Nonfollicular reactivation of bone marrow resident memory CD4 T cells in immune clusters of the bone marrow

Francesco Siracusa,a,1 Mairi A. McGrath,a,1 Patrick Maschmeyer,a Markus Bardua,b Katrin Lehmann,a Gitta Heinz,a Pawel Durek,b Frederik F. Heinrich,a Mir-Farzin Mashreghi,b Hyun-Dong Chang,a Koji Tokoyoda,b and Andreas Radbruch,a,2

The bone marrow maintains memory CD4 T cells, which provide memory to systemic antigens. Here we demonstrate that memory CD4 T cells are reactivated by antigen in the bone marrow. In a secondary immune response, antigen-specific T cells of the bone marrow mobilize and aggregate in immune clusters together with MHC class II-expressing cells, mostly B lymphocytes. They proliferate vigorously and express effector cytokines, but they do not develop into follicular T-helper cells. Neither do the B lymphocytes develop into germinal center B cells in the bone marrow. Within 10 days, the immune clusters disappear again. Within 30 days, the expanded antigen-specific memory CD4 T cells return to memory niches and are maintained again individually as resting cells. Thus, in secondary immune responses in the bone marrow T-cell memory is amplified, while in germinal center reactions of secondary immune response, immune clusters, clustering with antigen-presenting cells and proliferate vigorously. In the bone marrow (BM), memory CD4 T cells specific for systemic antigens are maintained, even when they are absent from spleen and lymph nodes in mice (1), or the blood in humans (4), arguing that these cells are bona fide residents of the BM. BM resident CD4 memory T cells have been shown to provide efficient cognate help for antibody class switching and affinity maturation of antibodies to B lymphocytes in secondary lymphoid organs upon adoptive transfer (1). In the memory phase of an immune response, such memory T cells rest in the BM in terms of proliferation and activation (1, 4) and are maintained individually in “memory niches” organized by mesenchymal stromal cells (5). Here we analyze the reaction of BM resident CD4 memory T cells to antigen. We show that upon rechallenge antigen-specific CD4 memory T cells proliferate within the BM, independently of immigrating cells, and express effector cytokines. The activated T cells migrate to and gather in immune clusters, clustering with MHC class II-expressing cells, mostly mature B lymphocytes. The activated T cells, however, do not show a follicular helper cell phenotype, nor do the B cells express a germinal center phenotype. Within 30 d after reactivation the immune clusters disappear and the amplified antigen-specific CD4 memory T cells rest again in terms of proliferation, individually dispersed throughout the BM. We show here that immunological CD4 memory is amplified within the BM in a nonfollicular fashion in secondary immune reactions.

Results

CD4 Memory T Cells Expand in the BM Following Antigen Reactivation. C57BL/6 mice were immunized with 100 μg lymphocytic choriomeningitis virus (LCMV) GP61–80 peptide conjugated with nitrophenol to mouse serum albumin (LCMV-NP-MSA) and with 100 μg poly(I:C) twice, on day 0 and day 14 (without NP-MSA) (Fig. L4). Antigen-specific CD4 T cells were detected either by MHC class II tetramer staining (LCMV.GP66–77) or according to expression of CD40L and the cytokine IFN-γ upon stimulation with the peptide in vitro. Both methods gave comparable results (Fig. S1 A and B). Sixty days after the second immunization, ~40,000 antigen-specific CD4 memory T cells were detected in the BM (Fig. 1C). More than 97% of these cells were resting in the G0 phase of the cell cycle, since they did not express Ki-67 (Fig. 1B). On day 60 after the second immunization mice were reactivated (boosted) or not with 100 μg LCMV-NP-MSA + 100 μg poly(I:C) and 3 d later the number of antigen-specific T cells in the BM was determined (Fig. L4). At this time point all of the antigen-specific CD4 T cells in the BM expressed Ki-67, indicating that they were in the G1 to M phases of the cell cycle (Fig. 1B). Their numbers increased from a mean of 36,182 (± SEM 9,924) to a mean of 959,230 (± SEM 252,243) at 14 d after reactivation.

Significance

The bone marrow (BM) harbors critical components of the adaptive immune system able to provide long-lasting protection against pathogens. Among those, CD4 memory T cells are potent helpers of immune reactions in secondary lymphoid organs. Here we analyze their reactivation in the BM in secondary immune reactions. The CD4 memory T cells form clusters with antigen-presenting cells and proliferate vigorously. Although these clusters contain many B lymphocytes, their formation is not dependent on them and no germinal centers develop. Rather, antigen-specific CD4 memory T cells are significantly amplified and, after termination of the immune reaction, they remain in the BM as resting cells. The BM thus provides a dynamic reservoir of CD4 memory T cells, adapting quantitatively to antigenic challenges.

Author contributions: F.S., M.A.M., and A.R. designed research; F.S., M.A.M., P.M., M.B., and K.L. performed research; G.H., P.D., F.F.H., M.-F.M., H.-D.C., and K.T. contributed new reagents/analytic tools; F.S. and M.A.M. analyzed data; and F.S. and M.A.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Data deposition: The RNA-sequencing data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE107413).

1F.S. and M.A.M. contributed equally to this work.

2To whom correspondence should be addressed. Email: radbruch@drfz.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1715618115/-/DCSupplemental.

1334–1339 | PNAS | February 6, 2018 | vol. 115 | no. 6 www.pnas.org/cgi/doi/10.1073/pnas.1715618115
and cells from BM after SEM 245,448) of antigen-specific CD4 T cells, compared with S2 Fig. S1 D SEM 14,818) (Fig. 2 SEM 14,818) to 634,352 cells (Fig. S1 F and G).

Reactivation of Memory CD4 T Cells in the BM is Independent of Immigrating Cells. In C57BL/6 mice which had been twice immunized with LCMV GP<sub>15-40</sub> and rested for 60 d the antigen-specific CD4 memory T cells of the BM were heterogenous with respect to expression of sphingosine-1-phosphate-receptor 1 (SIPR1), a chemokine receptor mediating egress into the blood (16), in that 32% of cells did not express it but rather expressed its antagonist CD69 (Fig. 2 A–C) (17). We used the SIPR<sub>1</sub> agonist FTY720 to block egress from and ingress into the BM of T cells, but also of antigen-presenting cells (APCs). Mice were treated with FTY720 (1 mg/kg, i.p.) 1 d before boost and for the following 3 d after boost (Fig. S2A). This treatment reduced the number of CD4 T cells in the blood by more than 90% (Fig. 2D) and MHC class II-expressing cells by more than 80% (Fig. S2B), while it did not affect the total cellularity of BM (Fig. S2C). In FTY720-treated mice, reactivated antigen-specific CD4 memory T cells expanded from 67,593 (± SEM 14,818) to 634,352 cells (± SEM 101,942), that is, ninefold (Fig. 2E). In control (saline–treated) mice, the reactivated cells expanded from 36,182 (± SEM 9,924) to 118,030 (± SEM 118,030), that is, 26-fold. This difference in expansion rates did not reach statistical significance, indicating that most of the expansion of CD4 T cells was autonomous to BM, with some, statistically not significant, contribution of migrating T cells. The data also do not indicate an accumulation of antigen-specific CD4 memory T cells in the BM of FTY720-treated mice at day 63, without boost (36,182 ± SEM 9,924 vs. 67,593 ± SEM 14,818) (Fig. 2F). BM autonomy in response to systemic antigens was further underlined by the efficient induction of Ki-67 expression in the antigen-specific CD4 T cells, in the presence of FTY720 (Fig. 2F).

Nonfollicular Reactivation of BM CD4 Memory T Cells. Three days after boost, antigen-specific CD4 memory T cells of the BM and spleen from three individual mice were isolated and their global transcriptomes were compared. While principal component 2, accounting for 13% of the differences, reflects experimental variation, reactivated T cells from spleen and BM differ fundamentally in principal component 1, accounting for 70% of the differences (Fig. 3A). Notably, expression of signature genes of follicular helper T cells (T<sub>FH</sub>) cells, such as Bcl6 and Cxcr5, were significantly less expressed in T cells of the BM, compared with those from spleen (Fig. 3B). In line with this, expression levels of genes described to be up- or down-regulated (18) in T<sub>FH</sub> vs. non-T<sub>FH</sub> cells were accordingly up- or down-regulated in reactivated antigen-specific CD4 memory T cells of the BM, compared with the spleen (Tables S1 and S2). According to protein expression of PD-1 and CXCR5, while a defined population of T<sub>FH</sub> cells could not be detected before the boost (Fig. S2D), about 30% of the reactivated antigen-specific T cells in the spleen qualified as T<sub>FH</sub> 3 d after the boost. In the BM, a defined population of T<sub>FH</sub> was not detectable, either before or 3 d after the boost (Fig. 3C and D and Fig. S2D). In line with this, expression of Bcl6 in reactivated T cells of the BM was lower than in reactivated splenic T cells, both at the level of mRNA and protein (Fig. 3B and D). Concurrent with the absence of T<sub>FH</sub> cells from BM after antigenic challenge we also did not detect B lymphocytes binding high amounts of peanut agglutinin (PNA<sup><sub>β</sub></sup>), that is, showing the phenotype of germinal center B cells, 10 d after reactivation,

118,030) within 3 d (Fig. 1C). Boosting the mice with 100 μg LCMV-NP-MSA only (i.e., without any adjuvant) resulted in a significant amplification of antigen-specific CD4 T cells as well, showing that the expansion of CD4 memory T lymphocytes is independent of the adjuvant (Fig. S1C). In comparison, CD4 memory T cells of the BM that were not specific for LCMV GP<sub>15-40</sub> did not expand significantly (Fig. 1D and Fig. S1D). A significant expansion was also observed for CD4 memory T cells specific for ovalbumin when reactivated by antigen (Fig. 1E and Fig. S1E).

These cells were reactivated 90 d after the second immunization and analyzed 3 d later. Reactivation resulted in a mean of 800,000 (± SEM 245,448) of antigen-specific CD4 T cells, compared with 120,000 (± SEM 8,092), without reactivation (Fig. 1E). Reactivation also mobilized antigen-specific T cells of the BM. On day 3 after boost the antigen-specific CD4 T cells had significantly up-regulated expression of filamentous actin (F-actin) (Fig. 1F) and were larger in size and polarized in shape (Fig. 1G), compared with resting memory T cells (Fig. 1H). Upon in vitro restimulation all of them expressed IFN-γ, with an increased geometric mean fluorescence intensity (GMFI) of 1,021 vs. 473, and about 20% also expressed TNF-α, in the examples given in Fig. S1 F and G.
while they were readily detectable in the spleen at that time (Fig. 3 E and F). Accordingly, PNA-hi-binding B lymphocytes were absent before the boost in both the organs (Fig. S2E).

Reactivated CD4 Memory T Cells Cluster with B Lymphocytes in Immune Clusters of the BM. Three days after boost, clusters of CD3+CD4+ T cells and MHC class II-expressing cells appeared in the BM (Fig. 4A). Large clusters of T cells and MHC class II-positive cells, with more than 20 CD4 T cells per cluster, were only observed in mice upon antigenic rechallenge. Approximately seven clusters, each one consisting of more than five CD4 T cells, formed in the femoral BM of the reactivated mice, while in the BM of mice which had not been challenged only one out of three femurs showed two clusters (Fig. 4B). In the clusters, most CD3+CD4+ T cells expressed the Vx2 TCR chain, a surrogate marker for LCMV-specific CD4 T cells (Fig. 4C), and more than 80% of them contacted MHC class II-expressing cells, while only 30% of the CD3+CD4+TCRVx2+ T cells outside of the clusters, and individually dispersed through the BM, contacted MHC class II-expressing cells (Fig. 4D). In the BM of boosted mice, expression of MHC class II was up-regulated by all MHC class II-expressing cells, around 60% of them B220−CD11c− B lymphocytes (Fig. 4 E and F). Among B220−MHC-II+ cells, 45% were IgM+IgD− naïve B lymphocytes with a minor component of IgM single-positive (14%) and IgG1- or IgG2a/b-expressing cells (0.3%) (Fig. S3 A and B), while no clear population of expanding NP-specific B lymphocytes could be detected (Fig. S3C). Accordingly, in the clusters around 80% of the MHC class II-expressing cells were B lymphocytes coexpressing IgM and IgD, while no expression of IgG1 or IgG2a/b could be detected

Fig. 2. Reactivation of memory CD4 T cells in the BM is independent of immigrating cells. (A) Representative dot plot of CD69 vs. LCMV.GP66–77 tetramer gated on B220+Gr1−CD3+CD4+CD44hi viable cells on day 63 without boost. (B) Frequency of BM LCMV.GP66–77-specific CD4 memory T cells expressing CD69 on day 63 without boost. (C) Relative mRNA expression of S1PR1 in FACS-sorted BM CD69hi (white bar) or CD69lo (black bar) LCMV.GP66–77-specific CD4 memory T cells on day 63 without boost. (D) Absolute numbers of CD3+CD4+ T cells per microliter of blood with (squares) and without (circles) FTY720 administration on day 63, without boost. (E) Absolute numbers of BM LCMV.GP66–77-specific CD4 memory T cells on day 63, with (closed symbols) and without (open symbols) boost and with (circles) and without (squares) FTY720 administration. (F) Proliferative capacity of BM LCMV.GP66–77-specific CD4 memory T cells as determined by up-regulation of Ki-67 on day 63 after boost, with (circles) and without (squares) FTY720 administration. Data in B represent the mean ± SEM of pooled results from three independent experiments, each with three to five mice per group. Data in C are from one experiment with three mice per group. Data in D and E represent the mean ± SEM of pooled results from three independent experiments, each with three to five mice per group. Data in F are from one experiment, representative of three independent experiments, each with three to five mice per group. **P < 0.01, ***P < 0.001, as determined by one-way ANOVA (E) or unpaired Student’s t test. Saline controls shown in E are the same control group as shown in Fig. 1C. ns, not significant.

Fig. 3. Nonfollicular reactivation of BM CD4 memory T cells. (A) Principal-component analysis (PCA) of transcriptomes of LCMV.GP66–77-specific CD4 memory T cells isolated from spleen and BM, 3 d after boost. Each dot represents an individual mouse for a total of three mice per group. (B) Heat map displays differentially expressed genes, which were up- or down-regulated in LCMV.GP66–77-specific CD4 memory T cells of the BM compared with their splenic counterpart, as determined by RNA sequencing. Shown are Z-score expression levels of selected genes with library-normalized read counts of a minimum of 15 and FC ≥ 1.3. Each column represents one individual mouse, for a total of three mice per group. (C–E) Three days after boost; representative dot plots of PD-1 vs. CXCR5 expression gated on B220+Gr1−LCMV.GP66–77+CD3+CD44hi viable spleen and BM cells. (D) Three days after boost; (Left) frequency of LCMV.GP66–77-specific CD4 memory T cells coexpressing PD-1 and CXCR5 in spleen (circles) and BM (squares) and (Right) expression level of Bcl6 in LCMV.GP66–77-specific CD4 memory T cells in spleen (circles) and BM (squares), as determined by geometric mean based on four mice per group. Data shown are from one experiment, representative of two independent experiments. (E) Ten days after boost; representative dot plots of PNA vs. forward scatter (FSC) gated on CD19+CD138+ Igd+ viable cells. (F) Ten days after boost; representative histology sections of spleen and BM with CD3 (green), B220- (red), and PNA− (blue) expressing cells. Images shown are from one mouse, representative of three. Brightness and contrast were similarly adjusted between spleen and BM images. Flow cytometric data in C and E are from one experiment and representative of three independent experiments, each with four to five mice per group. Data in D are shown as mean ± SEM. **P < 0.01, ***P < 0.001, as determined by unpaired Student’s t test.
On day 90, 30 d after boost, antigen-specific CD4 memory B lymphocytes assembled in the BM and E and to CD20, which were readily detectable in the BM of isotype-control antibody-treated mice. (Fig. S5A), had disappeared in anti-CD20-treated mice, but smaller clusters of CD3<sup>+</sup>CD4<sup>+</sup> T and MHC-II<sup>+</sup> cells were still detected (Fig. S5B).

**Discussion**

Here we have analyzed the reaction of CD4 memory T cells in the BM to antigen. We demonstrate that following antigenic challenge antigen-specific T cells were mobilized and proliferated within the BM. This reaction was autonomous to the BM, since it could not be blocked by the S1PR agonist FY720. While germinal centers did not form, antigen-specific CD4 memory T cells and IgD<sup>+</sup>IgM<sup>+</sup> B lymphocytes assembled in de novo formed immune clusters of the BM during the first days after reactivation. Ten days after reactivation the large immune clusters had dissolved again; 30 d after reactivation the antigen-specific memory T cells rested again in terms of proliferation, individually dispersed throughout the BM, and immune clusters were not detectable in femoral BM of the analyzed mice (Fig. 6D).

**B-Cell-Independent Expansion of Antigen-Specific CD4 Memory T Cells in the BM.** To investigate whether the B lymphocytes forming the immune clusters in the BM were responsible for the numerical expansion of the antigen-specific CD4 memory T cells, C57BL/6 mice which had been twice immunized with LCMV GP<sub>61-80</sub> and rested for 60 d were i.v. injected 3 d before the boost with a single dose of 250 μg anti-CD20 or isotype control, as shown in Fig. S4A. Efficient depletion of B lymphocytes was validated the day before the boost. B lymphocytes were reduced by 95% in the blood (Fig. S4). Three days after the boost, numbers of B220<sup>+</sup>MHC-II<sup>+</sup> B lymphocytes were also significantly reduced in the BM of anti-CD20-treated mice, compared with the control group (Fig. S5B and S5B). In contrast, numbers and frequencies of B220<sup>+</sup>MHC-II<sup>+</sup> cells expressing CD11c were not affected by the treatment (Fig. S4B-D). B-cell depletion did not affect the numerical amplification of antigen-specific CD4 memory T cells in the BM, with 461,229 (± 133,631 SEM) vs. 491,178 (± 82,031 SEM) reactivated cells in isotype- vs. anti-CD20-treated mice (Fig. 5C). The expanding antigen-specific CD4 memory T cells also up-regulated Ki-67 (Fig. S4E and F). The large immune clusters of T and B lymphocytes, readily detectable in the BM of isotype-control antibody-treated mice (Fig. S5A), had disappeared in anti-CD20-treated mice, but smaller clusters of CD3<sup>+</sup>CD4<sup>+</sup> T and MHC-II<sup>+</sup> cells were still detected (Fig. S5B).

**Fig. 4.** Reactivated CD4 memory T cells cluster with B lymphocytes in immune clusters of the BM. (A, Left) Representative histology section of immune clusters of the BM, 3 d after boost. MHC-II<sup>+</sup> cells are depicted in white, CD4 cells in blue, and TCR Vα2<sup>+</sup> cells in red. (Right) Zoomed-in area of cluster (Top) and noncluster (Bottom) of CD4<sup>+</sup> Vα2<sup>+</sup> and MHC-II<sup>+</sup> cells. (B) Number of immune clusters per section of BM calculated for three nonboosted (circles) and three boosted (squares) mice on day 63. (C) Frequency of LCMV GP<sub>61-80</sub> (tetramer<sup>+</sup> circles) and LCMV GP<sub>65-77</sub> (tetramer<sup>-</sup> squares) CD4<sup>+</sup>CD44<sup>+</sup> cells which express TCR Vα2. Data are from one experiment with five mice per group. (D) Frequency of TCRVα2<sup>+</sup> CD4 memory T cells contacting MHC class II-expressing cells inside (circles) or outside (squares) of immune clusters. Approximately 50 Vα2<sup>+</sup> cells in clusters and 130 Vα2<sup>-</sup> cells outside of clusters were counted per image for three individual mice and the percentage of cells contacting MHC class II<sup>+</sup> cells was calculated. (E) Expression level of MHC class II in MHC-II<sup>+</sup> lymphocytes of the BM with (squares) and without (circles) boost, as determined by geometric mean based on four to five mice per group. Data shown are from one experiment, representative of two independent experiments. (F) Representative dot plot of B220 vs. CD11c gated on MHC-II<sup>+</sup> viable cells, 3 d after boost. (G) Representative histology section of an immune cluster containing B220<sup>+</sup>IgM<sup>+</sup>IgD<sup>+</sup> B lymphocytes in the BM, 3 d after boost. B220<sup>+</sup> cells are shown in white, IgM<sup>+</sup> cells in blue, IgD<sup>+</sup> cells in red, and IgG<sup>+</sup> (IgG1 and IgG2a/b) in green. (F) Frequency of IgD<sup>+</sup>IgM<sup>+</sup> (circles) and IgD<sup>-</sup>IgM<sup>-</sup>IgG<sup>+</sup> (IgG1 and IgG2a/b) B220<sup>+</sup> B cells in immune clusters, 3 d after boost. Two images per mouse for a total of three individual mice were used to calculate the frequencies. Data are shown as mean ± SEM, **P < 0.01, ***P < 0.001, as determined by unpaired Student's t test. n.d., not detected.

Ten days after boost the immune clusters dissipated and the amplified memory T cells appeared to be dispersed throughout the BM (Fig. S3E). Although at this time the consequence of the colocalization of T and B lymphocytes in the immune clusters is not clear, their formation was clearly linked to cognate activation of the antigen-specific CD4 memory T cells, since they were present even when the mice were boosted only with antigen and without any adjuvant (Fig. S3D).

**Fig. 5.** B cell-independent expansion of antigen-specific CD4 memory T cells in the BM. (A) Absolute numbers of B220<sup>+</sup>MHC-II<sup>+</sup> cells per microliter of blood (open circles) and without (closed circles) anti-CD20 administration on day 59, before boost. (B) Absolute numbers of BM B220<sup>+</sup>MHC-II<sup>+</sup> cells with (open circles) and without (closed circles) anti-CD20 administration on day 63, with boost. (C) Absolute numbers of BM LCMV GP<sub>60-77</sub>-specific CD4 memory T cells on day 63 without boost (squares) and with boost with (open circles) or without (closed circles) anti-CD20 administration. Data are shown as mean ± SEM and are from one experiment with four to six mice per group. *P < 0.05, **P < 0.01, as determined by one-way ANOVA (C) or Mann-Whitney test. ns, not significant.

**Materials and Methods**

See SI Methods.
All mice were purchased from Charles River and maintained under specific pathogen-free conditions in the mouse facility of the German Rheumatism Research Center Berlin. Experiments were performed according to institutional guidelines and German federal laws on animal protection. Eight-week-old C57BL/6 male mice were immunized i.p. with 100 μg poly(I:C) (Invivogen) and 250 μg OVA (Invivogen) or 100 μg LCMV Gp66-77 (Genecust) coupled to MSA (Merck Millipore) and NP in 200 μL PBS. Two weeks after priming, mice were immunized i.p. again with 100 μg poly(I:C) and 250 μg OVA or 100 μg uncoupled LCMV Gp66-77. Either 60 or 90 d after the last immunization mice were or were not boosted i.p. with 100 μg poly(I:C) and 250 μg OVA or 100 μg LCMV Gp66-77-MSA-NP. Only when indicated, mice were boosted i.p. at day 60 after the last immunization with 100 μg LCMV Gp66-77-MSA-NP alone, without any adjuvant.

FTY720 and Anti-CD20 Treatments. When indicated, mice were treated i.p. with 1 mg/kg FTY720 (Cayman Chemical) or vehicle starting the day before last boost and for the following 3 d until the day of analysis. Normal saline solution was used to dissolve FTY720. When indicated, mice were treated i.v. with a single dose of
injection of 250 μg anti-CD20 (SA2712; BioLegend) or isotype control antibody (Rat IgG2b, c BioLegend) 3 d before the last boost. B-cell depletion was checked the day before the boost after bleeding the mice.

Flow Cytometry and Cell Sorting. Flow cytometry and cell sorting were performed as described (42). The following antibodies were used: LCMV.GP6-27 (DIYKGVYQFKSV) loaded tetramer or KCLIP control (Nih tetramer core facility), anti-CD55 (L138D7 or 2GB), anti-CD3 (17A2), anti-CD4 (RM4.4), anti-CD44 (IM7), anti-B220 (RA3.6B2), anti-CD8 (53-6.72), anti-Gr1 (RB6-8C5), anti-CD69 (H1.2F3), anti-PD1 (29F.1A12), anti-MHC class II (M5/114.15.2), anti-CD11C (N418), PNA (Vector Laboratories), anti-TCR Vß2 (B20.1), anti-CD10 (RM1-M), anti-iGd (11.26), anti-iGγG1 (56), anti-iGβa2b (57) or NiP (4-hydroxy-3-iodo-5-nitrophenylacety), anti-KI67 (B6), and Phalloidin AF647 (Thermo Fisher Scientific). Viability of cells was assessed via fixable live/dead dye aqua (Thermo Fisher Scientific). Stained samples were acquired on a BD Fortessa (BD Biosciences), MACSQuant (Miltenyi), or Attune NxT (Thermo Fisher Scientific) flow cytometer. For cell sorting, a FACs Aria I (BD Biosciences) cell sorter was used. Flow cytometric data were analyzed using FlowJo software (FlowJo LLC).

Immunofluorescent Staining and Confocal Microscopy. The following primary and secondary reagents were used: anti-CD3 (eBio500A2), anti-CD4 (YTS151.2; DRFZ conjugate), anti-CD44 (IM7; DRFZ conjugate), anti-B220 (RA3.6B2; DRFZ conjugate), anti-MHC II (MS5/114; DRFZ conjugate), anti-TCR Vß2 (B20.1; BioLegend), anti-iGm (M41; DRFZ conjugate), anti-iGd (11.26; DRFZ conjugate), anti-iGγG1 (56), anti-iGβa2b (57), PNA (Vector Laboratories), anti-CD11c (IM7), anti-CD69 (H1.2F3), anti-PD1 (29F.1A12), anti-MHC class II (M5/114.15.2), anti-CD11C (N418), PNA (Vector Laboratories), anti-TCR Vß2 (B20.1), anti-CD10 (RM1-M), and anti-iGd (11.26), anti-iGγG1 (56), anti-iGβa2b (57) or NiP (4-hydroxy-3-iodo-5-nitrophenylacety), anti-KI67 (B6), and Phalloidin AF647 (Thermo Fisher Scientific). Viability of cells was assessed via fixable live/dead dye aqua (Thermo Fisher Scientific).

ACKNOWLEDGMENTS. We thank Cassandra Steinkras, Daniel Schulz, Shintaro Hoyjo, Tuula Geske, Heidi Hecker-Kia, Heidi Schlimme, and Anette Peddinghaus for their technical help; Andreas Hutoft for scientific discussions; Toralf Kaiser and Jenny Kirsch for cell sorting; and Patrick Thiemann and Manuela Ohde for their assistance with animal care. This work was supported by the Leibniz ScienceCampus Chronic Inflammation (www.chroniche-entzuedung.org), European Research Council Advanced Grant IMMEO [ERC-2010-Aug-20100317 Grant 268987 (to A.R.); FFP Marie Curie Initial Training Network OT0118, Grant FP7-PEOPLE-2011-ITN-289150 (to F.S.), European Translational Training for Autoimmunity & Immune Manipulation Network (EUTRAIN) Grant FP7-PEOPLE-2011-ITN-289903 (to P.M.), and the state of Berlin and European Regional Development Fund ERDF 2014–2020, EFRE 1.8/11, Deutsches Rheuma-Forschungszentrum (to M.-F.M., P.M., F.-F. M., M.B., and M.A.M.).