Follicles in gut-associated lymphoid tissues create preferential survival niches for follicular $T_h$ cells escaping Thy-1-specific depletion in mice

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Abstract

Although a substantial number of T cells may escape depletion following in vivo mAb treatment in patients undergoing immunosuppression, their specific tissue location and phenotypic characteristics in different peripheral lymphoid tissues have not been analyzed in detail. Here we investigated the survival of CD4$^+$ T cells immediately following anti-Thy-1 mAb treatment in mice. We found a preferential survival of CD4$^+$ T cells expressing Thy-1 antigen in the Peyer’s patches (PP) and also in mesenteric lymph nodes (MLN), where the relative majority of the surviving CD4$^+$ T cells displayed CD44$^{high}$/CD62L$^{−}$ phenotype corresponding to effector memory T-cell features. These CD4$^+$ T cells also expressed CXCR5 and PD-1 (programmed cell death-1) markers characteristic for follicular $T_h$ cells ($T_{fh}$). We also demonstrate that the immediate survival of these cells does not involve proliferation and is independent of IL-7. Induction of germinal center formation in spleen enhanced while the dissolution of follicular architecture by lymphotoxin-$β$ receptor antagonist treatment slightly reduced $T_{fh}$ survival. Our results thus raise the possibility that the follicles within PP and MLN may create natural support niches for the preferential survival of $T_{fh}$ cells of the memory phenotype, thus allowing their escape during T-cell depletion.

Keywords: depletion, follicle, follicular $T_h$ cells, Peyer’s patches, Thy-1

Introduction

Several studies in animal models as well as observations in patients undergoing antibody-mediated T-cell depletion showed that a substantial number of T cells can escape in vivo exposure to antibody (either monoclonal or polyclonal), as determined by the specificity of the antibody employed. The survivor cells subsequently undergo fast homeostatic proliferation in order to reconstitute the T-cell pool (1–4). It has been repeatedly shown that after several days following depletion treatment, most surviving CD4$^+$ T cells display a CD44$^+$, CD62L$^{−}$ effector memory phenotype and function (2–4). Importantly, some of these cells are alloreactive memory T cells capable of mediating accelerated allograft rejection under both experimental and clinical settings (2, 5). As memory cells are less dependent on co-stimulation by CD28, survivor cells undergoing lymphopenia-induced proliferation can break peripheral tolerance, thus leading to alloreactivity and autoimmunity (6–8). Regulatory T cells can prevent allograft rejection, but their resistance to depletion and capacity to undergo homeostatic proliferation are still debated (1, 9–12).

Although previous studies have provided insights into the phenotype of the T cells escaping depletion and demonstrated their ability to promote acute allograft rejection, niches within the organism for their survival remain elusive. Whereas investigations in humans are limited to peripheral blood, experiments in murine models largely overlook the structural and functional differences between the peripheral lymphoid organs that host the bulk of mature T cells and, in particular, the specific location of surviving T cells within these organs.

Of all the structured lymphoid organs, the gut-associated lymphoid tissue (GALT) represents the largest collection of lymphocytes, containing 70% of all mature lymphocytes (13). As an important component of this set of lymphoid tissues, Peyer’s patches (PP) are organized lymphoid territories with defined T- and B-cell compartments. The bulk of PP in mice is formed by secondary follicles containing germinal centers (GC), whereas the interfollicular regions harbor primarily T cells. PP have a major role in protective immune responses against a variety of intestinal antigens and are also
essential in induction of immune tolerance to food protein and commensal bacteria (11, 14). Disruption of tolerance and an abnormal response toward microbiota have been associated with graft-versus-host disease and Crohn’s disease (15).

In this paper we demonstrate that various secondary lymphoid organs possess different capacities for rescuing CD4+ T cells following anti-Thy-1 (CD90) mediated T-cell depletion, with preferential CD4+ T-cell survival in PP and mesenteric lymph nodes (MLN). Interestingly, PP promoted almost exclusive survival of T cells localized within the follicles. The majority of these cells was positive for CXCR5 and PD-1, thus resembling to the majority of these cells was positive for CXCR5 and PD-1, resembling the phenotype of follicular T cells (FH cells). Furthermore, preferential survival of FH cells was seen in spleen after priming by thymus-dependent antigen, whereas acute disruption of follicular architecture by lymphotxin-β receptor (LTβR) antagonist treatment impeded the survival of GC FH cells.

Methods

Mice
BALB/c mice were obtained from the faculty’s specific pathogen-free breeding unit. C3 complement component deficient mice were kindly provided by Dr József Prechl, (Department of Immunology, Eötvös Loránd University, Budapest, Hungary) with permission from Dr Matyas Sandor (16). Mice were fed with a commercial pelleted diet and tap water in a regular 12h dark/light cycle at the temperature of 22°C. All procedures involving live animals were conducted in accordance with the guidelines set out by the Ethics Committee on Animal Experimentation of the University of Pécs (approved by #BA02/2000–1/2011 institutional ethical permit, Pécs, Hungary).

Antibodies and reagents
Rat mAb against Thy-1/CD90 (clone IBL-1, IgG1 isotype) was developed in our laboratory (17). Anti-CD44 IgG (clone KM81) was kindly provided by Dr Tibor Glant. Rat hybridoma cell lines secreting anti-CD3 (clone KT-3), anti-CD62L (clone MEL-14), anti–MAdCAM-1 (clone MECAM-367) and anti-B220 (clone RA3-6B2) mAbs were obtained from the American Type Culture Collection. For multicolor analysis, anti-CD44 IgG (clone 2G8), PE polyclonal goat anti-rat IgG, Cy5-Streptavidin and Alexa Fluor 647 (Invitrogen, Csertex Ltd, Budapest) were conjugated with FITC (Sigma-Aldrich Hungary, Budapest) according to standard procedures; anti-CD62L IgG was biotinylated with sulfo-N-hydroxysuccinimide biotin ester (Sigma-Aldrich Hungary). Cy5-streptavidin (Invitrogen, Csertex Ltd, Budapest), and non-labeled rat mAbs by PE-conjugated goat anti-rat IgG (Soft Flow Hungary Ltd, Pécs). For tissue immunofluorescence, rat anti-mouse CD4 mAb (clone YTS 191.1) was conjugated with Cy3 active ester (GE Healthcare, AP Hungary Ltd.).

Depletion and blocking protocols

Experimental T-cell depletion was induced by IBL-1 rat mAb against Thy-1/CD90. Mice received 600 μg of the Protein G-purified antibody intravenously as a single dose for kinetic studies or divided into two doses given at 0 and 24 h for end-point analysis. Mice were scarified at 0.5, 1, 2, 4, 6, 8, 24 or 48 h. For evaluating the effects of acute IL-7 withdrawal on the depletion efficiency, mice received simultaneously both anti-Thy-1 IgG (600 μg/recipient) and neutralizing polyclonal rabbit anti-mouse IL-7 (160 μg/recipient) blocking antibodies (R&D Systems, Budapest). Inhibition of LTβR was achieved by intravenous injection of recombinant LTβR-Ig fusion protein (kindly provided by Dr Jeff Browning, Biogen Idec) at the dose of 100 μg/recipient. Mice received 100 μg of LTβR-Ig or normal human IgG intravenously followed by the depletion protocol 48 h later. The effect of depletion and LTβR-Ig treatment was assessed on day 4 by immunofluorescence and flow cytometry. Control group mice received appropriate isotype control antibodies from rat, rabbit or human serum.

Induction of T-dependent immune response by immunization with human red blood cells
Human blood was collected into tubes containing EDTA, centrifuged and the supernatant discarded. 100 μl of a 10% suspension in PBS was applied intraperitoneally. Seven days later mice were subjected to T-cell depletion protocol, and the next day they were sacrificed and their spleens were processed for immunofluorescent staining or standard flow cytometric analysis.

Annexin V binding assay
Twenty four hours after induction of the depletion mice were sacrificed and their spleen and peripheral lymph nodes (PLN) were taken out. Single-cell suspensions were prepared and the cell numbers were adjusted to 10⁶ cells per sample. After completing the staining for flow cytometry, the last washing procedure and all the following steps were performed using Annexin V binding buffer (BD Biosciences from Soft Flow Hungary Ltd, Pécs) at room temperature. The reaction was stopped by 300 μl of annexin binding buffer, and the cells were immediately processed for flow cytometry analysis.

Flow cytometry

Lymphocytes were isolated from the spleen, PLN, MLN and PP by teasing apart the organs between the frosted ends of two microscopic slides and filtered through a 70-μm pore-size cell strainer from BD Biosciences (Soft Flow Ltd, Pécs). Blood samples were collected in tubes containing heparin. The cells were incubated with a mixture of fluorescein-labeled...
and biotinylated mAb for 20 mins on ice. The biotinylated mAbs were detected with Cy5-streptavidin. Erythrocytes in blood samples were lysed by using BD Biosciences’ lysis buffer. Intracellular staining for Foxp3 was performed according to the instructions provided by the vendors and BrdU measurement was performed following intraperitoneal injection of BrdU as recommended by the kit’s vendor. Dead cells were excluded based on their light scattering properties. At least 20 000 live cells were collected by a BD FACSCalibur cytometer and analysed using the CellQuest software.

Immunofluorescence

Immediately after sacrifice by cervical dislocation, tissue samples were collected, embedded in cryostat medium and kept frozen at −80°C until the staining procedure. Frozen and acetone-fixed sections were then blocked with 5% BSA in PBS for 20 mins. For multiple immunofluorescence first unlabeled rat mAb were detected using PE-conjugated anti-rat IgG, followed by saturation with 5% normal rat serum. Subsequently biotinylated antibody was applied, followed by the visualization with Streptavidin-Cy5 (18). After mounting, the sections were viewed under an Olympus BX61 fluorescent microscope. The acquisition of digital pictures with a charge-coupled device camera was performed using the analySIS software. Images were processed using Adobe Photoshop 6.0 with contrast and color adjustments applied on the whole images.

Statistical analysis

Student’s t-test or one-way ANOVA were used for comparison of two or more groups, respectively. Data distribution was examined by the Shapiro–Wilks normality test, and the Mann–Whitney U-test or Kruskall–Wallis test was employed where appropriate. SigmaPlot software (version 11.2, Systat Software, Inc., San Jose, CA, USA) was used and P < 0.05 was considered significant.

Results

Depletion efficiency and kinetics

Although anti-Thy-1 mediated depletion has been used for a long time to remove T cells in vivo, relatively little is known about its kinetics and how its efficiency varies between various lymphoid organs during the depletion phase. Therefore first we investigated the efficiency of depletion over time by comparing the decline of CD4+ T-cell frequency and cell numbers between different lymphoid tissues in the early period after the mAb injection and compared it with normal rat IgG effects administered at the same amount. In addition, absolute cell numbers were determined in spleen and PLN. Depletion kinetics were monitored by measuring frequencies of CD4+ T cells and B220+ B cells at 0, 1, 2, 4, 8, 24 and 48 h after the IBL-1 treatment.

We found that both the efficiency and kinetics varied significantly among the organs (P ≤ 0.001) (Fig. 1A). The highest level of depletion (measured by the frequency of CD4+ T cells at the start of the treatment and the end of experiment, respectively) was found in the blood (>95% reduction or 25-fold decrease). Of the structured lymphoid organs the highest level of depletion at the end of observation period was found in the spleen (7.8-fold decrease in frequency). PLN presented an intermediate level of depletion (2.3-fold), whereas the lowest level of depletion was found in MLN and PP (1.58- and 1.60-fold decrease, respectively). The first significant reduction of T-cell frequency appeared already as early as 1 h after the treatment in the spleen (P = 0.003), whereas in the PLN and PP it was detected after a delay, at 8 h. The decrease in CD4+ or CD3+ T-cell frequencies was coupled with a relative increase in B cells (Fig. 1B).

Parallel to the altered lymphocyte composition, we also found significant reduction in the absolute lymphocyte number in the PLN and spleen (P = 0.01 and P ≤ 0.001 respectively, Figure 1C). These results collectively show that the depletion kinetics vary between the lymphoid organs. The T-cell depletion proceeds fastest and is most extensive in the peripheral blood and the spleen, whereas PP and MLN show slow depletion with the highest number of survivor T cells.

We also assessed the occupancy of Thy-1 antigen by IBL-1 mAb in various peripheral lymphoid tissues and the remaining depleting antibody in serum. Using anti-rat IgG fluorescent conjugate we found that the binding of IBL-1-mAb increased over time and reached the maximum already at 4 h, with a higher level of binding in spleen and MLN, and a reduced intensity in PP and PLN, respectively (Supplementary Figure 1A, available at International Immunology Online). Furthermore, we also tested the remaining binding sites for IBL-1 anti-Thy-1 mAb by reacting lymphocytes from various lymphoid organs in treated mice with FITC-conjugated anti-Thy-1 mAb. In an inverse pattern a relatively higher level of unreacted IBL-1 epitope in the Thy-1 molecule could be detected in MLN, PLN and PP at 2 h post-treatment, whereas the antigen occupancy by IBL-1 mAb was rather high in spleen at this early period. In situ immunofluorescence also revealed strong T-cell labeling at 4 h following mAb injection (Supplementary Figure 1B, available at International Immunology Online). The parallel reduction of both antibody-bound Thy-1 antigen and residual unreacted Thy-1 indicates some shedding events as early as 6–8 h post-treatment, without any substantial reduction in CD4+ T-cell frequency as shown in Figure 1.

The residual IBL-1 mAb level in sera of treated mice ranged between 10 and 100 µg/ml IgG (data not shown). Our results thus indicate that the differences in depletion efficiency between the peripheral lymphoid organs are not a result of observed differences in the Thy-1 saturation level or antibody access to the lymphoid tissue.

T-cell depletion independent from complement activation and apoptosis induction

To determine the impact of complement mediated cell lysis in T-cell depletion we investigated the effect of anti-Thy-1 treatment in mice deficient for C3 complement component (16). Our results showed that the depletion in C3−/− mice was as efficient as in the wild-type controls, excluding the possibility of substantial role of the complement system in the IBL-1-mediated T-cell depletion (Supplementary Figure 2, available at International Immunology Online). Furthermore, Annexin V staining of CD4+ T cells in treated animals shortly after the mAb administration remained consistently negative.
In addition, \textit{in vitro} exposure of lymphocytes to IBL-1 mAb did not result in the exteriorization of phosphatidyl-serine, an early parameter associated with apoptosis (data not shown). These results were in the line with delayed onset of T-cell
number reduction, suggesting that the depletion elicited by anti-Thy-1 mAb treatment is not a fast event, mediated either by intracellular events or by complement-mediated lysis.

Variable Thy-1 expression on CD4+ T cells in GALT

PP in mice may harbor Thy-1− CD4+ T cells that may thus avoid anti-Thy-1 mAb-mediated depletion (19). Next we investigated the frequency and alteration of these cells in more detail in GALT. We also analyzed Thy-1 expression on CD4+ T cells in different secondary lymphoid organs 24 h after the IBL-1 treatment. The expression of Thy-1 antigen on the surviving cells was measured by detecting the bound mAb with anti-rat IgG.

We found that Thy-1− T cells were present in all lymphoid tissues (4–12% of all CD4+ T cells) with the highest frequency found in PPs (12%, n > 5) (Fig. 2A). Upon exposure to anti-Thy-1 mAb this frequency increased 2- to 3-fold in PP (P ≤ 0.007; Figure 2B). However, the majority of survivor CD4+ T cells was still Thy-1+, albeit a significant increase of Thy-1− CD4+ T cells is also detectable (Fig. 2B and C).

Preferential depletion of T cells from the T-cell zone in GALT

Despite using a repeated exposure and higher dosage of depleting antibody, we consistently detected survivor CD4+ T cells in peripheral lymphoid organs, particularly in PP and MLN. To investigate whether these depletion-resistant CD4+ T cells are positioned at any preferential localization within the lymphoid tissue, we performed immunofluorescence staining for B cells and residual CD4+ T cells followed by morphometric analysis. Immunofluorescent staining of PP and MLN revealed preferential depletion of extrafollicular T cells (Fig. 3), whereas morphometric analyses indicated that the number of CD4+ T cells increased within the follicles in both tissues (Table 1).

Fig. 2. Variable Thy-1 expression on CD4+ T cells. (A) shows frequency of Thy-1− CD4+ T cells in PLN, spleen, MLN and PP. Their relative increase 24 h after IBL-1 treatment in MLN and PP is depicted in (B). (C) shows representative plots of MLN and PP obtained from control and treated animal 24 h after IBL-1 injection (n > 10 mice/group). Thy-1 expression in control samples was determined with FITC-conjugated IBL-1 mAb, whereas the expression of Thy-1 on residual T cells in T-depleted animals was detected using FITC-conjugated anti-rat immunoglobulin. Data are presented as mean±SD and considered significant if P < 0.05; **P ≤ 0.001 (n > 12 mice/group).
Phenotype of surviving T cells in mucosal lymphoid tissues reveals $T_{FH}$ cells of the effector memory type

Next we analyzed the phenotype of surviving T cells in PP and MLN and compared them with those in spleen and PLN. Previous studies reported preferential survival of memory-like phenotype CD4$^+$ T cells, without providing data on PP or MLN (9, 20). Therefore, surviving CD4$^+$ T cells were stained for CD44 and CD62L to assess naive (CD44$^{low}$, CD62L$^{high}$), central memory (CD44$^{hi}$, CD62L$^{hi}$) and effector memory (CD44$^{hi}$, CD62L$^{lo}$) T cells. Our results revealed a moderate but statistically significant increase in effector memory phenotype among CD4$^+$ T cells in PLN. The GALT MLN showed a more robust tendency to preserve effector memory T cells ($P < 0.001$). However, PP presented almost exclusive survival of CD4$^+$ T cells with the effector memory phenotype ($P < 0.001$). In contrast, a small increase in effector memory T cells was recorded in the spleen ($P = 0.008$) (Fig. 4A and B).

As our histological observations indicated that CD4$^+$ T cells within the follicles largely remain unaffected after depletion, we hypothesized that these cells may include follicular $T_{FH}$ cells ($T_{FH}$), which are characterized by the expression of CXCR5 chemokine receptor and the inhibitory co-receptor (PD-1) (21–23). We found that, exclusively in PPs, a substantial fraction of surviving CD4$^+$ T cells display the $T_{FH}$ phenotype, including both CXCR5$^{hi}$PD-1$^{lo}$ pre-GC $T_{FH}$ cells and CXCR5$^{hi}$PD-1$^{hi}$ GC $T_{FH}$ cells ($P \leq 0.001$) (Fig. 4C). Furthermore, these cells displayed a CD44$^{hi}$/CD62L$^{lo}$ phenotype corresponding to effector memory T cells. In MLN 16.4% of CD4$^+$ T cells were positive for CXCR5 and PD-1, with a majority of cells displaying intermediate level expression of both markers, corresponding to pre-GC $T_{FH}$ cells and some recently primed CD4$^+$ T cells (24, 25). In addition, there was a significant increase in the frequencies of both GC and pre-GC $T_{FH}$ cells ($P \leq 0.001$, Figure 4D).

Previous studies have demonstrated that some CXCR5$^{hi}$, PD-1$^{hi}$ CD4$^+$ T cells localized in the follicles can also display Foxp3$^+$ phenotype and function as $T_{FH}$ cells and are resistant to depletion treatment (9, 14, 26). Therefore we also assessed Foxp3 expression on CD4$^+$ T cells harvested from PP of control and treated mice. Consistent with the general susceptibility of CD4$^+$ T cells to depletion, we found a significant reduction of total CD4$^+$, Foxp3$^+$ cells ($P = 0.034$), with no change in their frequency among the total number of survivor CD4$^+$ T cells ($P = 0.876$, Supplementary Figure 3A, available at International Immunology Online). The majority of Foxp3$^+$, CD4$^+$ cells was also CD44$^{hi}$, thus corresponding to effector memory CD4$^+$ T cells. The increase in the frequency of CXCR5$^+$ T cells was coupled with a significant increase in CXCR5$^+$, Foxp3$^+$ cells (1.24 ± 0.62% versus 3.43 ± 0.69%, $P = 0.015$) but with no effect on their frequency among total CXCR5$^+$ cells (3.79 ± 0.93% versus 4.87 ± 1.39%, $P = 0.372$). In contrast, the frequency of these CXCR5$^+$, Foxp3$^+$ cells among all Foxp3$^+$ cells increased significantly after anti-Thy-1 treatment (12.5 ± 5% versus 31.5 ± 7.6%, $P = 0.021$, Supplementary Figure 3B, available at International Immunology Online).

Resistence to anti-Thy-1 mediated depletion is defined by $T_{FH}$ characteristics in PP

In agreement with previous data (19), our observations indicated that a fraction of CD4$^+$ T cells in PP may escape anti-Thy-1 mediated elimination as a result of their lack of target antigen on the cell surface (Fig. 2). As our previous findings revealed a substantial increase of CD4$^+$ surviving cells expressing the $T_{FH}$ phenotype, we next investigated how the survival of CD4$^+$ T cells correlates with their $T_{FH}$ phenotype or surface Thy-1 expression. We found that in untreated mice or mice treated with normal rat IgG ~35% (in PP) and 4.3% (in MLN) of CXCR5$^+$ CD4$^+$ T cells lacked Thy-1 antigen (Fig. 5). Upon treatment this frequency increased significantly in MLN (up to 14%) ($P \leq 0.001$), whereas in PP we found no significant difference. As a result, the majority of survivor CXCR5$^+$CD4$^+$ T cells (>80% in MLN and 60% in PP) had Thy-1 molecules on their surface, as revealed through the detection of in vivo bound mAb. Thus the dominant fraction of the CXCR5$^+$ compartment of CD4$^+$ T cells within PP survive the anti-Thy-1 treatment, despite the presence of Thy-1 expression, whereas within the CXCR5$^-$ compartment the survival of CD4$^+$ T cells is facilitated by the lack of Thy-1, although the majority of survivor CD4$^+$ T cells expresses the target antigen. Our results thus suggest that resistance to anti-Thy-1 treatment is only partly attributable to the inherent and/or acquired lack of Thy-1 surface expression, and the survival of Thy-1$^-$ CD4$^+$ cells in PP strongly correlates with the CXCR5 expression.
Resistance of T<sub>FH</sub> cells to depletion within PP is independent from homeostatic proliferation and IL-7 availability

Previous studies investigating the course of experimental T-cell depletion demonstrated that T cells rapidly undergo homeostatic proliferation following the removal of T cells, starting as early as the second day following depletion treatment (1–4). To test whether the observed enrichment of T<sub>FH</sub> cells is due to an early-onset homeostatic proliferation, we tested their cell cycle status by in vivo BrdU labeling. The incorporation of BrdU was assessed in B and T cells of various lymphoid organs subdivided into L-selectin<sup>+</sup> and L-selectin<sup>-</sup> subsets (Fig. 6A). We found that there was no substantial proliferation among the CD4<sup>+</sup> T cells, whereas in PP a marginally increased BrdU uptake was observed within the L-selectin<sup>-</sup> subset, affecting ~3% of the residual CD4<sup>+</sup> T cells (Fig. 6B). In contrast, an extensive B-cell proliferation was detected in PPs among ~50% of the L-selectin<sup>-</sup> centroblast population (Fig. 6B).

As IL-7 plays a vital role in the homeostasis of CD4<sup>+</sup> T cells (4, 27), we also examined if the simultaneous blockade of IL-7 together with anti-Thy-1 mAb treatment can further...
increase depletion efficiency. We observed no significant difference between the groups treated with anti-IL-7 antibody and control animals (data not shown). Taken together, these results suggest that the enrichment of T<sub>FH</sub> cells 24 h after the initiation of anti-Thy-1-mediated depletion is not linked to their early homeostatic proliferation nor it affected by the availability of IL-7.

Resistance of T<sub>FH</sub> cells requires GC induction in an LTβR-dependent pathway

The natural presence of GC in PP and the tendency of T<sub>FH</sub> cells to survive in these structures raised the issue of whether other lymphoid organs may also support T<sub>FH</sub> survival if they harbor GC. Splenic GC formation was induced by immunization with human red blood cells as a T-dependent antigen followed by anti-Thy-1 mAb-mediated depletion. Seven days after immunization a strong induction of GC was observed (Fig. 7A), which coincided with an approximate 6-fold increase in CXCR5<sup>+</sup> memory phenotype CD4<sup>+</sup> T cell frequency (Fig. 7B). Subsequent treatment with IBL-1 anti-Thy-1 mAb resulted in a further 4-fold increase of both CXCR5<sup>+</sup>/PD-1<sup>+</sup> CD4<sup>+</sup> T cells. In addition, there was a 20-fold increase in GC T<sub>FH</sub> cells with CXCR5<sup>high</sup>/PD-1<sup>high</sup> phenotype and a 2-fold increase among effector memory CD4<sup>+</sup> T cells (P = 0.013) (Fig. 7C).

Formation of lymphoid follicles including a follicular dendritic cell (FDC) network and B-cell accumulation and their subsequent transformation into GC require the engagement of LTβR by a LTαβ<sub>2</sub> heterotrimer (28). To investigate whether LTβR activity can influence the relative resistance of GC T<sub>FH</sub> cells to anti-Thy-1 treatment, we used recombinant LTβR-Ig fusion protein to disrupt natural GC in PP. The in vivo effectiveness of LTβR-Ig treatment was assessed by staining for MAdCAM-1 in PP high endothelial venules (HEV) and splenic marginal sinus-lining cells (MS), respectively, and CR1.2 expression associated with FDC. The impact of treatment on GC formation was evaluated by PNA lectin binding.

Reduction of HEV- and MS-associated expression of MAdCAM-1 and FDC-associated display of CR1.2, which was associated with a reduced size of PNA<sup>+</sup> GC confirmed efficient blockade of LTβR activity (Fig. 8A). Compared with mice with preserved LTβR activity, we found a moderate reduction of T<sub>FH</sub> cells displaying CD44<sup>high</sup> and CXCR5<sup>−</sup>/CD44<sup>low</sup> CD4<sup>+</sup> T cells (P = 0.013) (Fig. 8C).

In the present study we demonstrate a highly divergent pattern for the survival of CD4<sup>+</sup> T cells in a mouse model, where preferential survival of CD4<sup>+</sup> T cells in the PP and MLN of BALB/c mice is demonstrated following anti-Thy-1 mAb-mediated T-cell depletion. Even though anti-Thy-1 treatment in mice has been used to deplete T cells in vivo for decades, the actual mechanisms of action and possible tissue influences that determine its efficiency are strikingly understudied. Our investigations indicate substantial differences both between peripheral lymphoid tissues and between different compartments within one lymphoid tissue that can influence the efficiency of depleting treatments. Furthermore, similar differences in antibody-mediated depletion can be expected to be present in human individuals as well.
Although extensive research efforts have yielded substantial knowledge on the phenotype of T cells surviving depletion treatment (1, 2, 5, 9), data on their specific tissue locations and the effects of depletion particularly within GALT are scarce. Our findings establish that upon anti-Thy-1 mediated T-cell depletion PP appear to be able to provide protective micro-environment for T<sub>FH</sub> cells. Our data are also consistent with previous reports that effector memory T cells are relatively resistant to depletion (1–3, 5). However, their frequency in spleen and PLN in our study was lower than reported earlier, as we evaluated the surviving CD4<sup>+</sup>T cells before homeostatic proliferation took place, as proven by the BrdU assay. Furthermore, the various degrees of lymphocyte depletion between different peripheral lymphoid organs may also reflect their different recirculation kinetics, which can contribute to the extent of local lymphocyte reduction.

Peripheral lymphoid tissues in mice may harbor T cells lacking surface Thy-1 molecules, partly associated with their activation and relocation into GC induced by T-dependent antigens (19, 29). Here we extended their phenotypic characterization and found that the bulk of treatment-resistant cells display T<sub>FH</sub> features. Although a substantial increase of Thy-1<sup>−</sup>CD4<sup>+</sup>T cells was observed in both lymphoid tissues, the majority of treatment-resistant cells retained Thy-1
antigen, indicating that their survival was not a mere escape from treatment, but probably the result of their activation or differentiation status, reflected in the expression of CXCR5 receptor. Thus, their specific phenotype and even more their specific location within secondary lymphoid organs could play an important role in providing them with survival niches.

Fig. 7. GC induction establishes a protective micro-environment for follicular T<sub>H</sub> cells in the spleen. (A) One week after immunization with human erythrocytes IBL-1 treatment was performed and the survival of T<sub>H</sub> cells assessed by FACS 48 h after mAb injection. (B) The GC reaction was identified by PNA lectin staining and lymphocyte markers as indicated. (C) The plots depict a representative example of a significant increase in CXCR5<sup>+</sup>CD44<sup>+</sup> and CD62L<sup>+</sup>CD44<sup>+</sup> cells corresponding to T<sub>H</sub> and effector memory CD4<sup>+</sup> T cells, respectively. Some of these cells also correspond to GC T<sub>H</sub> cells (n = 10 mice/group).
Tfh cells have only recently been recognized as a separate T lineage with distinct phenotype and function, specialized for providing help to B cells and establishing the capacity for long-lived, highly protective antibody responses (21–23, 30–33). Initial signals driving Tfh cells to commit to the Tfh lineage consist of high affinity TcR signaling and co-stimulation by CD28, ICOS, OX40, CD30 and SLAM associated protein (SAP) delivered by dendritic cells, lymphoid tissue inducer (LTi) cells and B cells (23, 31–33). Mice lacking B cells, such as μMT mice, do not form Tfh (21, 31). In addition, SAP plays an important role in enhancing TcR signals and prolonging T-cell–B-cell interactions. SAP−/− mice are unable to maintain GC and have reduced numbers of GC Tfh, however, CD4+ T cells providing B-cell help at extrafollicular foci are preserved in these mice (33–35). The up-regulation of the gene product of B-cell lymphoma 6 (Bcl-6), a transcriptional repressor, leads to final differentiation of pre-GC Tfh into GC Tfh (36). High expression levels of Bcl-6 but not T-bet, GATA3, RORγT or Foxp3 accompanied by CXCR5+, PD-1high, ICOShigh features are typically found in GC Tfh cells, together with the production of high levels of IL-21 and IL-4 and variable amounts of IL-17 and INFγ (21, 22, 29–32, 36).

Based on a set of phenotypic markers, several other cell types are distinguished from GC Tfh. These include pre-GC Tfh that interact with B cells and LTi cells at the T/B cell zone border, which can secrete various cytokines and initiate isotype switch, and display a CXCR5+, ICOS+, PD-1low/high, Bcl-6* phenotype (22, 23, 37). Subsequently, these cells can either

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**Fig. 8.** Acute disruption of follicular structure by LTβR-Ig. (A) Follicular architecture was acutely disrupted by LTβR-Ig treatment and its efficiency on PNA+ GC formation was verified by decreased MAdCAM-1 expression (red) correlated either with reduced GC size (PNA, blue) in PP or with ablation of follicular dendritic cell networks (CR1.2 staining, green) in spleen (n = three mice/group). (B and C) Twenty four hours following depletion treatment frequencies and phenotype of CD4+ T cells were assessed by FACS. (C) Survival of GC Tfh was significantly reduced inLTβR-Ig treated mice. Data are presented as mean±SEM and considered significant if P < 0.05 (n = six mice/group).
enter GC to become GC T<sub>FH</sub> or provide help to low affinity plasma cells at extrafollicular foci (22). In addition, a set of Foxp3 regulatory T cells that share the CXCR5<sup>−</sup>, PD-1<sup>high</sup>, ICOS<sup>high</sup> phenotype with GC T<sub>FH</sub> and express Foxp3 but not Bcl-6 can also be found in the GC (21, 26, 38). Finally, CD4<sup>+</sup> T cells that can provide help to B cells at extrafollicular foci can be identified by a CXCR<sup>−</sup>5, PD-1<sup>−</sup>, CXCR4<sup>−</sup>, ICOS<sup>−</sup>, Bcl-6<sup>−</sup> phenotype (22). Our findings show that it is the first two subsets that can preferentially be rescued.

A recent study indicated the role of OX40 in the preferential survival of CD4<sup>+</sup> T cells following anti-lymphocyte serum (ALS) treatment (9). Among the ALS-treatment-resistant CD4<sup>+</sup> T cells in the spleen and PLN two distinct subsets were identified, including effector memory T cells and Foxp3<sup>+</sup> T<sub>reg</sub> cells OX40 signalling promotes CXCR5 expression and T-cell localization to the follicles (39-41), facilitated by the enhanced expression of OX40 by T<sub>reg</sub> cells (42). Mice double deficient for OX40 and CD30 still form GC but the kinetics are different and they fail to maintain GC (43). They have impaired CD4<sup>+</sup> T cell memory formation, suggesting that the homing to follicles might also be important for normal T-cell expansion and generation of T-cell memory (21, 43, 44). Furthermore, Fas receptor expression in GC B cells also plays an important role in modulating the level of T-cell apoptosis via the Fas/FasL pathway, where activated B cells expressing high levels of Fas receptor can redirect the FasL expressed by T cells primed to undergo apoptosis (45, 46).

Taken together, these data indicate that delicate interplay between T<sub>FH</sub> cells and B cells or LT<sub>i</sub> cells could promote the survival and maintenance of T<sub>FH</sub> cells. This requirement for preserved GC architecture is further confirmed by our observation that fewer GC T<sub>FH</sub> cells survive after an acute disruption of follicular architecture by LT<sub>i</sub>R<sub>Ir</sub> fusion treatment. In line with this argument is our finding on the relative independence of T<sub>FH</sub> survival from the availability of IL-7, a cytokine that is primarily implicated in the survival of T cells in the T-zone fibroblast reticular cell micro-environment, as opposed to the distinct FDC-composed milieu within the follicles where T<sub>FH</sub> cells reside (47). It is thus important to appreciate that the T-cell depletion regimens do not simply reduce diverse T-cell population sizes in a biased manner along the naive-memory distinction but can also cause an important shift in the composition of lymphoid compartments, with potentially far-reaching consequences. As a possibly related phenomenon it has been shown that aberrant accumulation of T<sub>FH</sub> cells in sanroque mice leads to positive selection and expansion of self-reactive GC B cells and autoimmunity (48). This finding may correlate with the unexpected increase of proliferation of B cells in PP following anti-Thy-1 treatment that had resulted in T<sub>FH</sub> enrichment. These effects could be influenced by specific local survival signals for distinct subsets of T cells, including the follicular micro-environment in the GALT that may be favorable for the survival of the T<sub>FH</sub> cells of the memory phenotype.

Contribution

M.M. and P.B. performed the purification of IBL-1 mAb and treatment and produced various mAb conjugates necessary for flow cytometric or histological detection; M.M. and Z.K. isolated cells and performed cytometric assays and morphometric analyses of tissue immunofluorescence. M.M. and P.B. wrote the manuscript.

Supplementary data

Supplementary data are available at International Immunology Online.

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