rear footpads or into the right front paw. At intervals after immunization LN were removed and processed for FACS analysis or histology. To label cells in the S phase of the cell cycle, some mice received the thymidine analog BrdU (Sigma) administered i.p. as 200 μg in PBS given either 2 or 6 h before sacrifice as indicated in the results.

Flow cytometry and BrdU detection

Popliteal LN were digested for 20 min at 20°C with collagenase type II (1 mg/mL) (Lorne Laboratories, Reading, UK) and DNase I (0.15 mg/mL) (Sigma), followed by 5 min treatment with 10 mM EDTA (Sigma). Anti-CD45.1-PE (A20), CD4-PerCP-Cy5.5 (RM4–5), biotinylated anti-CD69 (H1.2F3), CD62L (MEL-14), CD25 (7D4), CD44 (IM7), CD11a (M17/4), CD45RB (16A), CD127/IL-7Ra (A7R34), CXCR5 (2G8) and streptavidin-APC were from PharMingen. BrdU incorporation was detected using BrdU APC flow kit (Becton Dickinson). Cell phenotype was assessed on a FACScalibur (Becton Dickinson). Final analysis and graphical output were performed using FlowJo software (Treestar, Costa Mesa, CA).

Confocal miscroscopy analysis of tissue sections

LN were embedded in OCT compound, snap frozen in liquid nitrogen and stored at 70°C until used. Five micrometer cryostat sections were mounted on four-spot glass slides. Sections were dried for 30 min at 20°C, fixed in acetone 20 min at 4°C, dried again for 20 min at 20°C and stored in sealed polythene bags at 70°C. Sections to be examined by confocal microscopy were stained with goat anti-mouse IgM-AMCA (Jackson Immunoresearch) and CD8α-APC (CT-CD8a) (PharMingen). Rat antibodies specific for PNAd (MECA-79) (PharMingen), Ki67 (a kind gift from Johannes Gerdes, Borstel, Germany), were detected using goat anti-rat-Cy3 (Zymed). OT-II cells were revealed using CD45.1-FITC (A20) (PharMingen). This signal was further amplified using rabbit anti-FITC (Dako, High Wycombe, UK) and goat anti-rabbit-FITC (PharMingen) antibodies. Stained sections were mounted with DABCO in glycerol at pH 8.6. Pictures were acquired and processed using LSM510 Zeiss confocal microscope and software.

Real-time semi-quantitative RT-PCR

Cell suspension mRNA was prepared using RNeasy columns from Qiagen. Reverse transcription was performed with random oligonucleotides (Promega) using MMLV reverse transcriptase (Invitrogen) for 1 h at 42°C. Relative quantification of specific IL-4 or processed γ1 GT cDNA species to β-actin or β2-microglobulin messenger was carried out in a duplex PCR using TaqMan chemistry (Applied Biosystems, Warrington, UK). As a prerequisite, the amplification efficiency was confirmed for each combination for the two sets of primers/probes. Probes for IL-4 and γ1 GT were detected *via* a 5′ label with FAM fluorescent label, while probe for β-actin or β2-microglobulin were 5′ labeled with VIC or NED fluorescent labels, respectively. TaqMan probes and primers were designed using Primer Express software (Applied Biosystems). Sequences for β-actin, β2-microglobulin, IL-4, γ1 GT, were published previously [16, 57]. Standard reaction conditions for the TaqMan RT-PCR or PCR were used on the ABI 7900. Relative quantification of the signal was achieved by setting thresholds within the logarithmic phase of the PCR for β-actin, or β2-microglobulin, and the test gene and determining the cycle number at which the threshold was reached (C_T). The C_T for the target gene was subtracted from the C_T for β-actin or β2-microglobulin. The relative amount was calculated as $2^{-\Delta C_T}$.

Ex vivo restimulation and intracellular cytokine staining

Various days after primary or secondary immunization popliteal or brachial LN cells were incubated at 10×10^6 cells/mL with 10 μM free 323–339 OVA-peptide for 5 h prior to cytokine detection. Intracellular FACS staining was performed using Cytofix/cytoperm kit (Becton Dickinson) according to the manufacturer's instructions. Anti- IL-4-APC (11B11) is from PharMingen.

Statistical analysis

All statistical analyses were performed using a two-tailed *t*-test or a non-parametric Mann–Whitney test. Values of $p < 0.05$ were considered significant.

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Abbreviations: **alumOVA:** alum-precipitated OVA · **d:** day · **distant LN:** Ag-free brachial LN outside the drainage area of the site of immunization · **draining LN:** popliteal LN draining the site of footpads immunization · **GT:** germline transcript · **NI:** non-immunized

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