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Neo-Lymphoid Aggregates in the Adult Liver Can Initiate Potent Cell-Mediated Immunity

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Abstract

Subcutaneous immunization delivers antigen (Ag) to local Ag-presenting cells that subsequently migrate into draining lymph nodes (LNs). There, they initiate the activation and expansion of lymphocytes specific for their cognate Ag. In mammals, the structural environment of secondary lymphoid tissues (SLTs) is considered essential for the initiation of adaptive immunity. Nevertheless, cold-blooded vertebrates can initiate potent systemic immune responses even though they lack conventional SLTs. The emergence of lymph nodes provided mammals with drastically improved affinity maturation of B cells. Here, we combine the use of different strains of alymphoplastic mice and T cell migration mutants with an experimental paradigm in which the site of Ag delivery is distant from the site of priming and inflammation. We demonstrate that in mammals, SLTs serve primarily B cell priming and affinity maturation, whereas the induction of T cell-driven immune responses can occur outside of SLTs. We found that mice lacking conventional SLTs generate productive systemic CD4+ as well as CD8-mediated responses, even under conditions in which draining LNs are considered compulsory for the initiation of adaptive immunity. We describe an alternative pathway for the induction of cell-mediated immunity (CMI), in which Ag-presenting cells sample Ag and migrate into the liver where they induce neo-lymphoid aggregates. These structures are insufficient to support antibody affinity maturation and class switching, but provide a novel surrogate environment for the initiation of CMI.

Introduction

Secondary lymphoid tissues (SLTs) are highly organized structures with defined compartments consisting of B and T cell areas. These distinct locations support the rapid circulation and concentration of Ag and the interaction of Ag-presenting cells (APCs) with lymphocytes. Prevailing dogma dictates that only if competent APCs transport Ag into SLTs, an adaptive immune response is initiated; otherwise, the Ag is ignored by the immune system [1]. For the initiation of humoral antibody (Ab)-mediated immunity in mammals, the formation of B cell follicles and germinal centers (GCs) appears to be a prerequisite. The dynamic nature of such GCs, including the interaction of follicular dendritic cells (FDCs) with B cells and Ag, was recently elegantly demonstrated by others [2]. However, in contrast to the B cell-dominated cortex, T cell areas, where T cells encounter mature APCs and their cognate Ag, are structurally ill defined. Whereas intravital confocal microscopy has provided compelling evidence for the capacity of SLTs to host T cell priming [3], definitive data supporting their absolute requirement for the initiation of T cell-mediated immunity (CMI) do not exist. In addition, cold-blooded vertebrates lacking conventional SLTs generate potent immune responses upon immunization. However, in the mammalian system, the apparent immunodeficiency of mice that lack SLTs strongly supports the notion that the initiation of effective immune responses requires the dedicated structures provided by SLTs [4–8]. Alymphoplasia (aly/aly) mice are characterized by a complete lack of lymph nodes (LNs) and Peyer’s patches, and structural alterations of the spleen and thymus due to a point mutation in the NFκB-inducing kinase (NIK) [9]. NIK is vital for the initiation of the noncanonical NFκB cascade, which appears to play a discrete role, for instance, in the function of CD40 and lymphotoxin-β receptor (LTβR) signaling in some cell types [10–12]. Aly/aly mice display impaired Ab responses and loss of CMI, demonstrated by their inability to reject allogeneic grafts or tumors [4,13,14]. The developmental deficits in aly/aly mutants are readily explained by the requirement of NIK in LTβR signaling. LTβR is vital for the development of SLTs, and LTβR−/− mice display similar developmental defects as do aly/aly mice or NIK−/− mice [12,15].

In this study, we describe that the immunodeficiency of aly/aly mice is not due to the absence of SLTs, but due to the impact of
Lymph nodes (LN) are believed to be the most important tissues initiating immune responses by facilitating the activation of T and B lymphocytes. Mice lacking such LN (called alymphoplastic) are severely immune compromised and resistant to immunizations. We discovered that the immune-deficiency of such alymphoplastic mice is actually not caused by the loss of LN, but rather by the underlying genetic lesion. Surprisingly, mice lacking all lymph nodes can still mount potent T cell-mediated immune responses. We also discovered that T and B cells have completely different structural requirements for their activation/maturation. Whereas B cells rely on LN to become efficient antibody-producing cells, T cells can be activated successfully outside of such dedicated tissues. So—in the absence of LN—antigens delivered by immunization are actively transported into the liver where cellular immunity is initiated. The mammalian fetal liver is responsible for the early formation of blood and immune cells, and we propose that the adult liver can still provide a niche for T cell–antigen encounters. During evolution, T and B cells emerged simultaneously, allowing cold-blooded vertebrates (which lack LN) to launch adaptive immune responses. The development of LN in mammals coincided with a drastic improvement in antibody affinity maturations, whereas T cells remain LN-independent to this day.

**Results**

**Autoimmunity Cannot Be Initiated in Aly/Aly Mice**

We first sought to determine whether LN are an absolute requirement for the induction of a complex T↓H1 cell-driven autoimmune disease as well as systemic CTL-mediated autoimmunity initiated through classical subcutaneous (s.c.) immunization/vaccination independent of SLTs. APCs present in the site of immunization migrate to and select the liver as a natural extra-lymphoid tissue for the initiation of CMI, which we propose to be as an evolutionary hard-wired pathway already found in cold-blooded vertebrates. This alternative pathway, undescribed to this day, can potentially drive CMI but fails to elicit B cell immunity, indicating that the immunization-induced T cell accumulation within conventional lymphoid organs mainly serves humoral immunity but that CMI can be initiated elsewhere.

**Induction of Productive T Cell Immunity in the Absence of SLTs**

The fact that $aly/aly$ mice do not develop T cell-driven autoimmune disease could be explained by their inability to prime self-reactive T cells (a) due to the lack of dedicated draining LN [5,6], or (b) due to a direct impact of the NIK mutation on immune cells [19,20]. In order to define whether their EAE resistance is due to the lack of LN or an intrinsic defect of $aly/aly$ mice to prime T cells, we generated a series of bone marrow (BM)-chimeric mice. Similar results were obtained with Ovalbumin (OVA) TcR Tg T cells (OTII) transferred into $aly/aly$ and $aly/+ $ mice (unpublished data), indicating that T cell expansion can be initiated independent of SLTs, whereas efficient effector function is dependent on the microenvironment provided by SLTs.
compared to the control mice aly/+ (Figure 2D), indicating that the resistance to EAE in the absence of NIK could be related to the function of NIK in T cell polarization. The mechanistic underpinnings of this phenomenon are currently being investigated, but it is clear that the loss of NIK signaling impairs the capacity of aly/aly mice to generate pathogenic TH cells regardless of their structural defects.

B and T Cells Have Different Structural Requirements for Priming and Maturation

Given the dogma that in mammals, CMI initiated by s.c. or intramuscular Ag-delivery requires the presence of SLTs, it is feasible that the remaining SLT (i.e., the spleen) in aly/+ aly/aly BM-chimeras compensates for the absence of LNs. In order to test this notion, we splenectomized aly/+ aly/aly BM-chimeras (aly/+ aly/aly) 14 d prior to the induction of EAE. Upon immunization, aly/+ aly/aly mice developed EAE with the same disease severity as control mice (Table 1). We noted a slight delay in disease onset when all SLTs are absent, while histopathological analysis of diseased mice revealed no difference between aly/+ aly/aly and aly/aly mice (Figure S1).

In contrast to T cell activation, we found that B cell activation requires the structural environment provided by SLTs. To investigate the impact of immunization on T versus B cell responses, we used Keyhole limpet hemocyanin (KLH) as a model of foreign Ag to elicit delayed-type hypersensitivity (DTH) responses. aly/aly as well as aly/+ mice were immunized with KLH, and 11 dpi, they were challenged by intradermal injection with KLH into the ear. As illustrated in Figure 2E, both groups were able to mount a solid DTH reaction measured by ear swelling, which was only marginally lower in aly/aly than in aly/+ mice. However, in contrast to ear swelling, which is indicative of CMI, aly/aly mice did not mount Abs against KLH when compared to aly/+ mice, demonstrating that the development of a humoral immune response is ablated in the absence of lymphoreticular structures (Figure 2F). We could reproduce
Figure 2. SLTs are crucial for B but not T cell-mediated immune responses. (A and B) EAE progression in BM-chimeras immunized s.c. with MOG35-55/CFA. (A) aly/+→aly/+; △, aly/+→aly/aly; △, aly/+→aly/+; △. (B) aly/aly→aly/+; △, aly/aly→aly/aly; △, aly/aly→aly/+; △. (C) EAE was induced by active immunization with MOG35-55/CFA of LTBR<sup>−/−</sup> mice (■) and wt mice (△). Shown are representatives of three individual experiments (n=5/group)±SEM. (D) LN-derived cells were obtained from aly/aly→aly/+ (black bars) and aly/+→aly/+ (grey bars) BM-chimeras 21 dpi with MOG35-55/CFA and rechallenged in vitro with 50 μg/ml MOG35-55 peptide to reveal IFNγ- and IL-17-secreting cells using Elispot. Shown is a representative of two individual experiments (n=3/group)±SEM. (E) DTH responses were induced by s.c. immunization with KLH/CFA of aly/aly and aly/+ mice. At 11 dpi, the mice were challenged by intradermal injection of KLH (grey bars), or PBS (black bars) into the ear. Swelling was measured 24 h postchallenge using a precision caliper, and shown is the increase of ear swelling over baseline of a representative of three independent experiments (n=2 mice/experiment)±SEM. (F) Sera were collected from KLH-immunized aly/aly (◇) and aly/+ mice (★) on 12 dpi and analyzed for the presence of total anti-KLH Abs by ELISA. Results are representative of three independent experiments (n=2 mice/group)±SEM.
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Table 1. Mice devoid of SLTs are fully susceptible to EAE.

| BM-Chimeras  | Incidence (%) | Mean Day of Disease Onset<sup>a</sup> | Mean Maximal Clinical Score<sup>a</sup> |
|---------------|---------------|---------------------------------|---------------------------------|
| aly/+→aly/+   | 94.7 (18 of 19) | 11.1 (±0.4) | 3.0 (±0.1) |
| aly/+→aly/+<sup>cl</sup> | 75 (9 of 12) | 11.8 (±0.2) | 3.0 (±0.3) |
| aly/+→aly/aly | 65.2 (15 of 23) | 13.7 (±0.7) | 3.3 (±0.0) |
| aly/+→aly/aly<sup>m</sup> | 64.7 (11 of 17) | 15.8 (±0.8) | 3.4 (±0.1) |
| aly/aly→aly/+ | 0 (0 of 10) | — | — |

<sup>a</sup>of diseased animals (±SEM).
doi:10.1371/journal.pbio.1000109.t001
functional DTH responses using other Ags including OVA and MOG35-55 (unpublished data). Similarly, in our EAE paradigm using BM-chimeras, whereas control mice (aly/+→aly/+ and aly/+→aly /aly ) elicit high Ab titers, anti-MOG Abs are virtually absent in mice without LNs (either aly/+→aly/aly or aly/+→aly /aly ) (Figure 3A). Analysis of isotype subtypes revealed that in splenectomized alymphoplastic mice, elevated anti-MOG IgM could be detected, which has previously been reported [7,23,24], whereas class switching to IgG could not be observed (Figure 3B). Taken together, and in agreement with the notion that SLTs are vital for B cell activation, highly organized SLTs are obligatory for the generation of high-affinity Igs and class switching, whereas potent cellular immunity can be induced successfully upon s.c. immunization even in the absence of SLTs.

In the Absence of SLTs, Subcutaneously Delivered Ag Is Transported into the Liver

Since the loss of SLTs in aly BM-chimeric mice does not hinder the development of T cell immunity, we wanted to determine at which alternative site T cell priming could take place and to which organ the Ag travels from the site of immunization (s.c.). Therefore, aly BM-chimeras were injected s.c. with yellow green (YG) carboxylate microspheres emulsified in CFA. At 7 dpi, various organs were isolated and analyzed for the presence of fluorescent cells by flow cytometry. Figure 4A shows that in control mice (aly/+→aly/+), fluorescently labeled APCs were exclusively detected in LNs upon s.c. immunization. It was previously shown that the BM has the capacity to drive an enriched population of high-affinity TcR Tg T cells in response to blood-borne Ag [25]. As expected, upon intravenous (i.v.) delivery of Ag, the vast majority of it accumulates in the spleen, BM, and liver, regardless of the presence of SLTs (Figure S2).

However, after (s.c.) immunization of aly/+→aly/aly spl BM-chimeras lacking SLTs, APCs carrying fluorescent microspheres migrate primarily to the liver and not the other organs analyzed (thymus, CNS, and gut; unpublished data) (Figure 4A). Only a small amount of Ag reaches the liver when draining SLTs are present. Next, we wanted to determine the means of the Ag transport from the s.c. reservoir to the liver. To determine whether the Ag diffuses to the liver or is actively transported by APCs, aly/+→aly/+ and aly/+→aly /aly chimeric mice were separated into two groups. One received YG microspheres/CFA in the left flank and polychromatic red (PR) microspheres/CFA in the right flank. The other group received a mixture of YG- and PR-coupled beads in both flanks (see scheme in Figure 4B). After 7 d, mice were sacrificed, perfused, and a single-cell suspension of livers, LNs, and spleens was generated for cytofluorometric analysis. We found that the mixture of PR/YG-coupled beads generated a large proportion of dual-labeled CD11b as well as CD11c-positive APCs. Conversely, the injection of either PR- or YG-coupled microspheres into each flank revealed merely single-labeled APCs in the liver. The presence of single-labeled cells within the liver strongly suggests that the Ag is delivered to the liver by the migration of APCs initially present at the site of immunization via the bloodstream to the liver cannot be fully excluded, but is evidently not the dominant means of Ag delivery. In addition, only a negligible amount of Ag reaches the liver when dedicated SLTs are present (Figure 4). We could also confirm these findings by using soluble FITC painted on shaved flanks (without the adjuvant CFA). Twenty-four hours after FITC skin painting, we found FITC+ APCs primarily in the liver, again supporting the notion that the liver can serve as an alternative Ag-presenting site when draining LNs are not available (Figure 4C).
Extra-Lymphoid Aggregates in the Liver Host T Cell/APC Encounters

In order to determine whether lymphoid-like structures can be found in the liver, we analyzed the livers of BM-chimeric mice immunized s.c. with MOG35–55/CFA by histology (7 dpi). Livers of aly/+/aly/alyR aly/aly control mice (Figure 5). Histological analysis displays dendritic cells (DCs) in close proximity to T cells in the infiltrated periportal areas of the liver, indicative of T cell priming by Ag-laden APCs (Figure 5B). In spite of the stroma’s inability to respond to LTα/β, detailed histological analysis revealed the presence of VCAM and ICAM in the infiltrates as well as B cells (Figure S3) and even the presence of CXCL13 transcripts indicative of aggregates ability to recruit B cells (unpublished data). However, no evidence for GC formation could be obtained (Figure S3).

We also transferred TcR Tg T cells from Luciferase-2D2 (Luc-2D2) mice into recipient BM-chimeras and observed the accumulation of Ag-responsive T cells in the liver 2 dpi with MOG35–55/CFA by bioluminescence imaging (Figure 6A). Figure 6B shows that the number of DCs (CD11c+) and adoptively transferred 2D2 T cells (CD4+/Vβ11+) is drastically increased in the liver in mice lacking SLTs. In order to demonstrate that the observed lymphocyte accumulations in the liver can support cell expansion, we injected naive (CD62L+) CD4+ T cells derived from 2D2 Tg mice into aly BM-chimeras and subsequently immunized them with MOG35–55/CFA. At 5 dpi, livers were analyzed for Ag-specific CD4+ T cell proliferation. Even in normal mice, we find a large number of expanded T cells within the liver (Figure 6C), but one could argue that they have immigrated from their initial priming site, the draining LN. However, in the absence of SLTs, the livers of aly/+ aly/aly aly/aly BM-chimeric mice are sufficient to propagate Ag-driven T cell expansion and accumulation. In order to confirm that Ag-specific T cell proliferation occurs in situ in the liver, we administered BrdU intraperitoneally (i.p.) into aly BM-chimeras 7 dpi with MOG35–55/CFA. Thirty minutes after BrdU injections, the mice were sacrificed, and livers were analyzed for proliferating (BrdU+) CD4+ T cells by flow cytometry. Figure 6D and 6E reveal the presence of BrdU+ cells in the livers of both aly/+ aly/+ and aly/+ aly/aly aly/aly BM-chimeras. The number of BrdU+ T cells in the liver is increased in aly/+ aly/aly aly/aly BM-chimeras compared to aly/+ aly/+ controls.
B Cells, Not T Cells, Depend on Lymph Nodes

Lymph node

Spleen

Liver

MOG immunized

CFSE

naive

MOG immunized

CD4

BrdU

Number of BrdU/cell/lin

w/o BrdU

naive

MOG

Number of cells/liver (x10^4)

CD11c^+

CD4^+

Vβ11^+

aly/+ → aly/+ (x 6.2)

aly/+ → aly/aly^spl (x 8.3)
to the controls. The fact that we found such a rapid (30 min) emergence of proliferating T cells even in normal mice in which SLTs are present, indicates that some degree of liver-initiated CMI occurs simultaneously to the priming within draining LNs.

The Adult Liver Can Support T Cell, But Not B Cell Priming

In contrast to our findings, which show that mice lacking SLTs do not generate high-affinity Ab-responses, intranasal influenza infection of splenectomized LT"a/a" mice reconstituted with wild-type (wt) stem cells, for instance, can initiate the formation of extra-lymphoid follicles within the lung, which support some degree of B cell maturation and Ab secretion [24,26]. One possible explanation for these contrasting observations regarding Ab production is that in our case, stroma cells such as FDCs cannot signal through LTβR due to the mutation within NIK and that this could be the reason for our inability to observe GC formation and Ab secretion, whereas Mozron-Quiroz et al. [24,26] used mice in which the stroma compartment can be engaged by LTα/β. To definitively address whether the stroma’s inability to signal through NIK is the reason for the weak B cell response, we obtained LN-deficient LTα/+ mice and reconstituted their hematopoietic system with wt stem cells. The resulting chimeras were splenectomized and lacked all peripheral SLTs (analogous to the aly/+→aly/aly"a/b"). Yet in contrast to aly/+→aly/aly"a/b", wt→LTα"a/a" chimeras have normal stromal cell function, and FDCs are capable of responding to LTβR. These mice were immunized s.c., and the formation of B cell maturation and Ab production was analyzed. Figure 7A demonstrates that these wt→LTα"a/a" chimeras behave exactly like aly/+→aly/aly"a/b" in regards to their inability to generate high Ab titers and to class switch.

In a comparative fashion, we analyzed the histological parameters of wt→wt, aly/+→aly/aly"a/b", and wt→LTα"a/a" chimeras (Figure 7B). Although only alymphoplastic mutants revealed the presence of lymphoid aggregates surrounding periporal areas of the liver, neither FDCs nor PNA-positive clusters could be found, again supporting the notion that the surrogate structures in the liver support T cell function but fail to initiate the formation of GCs needed for Ab-affinity cell maturation and class switching. Lastly, the large number of K67⁺ cells within the liver aggregates again support our conclusion, that active proliferation within the liver can be induced by s.c. immunization (Figure 7B).

Priming of Cytotoxic Antitumor T Cells Independent of SLTs

Although we have demonstrated the development of T cell-driven autoimmune disease in mice lacking SLTs, we wanted to elucidate whether these mice are also capable of inducing successful CTL immunity. We used the B16.F10 murine melanoma model, which represents a lethal and poorly immunogenic cancer. Irradiated GM-CSF expressing B16.F10 cells are used as s.c. vaccine to initiate potent CD8⁺-antitumor immunity against live parental B16.F10 tumor cells [27]. We injected irradiated B16.F10-GM-CSF cells s.c. into one flank of aly/+ and aly/+→aly/aly"a/b" chimeric mice. At 12 dpi, mice were challenged with parental B16.F10 cells injected into the opposite flank. Figure 8A shows that aly/+→aly/aly"a/b" chimeric mice can elicit potent antitumor CTL responses revealed by the inhibition of tumor growth. Next, we transferred CFSE-labeled MHC class I-restricted OVA-TcR Tg OTI T cells into aly/+→aly/aly"a/b" and aly/+→aly/aly"a/b" spl chimeras have normal stromal cell function, and subsequently injected irradiated B16.F10 cells expressing OVA. At 12 dpi, mice and, in control animals, also spleen and LNs were analyzed by FACS for Ag-specific CD8⁺ T cell expansion. As demonstrated in Figure 8B, proliferation of CD8⁺ OTI cells was detected in the liver of mice lacking SLTs. Hence, even under conditions in which the draining LNs are considered a compulsory site hosting the encounter of captured Ag and infiltrating CD8⁺ T cells, we can detect potent T cell responses, which originate in the liver when SLTs are absent.

Liver Follicles Are Induced by Immunization and Aberrant Homeostatic T Cell Migration

We next wanted to address the relevance of the liver to serve as an alternative priming site in a setting where LNs are present but T cell migration into LNs is defective. To this end, we analyzed plt/plt (paucity of LN T cells) mice, which display disturbed B cell zones but severely abrogated T cell zones due to the loss of CCL19 and CCL21, which results in the inhibition of both naive T cell and DC homing into SLTs [28]. We found that plt/plt mice also developed delayed but fulminating EAE after s.c. immunization with MOG₃₅-₅₅/CFA (Figure 8C). Examination of liver sections of immunized plt/plt mice again revealed lymphocyte aggregates consisting mainly of CD4⁺ T cells and DCs within the liver (Figure 8D).

Discussion

S.c. immunization instigates a situation in which draining LNs are widely held to be absolutely obligatory for the initiation of adaptive immunity. In the absence of such draining LNs, we found however, that APCs take up the Ag at the site of immunization and subsequently select the liver as an extra-lymphoid environment for the initiation of CMI. These findings are consistent with the propensity of alymphoplastic mice (NIK"a/a", LTβR"a/a", and LTBR"a/a") to develop abnormal lymphocyte infiltrates primarily in the liver [15,29]. The lymphocyte accumulation seen in the liver of naive alymphoplastic mice does not coincide with any overt tissue damage, nor do they develop any secondary sign of hepatic injury (M. Heikenwaelder, Zurich, Switzerland, personal correspondence). Such surrogate structures are evidently not as sophisticated as true SLTs and fail to support B cell priming, but are clearly sufficient to support CMI. Such neo-lymphoid structures in the liver are not restricted to alymphoplastic mouse
strains, but can be reproduced in mice in which T cells do not migrate into the LNs (plt/plt). The fact that we observe the rapid emergence of immunization-induced T cell expansion in the liver of normal mice supports the notion that the adult liver provides an efficient niche for the initiation of CMI. Moyron-Quiroz et al. [26] elegantly demonstrated that the lymphoid tissue in the lung (BALT) is sufficient to generate immunity against an infectious agent attacking the lung. In their experimental paradigm, peripheral SLTs are not compulsory for the initiation of protective immunity, and they could even observe some degree of B cell maturation. In our report, however, after s.c. immunization, the local APCs must sample the Ag and then actively migrate to and select the liver as a site for T cell priming, which then is even capable of driving autoimmune responses within the CNS. In our experimental paradigm, the site of Ag deposition, priming, and inflammation are distinct. The liver is thus not like the BALT or the NALT, a site where local immune responses can be initiated, but represents a niche for systemic T cell priming under conditions in which the draining LNs are widely held to be absolutely compulsory. The fact that Ag-laden APCs migrate from the site of immunization to the periportal areas in the liver could be explained by the presence of chemoattractive factors in the liver aggregates observed in SLT mutants. Alternatively, the extensive lymphatic network of the liver makes it an ideal niche for the accumulation of leukocytes as a reservoir when regular SLTs are inaccessible.

Figure 7. Surrogate liver aggregates support CMI, but not B cell maturation. Wt→wt and wt→LTα−/− spl BM-chimeric mice were immunized s.c. with MOG35–55/CFA. (A) At 11 dpi, titers of anti-MOG Abs (IgG, IgM and IgA) were determined from sera by ELISA (n=4 mice/group)±SD. (B) Liver sections from wt→wt, wt→LTα−/− spl, and aly/+→aly/aly spl BM-chimeras were stained with Abs against CD4, CD8, CD11b, CD11c, CD19, CD62L, CD68, FDC, ICAM, Ki67, PNA, and VCAM. Positively stained infiltrated areas of 14-mm² liver sections were counted (n=4 mice/group)±SD. doi:10.1371/journal.pbio.1000109.g007
B Cells, Not T Cells, Depend on Lymph Nodes

A

B

B16.F10-GM-CSF
aly/+ → aly/+ vacc
aly/+ → aly/+ non-vacc
aly/+ → aly/aly\textsuperscript{plp}
aly/+ → aly/aly\textsuperscript{plp} vacc
aly/+ → aly/aly\textsuperscript{plp} non-vacc

B16.F10-GM-CSF / B16.F10-OVA
aly/+ → aly/+ prl
aly/+ → aly/aly\textsuperscript{plp}

Lymph node
Liver

C

Clinical score

D

CD11c
CD4
CD11b

PNA
FDC
B220
Although the induction of CMI is not a function traditionally attributed to the adult liver, the fetal liver is a primary lymphoid organ hosting early hematopoiesis. Our findings suggest that the liver has the potential to "remember" its lymphoid function. The phenomenon, that, for instance, food allergies can be transferred by liver transplantation of livers from an allergic donor to a previously nonallergic recipient [30], can be explained by our findings. Such transplant-acquired food allergy has only been described for the liver and not for other transplanted organs of the same donor [30]. It has been hypothesized that this occurrence is due to donor-derived allergen-specific lymphocytes residing in the liver. In support of this, Klein and Crispe [31] reported recently that after liver transplantation in a mouse in which Ag presentation was restricted to resident cells of the liver grafts, efficient CD8<sup>+</sup> T cell priming can be induced locally in the transplanted liver.

The situation also is reminiscent of the effect of immunizations on some cold-blooded vertebrates that are much more primitive than mammals in their SLT organization (i.e., lacking GCs and showing only minimal affinity maturation). Frog tadpoles (Alytes obstetricans) immunized with rabbit serum in CFA developed a large accumulation of lymphocytes in the liver visible 2-3 wk after injection (L. Dupasquier, Basel, Switzerland, personal correspondence). Interestingly, during evolution, the emergence of RAG was permissive for the development of adaptive immunity in jawed fish [32]. RAG mediates somatic recombination and is required for the formation of both B and T cell receptors, which appear to have emerged simultaneously during evolution. However, whereas the adaptive immune system is well developed in the oldest jawed vertebrates (cartilaginous fish, e.g., sharks), potent affinity maturation, Ig-class switching, and GC formation are lacking. Class switching only appeared at the time of the divergence of amphibians [33]. The fact that CMI evolved earlier than modern humoral immune responses corroborates our discovery that T cells can function outside of dedicated lymphoreticular structures.

In summary, we demonstrate that the structural requirements for the initiation of B and T cell responses differ significantly. We found that B cells are dependent on the topography of dedicated lymphoid tissues, whereas CD<sup>4</sup><sup>+</sup> as well as CD<sup>8</sup><sup>+</sup> T lymphocytes retain the capacity to recognize Ag in a structure-independent fashion. This finding has obvious implications for our understanding of adaptive immunity and vaccination. As for the development of autoimmune diseases, our findings show that self-reactive T cells may not need to be primed in tissue-draining LNs, but could occur at the inflammatory site or even in organs distant to the target tissue.

**Materials and Methods**

**Mice**

C57BL/6 mice were purchased from Janvier Laboratories. A lymphoplasia (aly/aly) mice were obtained from Clea Laboratories and bred in-house under specific pathogen-free (SPF) conditions. Heterozygous aly/aly<sup>+</sup> mice were used as controls for homozygous aly mice (aly/aly). 2D2 (MOG-TCR) Tg mice were provided by V. Kuchroo (Harvard Medical School, Boston, Massachusetts); LT<sup>B<sub>RC</sub></sup><sup>+/−</sup> and LT<sup>B<sub>TC</sub></sup><sup>+/−</sup> mice were provided by A. Aguzzi and M. Heikenwalder (University Hospital Zurich, Zurich, Switzerland); and OTI and OTII mice were purchased from Jackson Laboratories. Luciferase (pAActin-Luciferase) Tg mice were obtained from C. Contag (UCSF) and crossed to the 2D2 mouse (Luc-2D2). Pit/plt mice were obtained from V. Kuchroo (Kantonsspital St. Gallen, Switzerland). All mice were bred in-house under SPF conditions. BM-chimeras were generated as described previously [34]. Mice were splenectomized as described previously [35]. Animal experiments were approved by the Swiss veterinary office (68/2003, 70/2003, 10/2006, and 13/2006).

**Induction of EAE**

MOG<sub>35-55</sub> peptide (MEVGWYRSPFSVHVLYRNGK) was obtained from GenScript. EAE was induced as described previously [34] with the modification that BM-chimeras were generally not boosted with pertussis toxin. For adoptive transfer, MOG-reactive lymphocytes were generated as described [34]. Each time point shown is the average disease score of each group±the standard error of the mean (SEM).

**Leukocyte Isolation**

Mice were euthanized with CO<sub>2</sub>, and various organs were removed to isolate leukocytes: For isolating lung cells, lungs were incubated with DNase (0.5 mg)/Liberase (1 mg/ml) (Roche) for 30 min at 37°C. Spleen, LNs, thymus, and lung were homogenized, and BM and cells were isolated by flushing the bones with PBS. Cells were strained through a 100-μm nylon filter (Fisher) and washed. Erythrocytes of whole blood, BM, and spleen were lysed. For isolating hepatic nonparenchymal cells, the liver was incubated with DNase/Liberase for 30 min at 37°C, homogenized, and then centrifuged at room temperature (RT) for 2 min at 50g. The supernatant was then centrifuged at 1,500 rpm for 10 min, and the pellet was resuspended in 30% Percoll (Pharmacia) and centrifuged at 12,000 rpm for 30 min at 4°C. The interface cells were collected and washed. For isolating intestinal lymphocytes, intestines were opened longitudinally, washed, and then cut into small pieces. Tissues were then incubated with DNase/Liberase and leukocytes were isolated using a percoll gradient as described above. Isolation of CNS lymphocytes has been described previously [35].

**Proliferation Assay**

Mice were injected i.v. with 2×10<sup>5</sup> CFSE (carboxylfluorescein diacetate succinimidyl ester)-labeled (Invitrogen/Molecular Probes) (10 μM) splenocytes obtained from either 2D2, OT-II, or OT-I TcR Tg mice or with 8×10<sup>6</sup> CFSE-labeled naive CD4<sup>+</sup> 2D2 Tg T cells (isolated with CD4<sup>+</sup>CD62L<sup>+</sup> isolation kit from Miltenyi). Mice were subsequently immunized s.c. with 200 μg of MOG<sub>13-35</sub>/CFA (Adjvant complete H37 Ra.; DIFCO) (for 2D2), OVA<sub>323-339</sub>/CFA (for OT-II), or with a 1:1 mix of irradiated 2×10<sup>6</sup> B16.F10-GM-CSF/B16.F10-OVA cells (for OT-I). At 4 or 5 dpi (12 dpi for OT-I), mice were sacrificed.
and spleen, LN(1s if present), and livers were analyzed by fluorescence-activated cell sorting (FACS) for the proliferation of CD4\(^+\) T cells using the clonotypic TCR and CFSE fluorescence (2D2: TCR V\(_{32}\) Ab; OT-II and OT1: V\(_{x2}\) Ab).

**Histology and Flow Cytometry**

Tissues were freshly snap-frozen in liquid nitrogen. To determine infiltration of inflammatory cells, tissue sections were stained with hematoxylin and cosin (H&E) or with the following mouse-specific Abs as previously described [31]: anti-CD11c (Jackson ImmunoResearch Labs), anti-CD11b (BMA Biomedicals), anti-CD3, anti-CD4, anti-CD19, anti-FDC M1, and anti-Thy1.1 (BD-Pharmonic), anti-ICAM, anti-VCAM, and anti-CD8 (Sero-tech). GC cells were stained with peanut agglutinin (PNA; Vector Laboratories).

For FACS analysis, the following Abs were used: anti-CD11c, anti-CD4, anti-CD8, anti-CD11b, anti-VC3,2, anti-Vx3, and anti-Thy1.1 (BD-Pharmonic). The cells were analyzed using a FACS-Canto (BD) with Cell DIVA software. Postacquisition analysis was performed using FLOWJO software. To trace the distribution of Ag after immunization, mice were injected s.c. with 200 \(\mu\)l of yellow-green (YG) or polychromatic red (PR) 1.0-\(\mu\)m microspheres (Polysciences) emulsified in CFA. At 7 dpi, mice were euthanized with CO\(_2\), and organs were removed to isolate lymphocytes as described above. Single-cell suspensions were analyzed by FACS for the presence of fluorescein isothiocyanate (FITC\(^+\)) or PE\(^+\) cells.

For FITC skin painting, mice were painted on the shaved flanks with 100 \(\mu\)l of 5 mg/ml FITC (Molecular Probes) dissolved in 1:1 acetone:dibutylphthalate. On day 1, mice were euthanized with CO\(_2\), and organs removed and analyzed by FACS as described above.

**Delayed-Type Hypersensitivity (DTH) Assay**

Mice were immunized s.c. with 100 \(\mu\)g/flank of KLH (Sigma) emulsified in CFA. At 11 dpi, mice were challenged by injecting 10 \(\mu\)g/10 \(\mu\)l KLH, PBS into the dorsal surface of the ear. DTH responses were determined by measuring the ear thickness using a caliper micrometer (Mitutoyo) 24 h after challenge, and \(\Delta\) ear swelling was established by the increase in ear thickness over baseline (prechallenge ear thickness).

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Plates were coated with 10 \(\mu\)g of rMOG\(_{35-55}\) in 0.1 M NaHCO\(_3\) (pH 9.6) at 4°C overnight or KLH (Sigma), and blocked with 1% (w/v) bovine serum albumin (BSA). Diluted sera were incubated for 2 h at RT. After washing, peroxidase-conjugated antibodies to mouse immunoglobulins, IgG, IgA, and IgM (Sigma) were added (1:1,000 diluted) and incubated for 1 h at RT. Plates were washed, and chromogen (Biosource) was added. Absorbance was measured on a microplate reader (450 nm) (Bio-Rad).

**Enzyme-Linked Immunospot Analysis (Elispot)**

A total of 2\(\times\)10\(^5\) cells were plated in medium containing 10% FCS and 50 \(\mu\)g/ml of MOG\(_{35-55}\) in 96-well plates (Millipore) coated with the capture Ab against either IFN\(\gamma\) or IL-17A [36]. Elispots were revealed as described previously [36] and subsequently analyzed on an Elispot reader (CTL immunospot).

**Bioluminescence Imaging**

To visualize Luc-2D2 cells, mice were injected i.p. with 3 mg of luciferin (Xenogen) prior to bioluminescence imaging using an IVIS100 imaging station (Xenogen). The luminescent image was overlaid on the photographic image.

**Bromodeoxyuridine (BrdU) Treatment**

Mice were immunized s.c. with MOG\(_{35-55}\)/CFA. At 7 dpi, BrdU (BD Pharmingen) (2.5 mg) was injected i.p. 30 min before the mice were sacrificed and analyzed for proliferating (BrdU\(^+\)) CD4\(^+\) T cells by flow cytometry with anti-BrdU Ab (Bioscience).

**Tumor Induction**

Mice were s.c. vaccinated into one flank with irradiated (6,000 rads) 1\(\times\)10\(^6\) B16.F10-GM-CSF cells. At day 12 after vaccination, mice were injected with live 2\(\times\)10\(^7\) B16.F10-Luc cells into the opposite flank. Each time point shown is the average tumor size of each group±SEM, measured using a caliper.

**Supporting Information**

**Figure S1** Inflammatory lesions in the CNS of mice lacking SLTs. H&E stainings of spinal cord sections of diseased aly/+→aly/+ or aly/+→aly/aly\(^{BM}\) BM-chimeras. Lower row represents a higher magnification of the insert in upper row. Bar in upper row indicates 200 \(\mu\)m and in lower row 50 \(\mu\)m. Found at: doi:10.1371/journal.pbio.1000109.s001 (3.79 MB TIF)

**Figure S2** Intravenously delivered Ag accumulates in the spleen, BM, and liver. A\(\text{ly}\)-BM-chimeras were injected i.v. with YG microspheres, and various organs were analyzed by FACS for the presence of fluorescently labeled APCs 7 dpi. Data represent one of three individual experiments. Found at: doi:10.1371/journal.pbio.1000109.s002 (0.63 MB TIF)

**Figure S3** Expression of lymphoid structure markers in livers of aly\(\text{BM}\)-chimeric mice. Liver cryosections from aly\(\text{BM}\)-chimeras immunized s.c. with MOG\(_{35-55}\) (d11) were stained with antibodies against CD4, CD8, CD11b, CD11c, CD19, CD62L, CD68, FDC, ICAM, Ki67, PNA, and VCAM. Bar indicates 200 \(\mu\)m. Found at: doi:10.1371/journal.pbio.1000109.s003 (17.11 MB TIF)

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**Author Contributions**

The authors have made the following declarations about their contributions: Conceived and designed the experiments: MG JH BB. Performed the experiments: MG JH. Analyzed the data: MG JH BB. Wrote the paper: MG JH BB.

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