European Journal of Immunology

Recirculating CD4 memory T cells mount rapid secondary responses without major contributions from follicular CD4 effectors and B cells

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Received 5/8/06
Revised 29/1/07
Accepted 10/4/07

[DOI 10.1002/eji.200636573]

For weeks after primary immunization with thymus-dependent antigens the responding lymph nodes contain effector CD4 T cells in T zones and germinal centers as well as recirculating memory T cells. Conversely, remote nodes, not exposed to antigen, only receive recirculating memory cells. We assessed whether lymph nodes with follicular effector CD4 T cells in addition to recirculating memory CD4 T cells mount a more rapid secondary response than nodes that only contain recirculating memory cells. Also, the extent to which T cell frequency governs accelerated CD4 T cell recall responses was tested. For this, secondary antibody responses to a superantigen, where the frequency of responding T cells is not increased at the time of challenge, were compared with those to conventional protein antigens. With both types of antigens similar accelerated responses were elicited in the node draining the site of primary immunization and in the contralateral node, not previously exposed to antigen. Thus recirculating memory cells are fully capable of mounting accelerated secondary responses, without the assistance of CD4 effector T cells, and accelerated memory responses are not solely dependent on higher T cell frequencies. Accelerated memory CD4 T cell responses were also seen in B cell-deficient mice.

Key words: B lymphocytes · Cell trafficking · Lymph nodes · Memory · T lymphocytes

Introduction

Primary immunization with thymus-dependent antigens results in specific T and B cell responses in draining lymph nodes (LN). Naive CD4 T cells enter cell cycle after being primed by cognate interaction with dendritic cells in the T zones. This results in a strong expansion of antigen-specific lymphocytes within the LN [1]. The high number of antigen-responding CD4 T cells typically is not sustained, and only a small proportion of the cells persists [2, 3]. Nevertheless, CD4 memory T cells can survive for long periods without continued contact with antigen [4–6], although it has been reported that such antigen-deprived cells may lack competence on reactivation *in vivo* [7].

Antigen-experienced CD4 T cells have been classified into central memory and effector memory cells [8]. Central memory T cells have a slow turnover and express high levels of CCR7 and CD62L, which are both associated with homing to T zones of lymphoid tissues. Conversely, effector memory T cells home to inflamed tissues, have a rapid turnover, and are $CCR7^{-}$ $CD62L^{low}$. Both effector and central memory populations *in vitro* respond rapidly to low doses of antigen, but while

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Abbreviations: **CGG:** chicken gamma globulin · **LCMV:** lymphocytic choriomeningitis virus · **MMTV(SW):** Swiss-type mammary tumor virus · **NP:** 4-hydroxyl-3-nitrophenylacetyl · **PCC:** pigeon cytochrome C

central memory cells are reported to produce mainly IL-2, peripheral effector memory cells produce effector cytokines including IFN-γ, IL-4, and IL-5 within hours of restimulation *in vitro* [9, 10]. This concept has been supported by studies *in vivo*: long-term-surviving CD8 T cells show cytolytic activity when isolated from non-lymphoid tissues, while CD8 T cells from lymphoid tissue do not have immediate *ex vivo* effector function [11]. Also memory CD4 T cells from non-lymphoid tissues respond to restimulation with peptide with rapid IFN-γ production [12]. Central memory T cells can efficiently protect against *Leishmania* or lymphocytic choriomeningitis virus (LCMV) during weeks after infection [13, 14]. LCMV-specific central memory CD8 T cells were shown to produce effector cytokines rapidly upon restimulation [14].

In lymphoid tissues, CD4 memory T cells and B cells interact in the outer T zone within hours of secondary immunization [15]. It is unclear whether the responding CD4 T cell in this situation is a central memory T cell [16, 17], or whether effector T cells, like the T cells that colonize follicles in LN responding to antigen, play a major role. Effector T cells in germinal centers select B cells that have undergone hypermutation [17, 18] and these T cells can persist in follicles for months [19]. We have approached this question by assessing whether, in secondary responses, recirculating memory CD4 T cells in LN not involved in the primary response produce cytokines and activate B cells with different speed and efficiency compared to those T cells in LN that participated in the primary response.

LN draining the site of immunization develop substantial populations of antigen-specific CD4 effector T cells located in the follicles and outer T zone; these are not found in the remote LN. After immunization with limiting amounts of antigen, most antigen is trapped in the LN draining the site of immunization (draining LN) and lymphocyte activation is limited to this site. Several weeks after immunization draining LN will contain effector and memory CD4 T cells. In contrast, LN remote from the site of primary immunization (remote LN) will only have received recirculating memory T cells. Naive antigen-specific CD4 T cells in these remote LN are not activated by antigen locally and do not enter cell cycle in this site [20, 21]. Using this system we compared recall antibody responses *in vivo* in LN containing only recirculating memory T cells with those in LN that contained effector T cells as well as recirculating memory T cells. These experiments show that recirculating memory T cells are fully capable of inducing secondary B cell responses.

Superantigen was used in some studies to enable us to test the extent to which the speed of the secondary response is attributable to the frequency of antigen-reactive memory T cells. This is possible because antigen-responsive CD4 T cells in LN not exposed to antigen fall after immunization. The opposite typically occurs in responses to a conventional antigen. These experiments demonstrate that accelerated secondary responses are not simply dependent on higher T cell frequencies. Further studies were carried out in B cell-deficient mice to test whether B cells play a significant role either in the production of CD4 memory T cells in primary responses or through presenting antigen to the memory T cells in secondary responses.

Results

Rate and extent of replacement of naive with antigen-experienced CD4 T cells

The rate and extent of replacement of naive CD4 T cells with antigen-experienced effector and memory T cells was first studied in a superantigen-dependent response. The frequency of superantigen-responsive $V\beta6^+$ CD4 T cells in the draining popliteal LN and the contralateral remote LN were assessed after infection with Swiss-type mammary tumor virus [MMTV(SW)] in one foot (Fig. 1A). To exclude effects from infection with live virus, parallel experiments were performed in which BALB/c mice were immunized in one foot with splenocytes from BALB.D2 mice (Fig. 1B). BALB.D2 B cells constitutively express the endogenous Vβ6-specific superantigen (Mtv-7) but do not produce virus [22, 23].

In both responses before infection or immunization, 12–14% of CD4 T cells ($\sim 10^5$ CD4 T cells per LN) were $V\beta6^+$ (Fig. 1A, B, day 0). In the draining LN the frequency and number of $V\beta6^+$ cells rose sharply from day 3 after injection of superantigen, as previously described [1]. Between day 7 and 10, $V\beta6^+$ CD4 T cell numbers in the draining LN fell dramatically. This was followed by a long phase with a slow decline in the number of these cells. Five weeks after immunization, $V\beta6^+$ CD4 T cells in the draining LN were at pre-immunization levels. Total numbers on day 35 were still fivefold above pre-immunization levels because the draining LN at this stage is still enlarged.

Seven months post-infection, the frequency of memory $V\beta6^+$ CD4 T cells that remained in the draining LN was only 7% of CD4 T cells, about half of that found in LN before infection (Fig. 1A). Remote LN, by contrast, did not show an increase in $V\beta6^+$ CD4 T cell frequency following primary infection (Fig. 1A); instead there was a slow loss of these cells. After 6 months, approximately 2% of the CD4 T cells in the remote LN were $V\beta6^+$. Antigen-dependent activation does not occur in the remote LN [12, 21, 24]. Nevertheless, recirculating naive $V\beta6^+$ T cells will continue to enter the draining LN. For

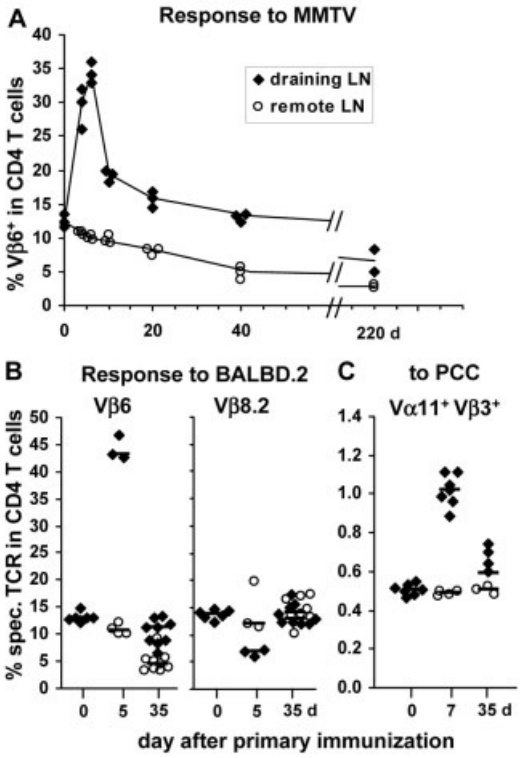

Figure 1. Increase and subsequent decline of antigen-specific T cell numbers during primary responses in draining and remote LN. Mice were immunized into one hind foot and the T cell frequencies were determined in popliteal LN draining the site of immunization (draining LN) or contralateral popliteal LN (remote LN). (A) Proportion of Vβ6$^+$ cells in CD4 T cells in the draining LN (filled symbols) and remote LN (open symbols) after primary foot infection with MMTV(SW), measured by flow cytometry; lines are drawn through the medians. (B) Similar changes after priming of BALB/c mice with congenic BALB.D2 cells expressing the Mtv-7 superantigen. Vβ8.2 CD4 T cells not reacting with the superantigen are shown as a control. The apparent decline of Vβ8.2 T cell frequencies in draining LN on day 5 is due to dilution of Vβ8.2 T cells by the expanding Vβ6$^+$ T cells. (C) Similar changes in Vα11$^+$ Vβ3$^+$ CD4 T cells in draining and remote LN after immunization with alum-precipitated PCC with higher frequency of antigen-specific CD4 T cells in the draining LN 5 wk after immunization ($p<0.05$). Each symbol represents one LN, bars indicate median values.

as long as superantigen continues to be available in this LN, these cells will be lost from the naive Vβ6$^+$ recirculating CD4 T cell pool. The extent to which this occurs is addressed in *Distribution of antigen-experienced T cells in different lymph nodes*.

The frequency of antigen-specific CD4 T cells was also followed in a T cell response to a conventional protein antigen, pigeon cytochrome C (PCC). In this response antigen-reactive CD4 T cells are prominent among Vα11$^+$ Vβ3$^+$ T cells [16], allowing the T cell response to be traced by following the number and distribution of Vα11$^+$ Vβ3$^+$ CD4 T cells. Although there are far fewer PCC-responsive CD4 T cells than super-antigen-responsive Vβ6$^+$ cells, the pattern of recruitment, expansion, and survival of PCC-responsive cells is similar to that seen for superantigen-responsive cells following infection with MMTV(SW) (Fig. 1C).

Distribution of antigen-experienced T cells in different lymph nodes

Five weeks after foot immunization with BALB.D2 splenocytes, the follicles of draining LN still contained germinal centers (Fig. 2A, B, left panels). These were absent from remote LN (Fig. 2A, right). The abundant follicular CD4 T cells, *i.e.* cells in germinal centers and follicular mantle, were predominantly Vβ6$^+$ superantigen-responsive cells (Fig. 2A, centre; Fig. 2B, right). By contrast, there were few follicular T cells in remote LN (Fig. 2A, right) and among those present there was no bias towards Vβ6 expression (Fig. 2B, right). In the T zone of both the remote and draining LN the proportion of Vβ6$^+$ cells was low (Fig. 2B, right). This

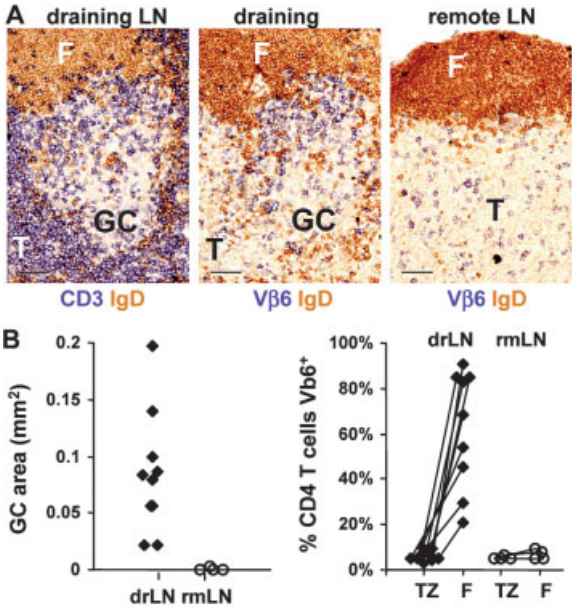

Figure 2. Long-term accumulation of T cells in germinal centers 5 wk after immunization with BALB.D2 cells. (A) Draining LN 5 wk after immunization with BALB.D2 cells. Staining for CD3 (blue) to show the T zone (TZ), and IgD (brown) to show the follicle (F). A large germinal center (GC) can be identified by its lack of IgD$^+$ B cells and presence of T cells in the germinal center light zone. Adjacent section showing T cells in the light zone to be largely Vβ6$^+$. (right) Remote LN showing recirculating Vβ6$^+$ T cells (blue). These are restricted to the T zone. Scale bars indicate 50 μm. (B) Germinal center area in mm^2 on tissue sections of draining LN (drLN) and remote LN (rmLN) 5 wk after immunization shows that germinal centers only appear in the antigen-containing draining LN ($p<0.05$). (right) Frequency of Vβ6$^+$ T cells 5 wk after immunization with BALB.D2 cells compared in T zone and germinal centers or follicles of draining LN (filled symbols) and remote LN (open symbols). Lines connect data from the same LN.

suggests that at 5 wk, while superantigen continues to be available in germinal centers of the draining LN, its presentation by dendritic cells to recirculating cells entering the T zone may be limited.

Five weeks after immunization with BALB.D2 splenocytes most superantigen-responsive CD4 T cells should have had contact with antigen by recirculation through the draining LN [25]. The extent to which naive Vβ6$^+$ cells were lost and memory cells colonized draining and remote LN was investigated using flow cytometry. Fig. 3 shows the expression of a number of markers associated with antigen experience on Vβ6$^+$ CD4$^+$ T cells 5 wk after injection of BALB.D2 cells into one foot. As a control the expression of these markers on non-superantigen-responsive Vβ6$^-$ CD4 T cells was assessed. Vβ6$^-$ CD4 T cells express similar surface markers to Vβ6$^+$ T cells from LN of non-immunized naive animals (data not shown). In both the draining and remote LN the majority of the Vβ6$^-$ CD4 T cells have a naive phenotype. Evidence of antigen experience is provided by decreased CD45RB and increased CD11a and CD44 expression among Vβ6$^+$ CD4 T cells. The proportion of cells expressing these markers is higher in draining than in remote LN (Fig. 3B). Interestingly, similar proportions of Vβ6$^+$ CD4 T cells in draining and remote LN show loss of CD62L and CCR7 expression.

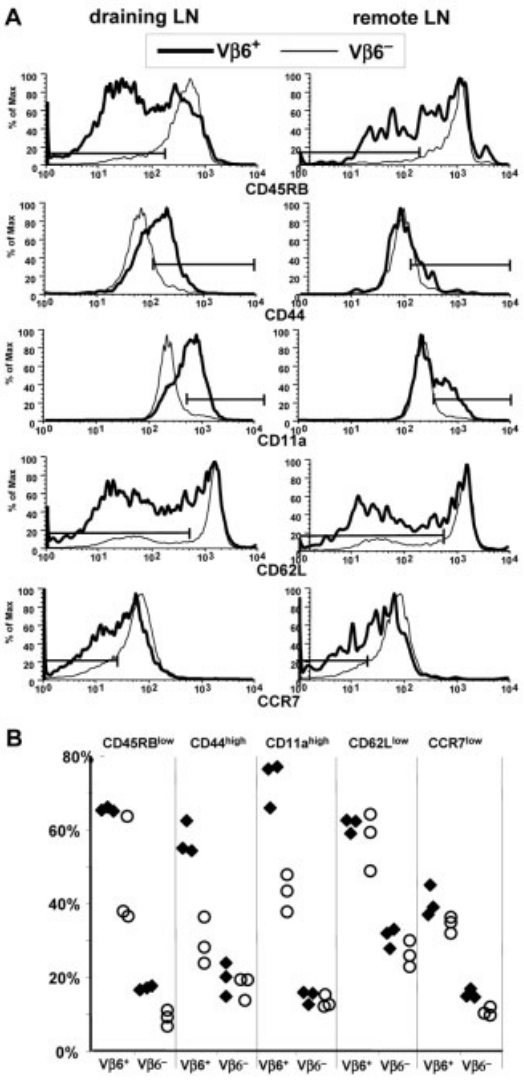

Figure 3. Activation/memory markers on CD4 T cells in draining and remote LN 5 wk after injection of BALB.D2 cells. (A) Expression of surface markers on Vβ6$^+$ CD4 T cells (bold line) compared with Vβ6$^-$ cells (thin line) at 5 wk after immunization. Profiles on Vβ6$^-$ cells were similar to naive CD4 T cells. (B) Fraction of cells with expression markers within the gates indicated in (A) for draining LN (filled symbols) or remote LN (open symbols).

Secondary responses are accelerated in both the draining LN and remote LN

As is shown above the draining LN contains follicular effector cells as well as recirculating memory T cells, while remote LN contain only recirculating memory T cells. To assess whether this follicular effector population is important for achieving accelerated secondary responses, mice were primed by immunization with BALB.D2 cells in one foot and rechallenged 5 wk later in both feet. The time of onset of T cell activation between the two popliteal LN was then compared. This was assessed by the rate of induction of synthesis of IL-4 mRNA, which in responses of this sort of antigen is produced by antigen-specific CD4 T cells [26]. Further, B cell-derived IgG H chain germ-line transcripts were detected as markers for CD4 T cell-dependent B cell activation [15, 27].

The recall response in both LN was clearly more rapid than that seen after primary immunization (Fig. 4). While the primary response is detectable from day 3 (inducing mainly Th1-like cytokines such as IFN-γ and Ig class switching to IgG2a; data not shown, see [24]), the secondary response showed accelerated up-regulation of IL-4 mRNA and induction of IgG1 germ-line transcript production within 36 h in both draining and remote LN (Fig. 4). The secondary response in the draining LN starts with higher background levels of IL-4 mRNA and H chain germ-line transcripts compared to those in remote LN. This is likely to be due to persistent CD4 T cell activity in germinal centers (Fig. 3) and corresponds well with the higher numbers of antigen-specific T cells in the draining LN (Fig. 1B). The superantigen-dependent system shows that the accelerated memory response in LN is due to an increase in responsiveness of the T cells and not simply to an increase in the frequency of responsive T cells, for the frequency of Vβ6$^+$ CD4 T cells in the remote LN (Fig. 1B) falls rather than rises after primary immunization.

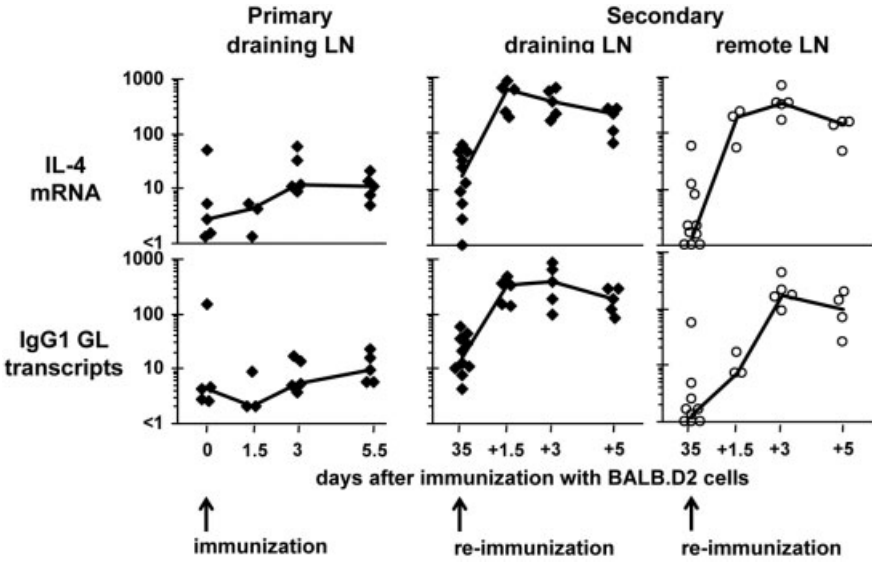

Figure 4. Primary and secondary T cell activation in response to BALB.D2 cells. Mice were immunized into one hind foot with BALB.D2 cells and 5 wk later rechallenged into both hind feet. T cell cytokine mRNA expression and IgG1 H chain germ-line transcript expression were used as indicators of successful *in vivo* cognate T cell-B cell interaction in the popliteal draining LN or remote LN (which was only exposed to antigen after secondary immunization). Secondary immunization induces an accelerated response in the draining LN and remote LN with a significant increase within 36 h of IL-4 mRNA and IgG1 germ-line transcript ($p \leq 0.01$, IgG1 germ-line transcript in remote LN $p < 0.05$). Symbols show the relative amount of the specified mRNA per tissue section, determined by real-time RT-PCR.

Recirculating memory CD4 T cells produce accelerated responses to conventional protein antigens

Secondary responses were also followed after immunization with 4-hydroxy-3-nitrophenylacetyl (NP)-chicken gamma globulin (CGG), a hapten-protein conjugate that is presented in a conventional way. This activates fewer T cells and enables the detection of activation of antigen-specific B cells. Mice were primed with CGG in alum into one foot and 5 wk later challenged with soluble NP-CGG in both feet. The change in IL-4 mRNA and IgG1 germ-line transcripts in the left and right LN were comparable (Fig. 5A, B). The early T cell-B cell interaction induces B cell differentiation that leads to NP-specific plasmablast development at similar kinetics in draining and remote LN (Fig. 5C). Again, 5 wk after primary immunization, germinal centers that contain follicular effector T cells are only found in draining LN (Fig. 5D).

To test whether inflammation and preactivation of innate immune cells had a role in the different activation levels of lymphocytes in draining and remote LN, the remote LN was primed with an irrelevant antigen (heat-killed *Escherichia coli*). This produced significant swelling of the remote LN that lasted until secondary immunization at 5 wk after priming, but did not accelerate the secondary response to NP-CGG (Fig. 5A, B, squares).

B cells are not necessary for accelerated T cell recall responses

It has been reported that B cells may have a role accelerating memory T cell responses [28]. Memory CD4 T cells may be better able to interact with and be activated by B cells than naive CD4 T cells [29]. In addition, an increase in the number of antigen-specific B cells following primary immunization may increase the chance of B cells, as opposed to dendritic cells, activating naive or memory CD4 T cells. Finally, B cell follicles provide a niche for a proportion of CD4 T cells recruited into antibody responses (Fig. 3). Influence of these factors on the rate of cytokine induction in secondary responses was tested in wild-type (BALB/c) and congenic B cell-deficient (μMT) mice. Mice were primed with alum-precipitated CGG in both hind feet and challenged 5 wk later in the same sites with soluble NP-CGG. The rate of induction of IL-4 mRNA in the draining LN was compared at intervals after challenge (Fig. 6). The rate of IL-4 mRNA induction in B cell-deficient mice was similar to that in wild-type mice.

Discussion

This study shows that CD4 T cells from antigen-containing draining and antigen-free remote LN have a similar capacity to induce secondary antibody

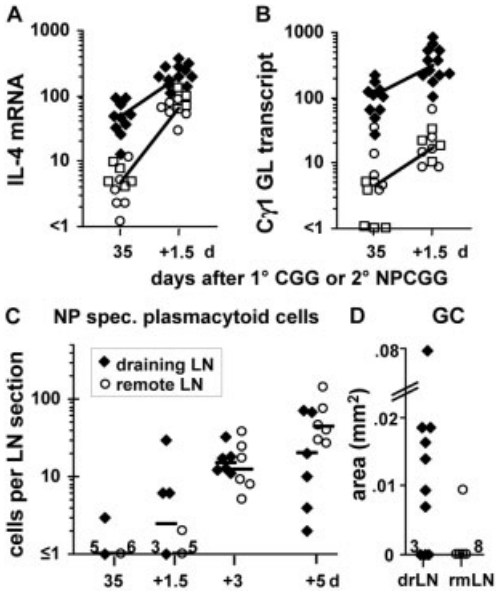

Figure 5. Primary and secondary activation in draining LN and remote LN after immunization with protein. Mice were primed with CGG into one hind foot and 5 wk later rechallenged with NP-CGG into both feet. Secondary immunization induces a fast and equivalent onset of (A) IL-4 mRNA production ($p<0.001$) and (B) T cell-dependent IgG1 germ-line transcript production by B cells in draining LN (filled symbols) and remote LN (open circles) ($p<0.005$). Remote LN primed with heat-killed *E. coli* as a control for effects of inflammation are labeled with open squares (C) Similar kinetics and magnitude of NP-specific plasmablast proliferation in draining and remote LN after rechallenge with NP-CGG. Points show the number of cells specific for NP per draining and remote LN section. Numbers next to symbols denote the number of points on the axes. (D) Five weeks after primary immunization, small germinal centers are present exclusively in draining LN ($p<0.01$). Data show germinal center area in mm^2/tissue section.

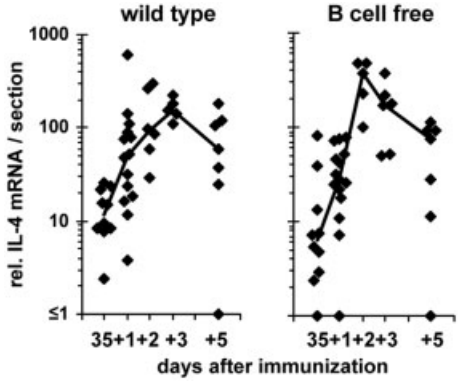

Figure 6. Secondary rapid induction of IL-4 synthesis is not dependent on early cognate T cell interaction with B cells. Wild-type and B cell-deficient (μMT) mice were primed with CGG in alum with *B. pertussis* and 5 wk later rechallenged with soluble NP-CGG. B cell-deficient mice have an equivalent fast onset of IL-4 mRNA expression with significant induction of IL-4 mRNA within 24 h ($p<0.01$). Data merged from three similar experiments are shown, assessed by real-time PCR.

responses. Several studies indicate that central memory T cells have a lower capacity than effector memory T cells to produce effector cytokines rapidly on restimulation [8, 11, 12]. More recently, central memory T cells have been found to protect efficiently against reinfection with *Leishmania* [13] or LCMV [14]. LCMV-specific central memory CD8 T cells were shown to be able to express effector function rapidly on restimulation [14].

We show here that recirculating CD4 memory T cells in remote LN efficiently activate B cells *in vivo*, and that the onset of cytokine induction and B cell activation occurs at a similar speed to that in LN that contain effector T cells as well recirculating CD4 T cell memory. Since these responses are equivalent, recirculating memory T cells appear to be the main T cell population that induces rapid secondary antibody responses. Effector T cells such as follicular T cells from draining LN do not appear to play a major role in the efficient recruitment of B cells into secondary responses. The less efficient reactivation of central memory T cells *in vitro* [8] as opposed to recirculating memory cells *in vivo* may be due to a stricter requirement for accessory cells only available in an intact LN structure [30].

The other major conclusion, which can be drawn from the experiments with superantigen, is that the accelerated secondary T cell response is not simply a function of a higher frequency of antigen-specific T cells. Superantigen-specific secondary responses in draining as well as remote LN develop rapidly despite lower frequencies of antigen-reactive T cells in both LN compared with the frequency in the LN of naive mice. MMTV induces a strong expansion of superantigen-reactive CD4 T cells within the first week after immunization. This is followed by a similarly quick loss of antigen-specific T cells to levels close to those seen in naïve LN. A slower prolonged depletion phase finally leads to lower levels of antigen-specific T cells than there were present before immunization. T cells in LN remote from the site of immunization are depleted from the beginning of the immune response.

Earlier studies have concluded that primary immunization with superantigen leads to a state of T cell anergy. This was based on finding that the T cells remaining after superantigen exposure are not easily induced to proliferate *in vitro* and that re-immunization leads to little increase in T cell numbers [31–33]. Nevertheless, in the present *in vivo* analysis re-exposure to Mtv-7-expressing cells induced strongly accelerated T cell cytokine production and T-dependent B cell activation. These secondary responses were comparable to those to conventional protein antigens.

A considerable number of the recirculating memory T cells in remote LN express low levels of CCR7 and CD62L. Does this mean that recirculating memory T cells are effector memory T cells [8]? Although CCR7 and

CD62L expression are important for lymphocyte entry into LN *via* high endothelial venules [34, 35], even naive LN have considerable numbers of $CCR7^{low}$ and $CD62L^{low}$ cells (equivalent to the $V\beta6^-$ cells, Fig. 3). This loss of expression may reflect the change in responsiveness to chemokines and binding to adhesion molecules while recirculating lymphocytes move in and out of secondary lymphoid tissues. This change seems to be more pronounced in the preactivated memory T cells.

The progressive loss of T cells from draining and remote LN might reflect differences between the number of naive and memory T cells that can be sustained. Memory/effector CD4 T cells are intrinsically more vulnerable to apoptosis [36] than naive T cells although their survival can be maintained by factors not specific for antigen released from inflammatory stroma, such as IL-2 family cytokines [37, 38], or type I IFN [39]. At later stages of the immune response, when less stimulation from antigen-presenting cells is available, numbers of antigen-specific memory T cells that are sustained in non-immunized LN may fall below those of naive T cells in those LN. The same is seen from the earliest stages of the response in remote LN. Remote LN in the response to MMTV are never exposed to antigen, because production of neutralizing antibody in the draining LN prevents the infection from spreading to remote LN [21, 40]. Also injection of BALB.D2 cells or alum-precipitated protein does not induce proliferation in remote LN (Fig. 1B).

Nevertheless, continued recirculation of naive CD4 T cells into the draining LN [25] results in a gradual replacement of the superantigen-specific naive recirculating T cell pool by recirculating memory T cells. As there is no stimulation from antigen-presenting cells in remote LN, the frequency of antigen-specific T cells will fall here from the earliest stages after immunization. Draining LN, by contrast, provide additional environments where antigen-dependent stimulation is provided: follicles with germinal centers [16, 41, 42] containing follicular dendritic cells that hold long-term depots of antigen and also B cells that provide cognate interactions.

We also investigated whether B cells have a role in sustaining memory T cells in the responses studied. B cells have been shown to be efficient inducers of cytokine production by memory and effector T cells *in vitro* [43]. Also, B cells are essential to form the germinal centers, and memory B cells might be able to take up antigen very quickly and present this to T cells. Surprisingly, the present study shows that in secondary responses, B cell-deficient mice produce T cell effector cytokines at just the same speed and magnitude as wild-type mice. Other studies have shown that memory T cells from B cell-deficient mice produce less cytokine after restimulation with antigen-pulsed splenocytes [28, 44, 45]. This may reflect the lack of follicular effector T cells in B cell-deficient mice that would interact with antigen-presenting cells *in vitro*.

However, as shown here, these cells are not necessarily the cells that induce rapid secondary antibody responses. The fast onset of secondary T cell cytokine production in B cell-free mice *in vivo* shows not only that B cells are unnecessary for long-term survival of recirculating memory T cells, but also that the interaction of memory T cells with dendritic cells is sufficient to induce fast secondary T cell activation response. This confirms the conclusion drawn from the experiments studying responses to superantigen that memory T cell precursor numbers are not the only factor determining the rate of secondary responses. Qualitative differences between primary and antigen-experienced T cells [43] play a key role in the rapid activation of recirculating memory T cells without the prolonged interactions necessary for primary activation of naive T cells [10].

Materials and methods

Mice

BALB/c and C57BL/6 mice were purchased from HO Harlan OLAC Ltd. (Bicester, UK). MMTV(SW)-infected BALB/c mice were bred from breeding pairs originally purchased from IFFA Credo (L'Arbesle, France). BALB.D2 mice were bred from breeding pairs originally obtained from H. Festenstein [22]. B cell-deficient μMT mice were bred from breeding pairs obtained from K. Rajewsky [46]. Mice were injected at 5–12 wk of age. All procedures were approved by the Birmingham University Ethical Reviews Sub-Committee.

Immunizations and antigens

MMTV-containing milk was obtained from lactating female mice as previously described [23]. Approximately 10^8 MMTV particles (0.1–0.5 μL milk) were diluted in phosphate-buffered saline (final volume of 10 μL) and injected subcutaneously into the plantar surface of one or both hind feet. Alternatively, 5×10^6 BALB.D2 cells obtained from spleen and LN were injected subcutaneously into the foot. Secondary injection of BALB.D2 cells was done 5 wk later *via* the same route.

Protein antigens CGG, NP-CGG, and PCC (Sigma-Aldrich, Poole, UK) were prepared as described [15, 16]. Primary immunization was done with 25 μg alum-precipitated protein per 20 μL saline. If mentioned in the text, 5×10^8 chemically killed *Bordetella pertussis* bacteria (Evans Medical, Liverpool, UK) were added during primary immunization. To control for the effects of inflammation *per se*, some remote LN were primed with 5×10^6 heat-killed *E. coli* as an irrelevant antigen. Between 10 and 20 μL were injected subcutaneously into the plantar surface of the foot [24]. Secondary immunization with 25 μg soluble protein subcutaneously above both hind feet was performed 5–7 wk after primary immunization. At intervals after immunization, LN were removed and processed for FACS

analysis, histology, or real-time RT-PCR. LN draining the primarily immunized foot were designated draining LN. LN draining the foot contralateral to the site of primary immunization (in some experiments this foot received a secondary immunization) were designated remote LN.

Flow cytometry

At the indicated time points, popliteal or brachial LN were harvested and dilacerated. Cell suspensions were incubated with collagenase type II (Lorne Laboratories Ltd., Reading, UK) and DNase (Sigma-Aldrich). Cells were then stained for flow cytometry. The following antibodies were used for flow cytometry: anti-Vβ6-FITC (44.22.1) [47], anti-CD4-PE or -PerCP-Cy5.5 (H129.19 or RM4-5), anti-CD45.1-PE (A20), anti-CD45RB-biotin (C363.16A), anti-CD11a-biotin (M17/4), anti-CD62L-biotin (Mel-14), anti-CD44-biotin (1M7), and streptavidin-allophycocyanin (Pharmingen, San Diego, CA). The Vβ6-specific antibody was coupled to FITC in our own laboratory. All other antibodies were from Pharmingen. CCR7 was labeled by binding with CCL19-Fc [48] followed by goat anti-human Ig-biotin (Jackson ImmunoResearch Laboratories, West Grove, PA) and streptavidin-PE-Cy5.5 (eBioscience, San Diego, CA). Analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA) using FlowJo software (Treestar, Costa Mesa, CA).

RT-PCR

Semiquantitative real-time RT-PCR was done from two 25 μm-thick frozen LN sections adjacent to sections used for immunohistology as described [49]. Briefly, RNA was prepared from frozen tissue sections using RNAzol B (Biogenesis, Poole, UK). This was reverse-transcribed using random oligonucleotide hexamers (Amersham Biosciences, Chalfont St. Giles, UK). Relative quantitation of specific cDNA species message was carried out on the ABI 7700 (Applied Biosystems, Warrington, UK) using TaqMan chemistry (Applied Biosystems) in a multiplex PCR with primers and probes for the target gene and β-actin cDNA. Probes for cytokines and IgG1 H chain germ-line transcripts were detected *via* a 5′ label with FAM (Applied Biosystems), while probes for β-actin were 5′-labeled with VIC (Applied Biosystems). Sequences were as described earlier [49].

Relative signal per cell was calculated by setting thresholds within the logarithmic phase of the PCR for β-actin and the target gene and determining the cycle number at which the threshold was reached (C_t). The C_t for β-actin was subtracted from the C_t for the target gene. The relative amount was calculated as $2^{-\Delta Ct}$. To calculate mRNA per LN section, the relative amount was multiplied by the section area.

Immunohistology

Immunohistology was performed as described previously [1, 15, 16]. An additional antibody used was rabbit anti-mouse Ki-67 antiserum (gift from Johannes Gerdes, Borstel, Germany) labeled with swine anti-rabbit Ig antiserum (Dako, Glostrup, Denmark), followed by rabbit PAP (Dako). Sections were developed as described [15].

Germinal center sizes were determined from CD3-, IgD-stained tissue sections by point counting [15] Germinal centers were taken as IgD^- areas with few T cells surrounded by IgD^+ follicles and $CD3^+$ T zones. $V\beta6^+$ germinal center T cells were counted on adjacent sections stained for Vβ6 and IgD.

Statistical analyses

Statistical analysis was carried out using the Mann–Whitney non-parametric sum of ranks test.

Acknowledgements: This study was supported by a Medical Research Council program grant and the Swiss National Science Foundation grant No. 31-59165.99. S.A.L. was funded by the Roche Research Foundation, Emma Muschamp Foundation, and the Swiss National Science Foundation grant no. PP00A-68805.

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