Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation

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Contributed by Tak W. Mak, April 11, 2012 (sent for review March 2, 2012)

Follicular T-helper (T_{FH}) cells cooperate with $GL7^+CD95^+$ germinal center (GC) B cells to induce antibody maturation. Herein, we identify the transcription factor IRF4 as a T-cell intrinsic precondition for T_{FH} cell differentiation and GC formation. After immunization with protein or infection with the protozoon *Leishmania major*, draining lymph nodes (LNs) of IFN-regulatory factor-4 ($Irf4^{-/-}$) mice lacked GCs and GC B cells despite developing normal initial hyperplasia. GCs were also absent in Peyer's patches of naive $Irf4^{-/-}$ mice. Accordingly, $CD4^+$ T cells within the LNs and Peyer's patches failed to express the T_{FH} key transcription factor B-cell lymphoma-6 and other T_{FH}-related molecules. During chronic leishmaniasis, the draining $Irf4^{-/-}$ LNs disappeared because of massive cell death. Adoptive transfer of WT $CD4^+$ T cells or few *L. major* primed WT T_{FH} cells reconstituted GC formation, GC B-cell differentiation, and LN cell survival. In support of a T-cell intrinsic IRF4 activity, $Irf4^{-/-}$ T_{FH} cell differentiation was not rescued by close neighborhood to transferred WT T_{FH} cells. Together with its known B lineage-specific roles during plasma cell maturation and class switch, our study places IRF4 in the center of antibody production toward T-cell–dependent antigens.

interleukin-21 | inducible costimulator | CXC-chemokine receptor 5 | apoptosis

Apart from Th1 and Th2, the family of Th subsets now includes Th17 and Th9 (1). In addition, follicular T-helper (T_{FH}) cells are defined based on their location within germinal center cells (GCs) of lymphoid organs (2, 3). Here, these cells produce cytokines that normally define other subsets, such as the Th2 product IL-4 (4, 5) or the Th17 product IL-21 (6), which is involved in GC B-cell generation (7–10). T_{FH} cell propagation is supported by the transcription factor B-cell lymphoma (BCL)-6 and suppressed by Blimp1 (11–13). Further markers used to define T_{FH} cells include inducible costimulator (ICOS), programmed death-1 (PD-1), and CXC-chemokine receptor 5 (CXCR5), which mediate their migration into GCs (2, 14–16).

The IFN-regulatory factor (IRF) family of transcription factors includes nine members in mammals that bind to related target-gene sequences (17). We and others have described important roles of IRFs during Th cell differentiation. In particular, IRF1 is decisive for Th1 cell generation because it is ubiquitously expressed and redundantly addresses many genes with independent Th1-supporting function (17–19). In contrast, IRF4 controls Th2 and Th17 cell differentiation (20–23), with ensuing total resistance of $Irf4^{-/-}$ mice in a Th17-dependent mouse model of multiple sclerosis (24). In addition, regulatory T-cell (Treg)-specific IRF4 deficiency or lack of IRF4 binding protein lead to a generalized autoimmune syndrome (25, 26). Finally, we reported on the role of IRF4 during Th9 differentiation (27). Remarkably, IRF4 is also a B-cell intrinsic prerequisite for class switch and plasma cell maturation (28, 29).

Given these pleiotropic activities of IRF4 on B and T cells, we wondered whether IRF4 also contributes to the interaction of T_{FH} and GC B cells. Herein, we use chronic leishmaniasis, a model infection with prominent T- and B-cell interactions (30) to prove a decisive T-cell intrinsic role of IRF4 for murine T_{FH} cell development.

Results

***$Irf4^{-/-}$* Mice Fail to Generate GCs.** To study the development of T_{FH} cells in vivo, we infected $Irf4^{-/-}$ mice and *Irf4*-competent control mice with *Leishmania major* (30). Two weeks later, draining popliteal lymph nodes (LNs) were analyzed (Figs. 1 and 2). By immunohistology, prominent GC formation was observed in WT and $Irf4^{+/-}$ LNs, including presence of $GL7^+$ GC cells (Fig 1*A*). In contrast, GCs were totally absent in $Irf4^{-/-}$ LNs and few $GL7^+$ cells were dispersed throughout the LN. However, $Irf4^{-/-}$ LNs did contain normal B and CD4 T-cell areas (Fig. 1*A*, *Bottom Right*). We confirmed the lack of GCs in $Irf4^{-/-}$ mice that were immunized with the myelin oligodendrocyte glycoprotein (MOG) peptide instead of *L. major* infection (Fig. S1) and in Peyer's patches (PP) from naive mice (Fig. 3*A*).

A strong reduction in GC B cells coexpressing the markers GL7 and CD95 (31, 32) was verified by flow-cytometry of single LN cell suspensions from *L. major* infected $Irf4^{-/-}$ mice (Fig. 1*B*, note the compiled data of three different experiments). Again, the finding was reproduced in PP from naive mice (Fig. 3*B*). In control FACS-stainings, GL7 was only weakly expressed on WT and $Irf4^{-/-}$ $CD4^+$ cells. Thus, $Irf4^{-/-}$ mice form the architecture of normal LNs, but lack GC formation. Furthermore, the ICOS ligand (ICOSL) molecule was strongly up-regulated on $Irf4^{-/-}$ B compared with WT B cells (Fig. 1*C*), possibly because of a feedback-loop between ICOSL and its partner ICOS (33) expressed on T_{FH} cells. Together with missing GC formation, these findings suggest a defect in T_{FH} cells within $Irf4^{-/-}$ mice.

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

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1205834109/-/DCSupplemental.

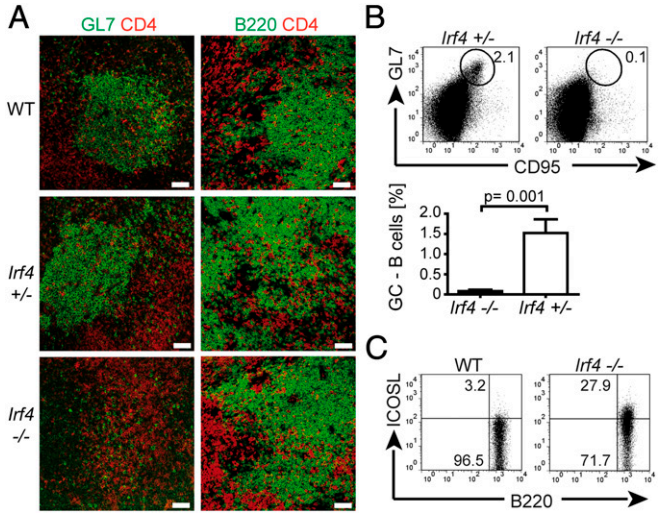

Fig. 1. Lack of GC formation in *Irf4*$^{-/-}$ mice. Mice of the indicated genotypes were infected with *L. major*. Two weeks later, popliteal LNs were prepared. (*A*) Tissue sections were stained for CD4 (red), GL7 (green, *Left*), or B220 (green, *Right*), and analyzed by fluorescence-microscopy (20× magnification). (Scale bars, 50 μm.) (*B* and *C*) LN cell suspensions were stained for the indicated surface molecules and analyzed by flow cytometry. Numbers indicate percentages of positive cells in the respective circles or rectangles. Panels are from one representative mouse per group. Three different experiments, each with two or three mice per group. (*B*) B220^{+} B-cell gate. Percentages (mean ± SD) of GL7^{+}CD95^{+} B cells compiled from all mice tested.

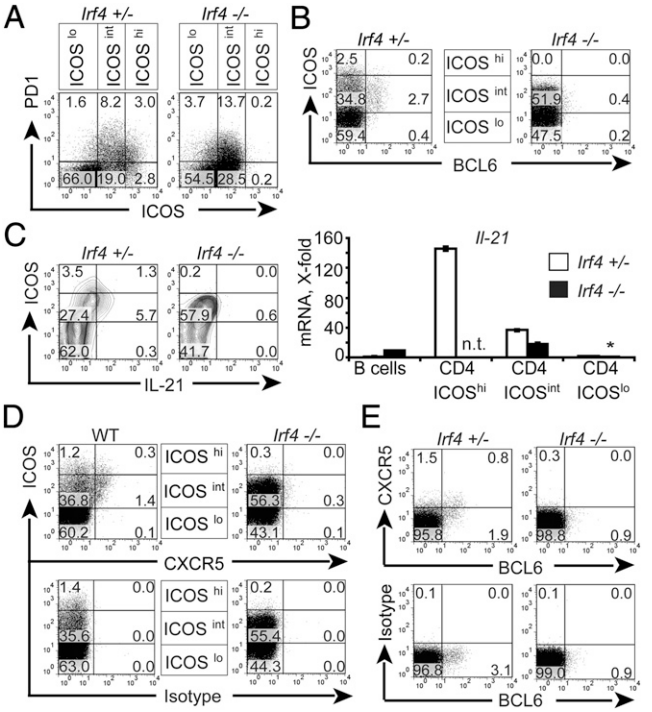

Fig. 2. Lack of T$_{FH}$ cells in *L. major* infected *Irf4*$^{-/-}$ mice. *Irf4*$^{-/-}$, WT mice, and *Irf4*$^{+/-}$ mice (three per group) were infected with *L. major* and their popliteal LN cells analyzed 2 wk later for expression of extracellular ICOS, CXCR5, and PD-1 (*A*) and intracellular BCL-6 (*B*) and IL-21 (*C*). (*A; B; C, Right; D;* and *E*) Direct ex vivo analysis. (*C*) Analysis after restimulation for 4 h with PMA and ionomycin (*Left*). (*Right*) CD4^{+} cells from pooled LN cells of all mice per group sorted according to ICOS expression (see *A*), and analyzed for IL-21 compared with HPRT (hypoxanthine-guanine phosphoribosyl transferase) expression by RT qPCR. Bars denote the SD of duplicate qPCR determinations of each sample. The asterisk signifies that this value was set to one. n.t., not tested. (*D* and *E*) Single CD4^{+} cells were gated similar to gate B of Fig. S2*B*. Numbers in *A–E* indicate percentages of cells in the respective rectangles. Data are from one representative mouse per group. Three experiments with similar outcomes.

***Irf4*$^{-/-}$ Mice Fail to Generate T$_{FH}$ Cells.** To directly test this theory, LN cells of *L. major* infected mice were analyzed for expression of T$_{FH}$ marker molecules. In *Irf4*$^{+/-}$ mice, T$_{FH}$ cells expressing BCL-6, IL-21, and PD-1 were present and coexpressed ICOS at high (ICOS^{hi}) or intermediate (ICOS^{int}) levels. Importantly, *Irf4*$^{-/-}$ CD4^{+} cells totally lacked ICOS^{hi} cells, although ICOS^{int} cells were present at even enhanced frequency (Fig. 2*A*). Both findings were confirmed in PP of naive mice (Fig. 3*C*). As for PD-1, its expression was comparable in CD4^{+} cells of infected *Irf4*$^{-/-}$ and *Irf4*$^{+/-}$ mice (Fig. 2*A*), but was also reduced in *Irf4*$^{-/-}$ CD4^{+} cells of naive PP (Fig. 3*C*). Furthermore, amounts of BCL-6 protein, a central molecule for T$_{FH}$ cell function (11–13), were considerably lower in *Irf4*$^{-/-}$ than in *Irf4*$^{+/-}$ CD4^{+} cells (Fig. 2*B*).

An even stronger defect was noted with respect to IL-21 production (Fig. 2*C*): after short-term restimulation, IL-21 protein was synthesized by many of the ICOS^{hi} and ICOS^{int} *Irf4*$^{+/-}$ control cells (thus further characterizing them as the source of T$_{FH}$ cells), but not by *Irf4*$^{-/-}$ CD4^{+} cells (Fig. 2*C*, *Left*). To measure expression of *Il-21* at the mRNA level, we performed quantitative PCR (qPCR) directly ex vivo (Fig. 2*C*, *Right*) after sorting ICOS^{hi} (only control mice), ICOS^{int}, and ICOS^{lo} cell populations (Fig. 2*A*), Again, most *Il-21* mRNA was detected in ICOS^{hi} *Irf4*$^{+/-}$ control cells, but the lower levels in ICOS^{int} cells were further reduced in their *Irf4*$^{-/-}$ counterparts. These data demonstrate a striking defect of *Irf4*$^{-/-}$ CD4^{+} cells to express T$_{FH}$ cell markers.

Analysis of CXCR5 Expression. Expression of the CXCR5 molecule permits T$_{FH}$ cell migration into GCs (16), but is found at even higher levels in B cells (15). Recently T–B conjugates have been described in FACS analyses of LN cell preparations (5). These conjugates might contain T$_{FH}$ cells tightly interacting with B cells and complicate testing of CXCR5 expression on T cells. Indeed, we identified CD4^{+} events with considerable CXCR5 costaining (Fig. S2*A*) as aggregates of CD4^{+} T and B220^{+} B cells (Fig. S2*B*). In contrast, CXCR5-staining was weaker on single CD4+ T cells, despite nice staining of single B cells within the same sample (Fig. S2*B*). Mechanical dissociation and reanalysis confirmed lower CXCR5 staining on T cells. Thus, outgating of B–T conjugates did not remove any T$_{FH}$ cells with particularly strong CXCR5 staining, and was routinely used during data acquisition (with the exception of Figs. S2 and S3). We detected B–T conjugates not only in the *Leishmania* model, but also in PP of naive mice (Figs. S2 and S3) or after MOG immunization. Although CXCR5 clearly remains a marker of T$_{FH}$ cells, these T–B conjugates suggest critical care during its staining on T cells.

When we now compared CXCR5 expression on *Irf4*$^{-/-}$ and WT cells, we found that CXCR5 staining was totally absent in *Irf4*$^{-/-}$ CD4^{+} cells (Fig. 2*D*), confirming their lack of T$_{FH}$ cells. Importantly, this conclusion required exclusion of B–T conjugates from the analysis (Fig. S3, gate B), because gating on all viable CD4^{+} events revealed CD4^{+}CXCR5^{+} events (Fig. S3*A*) as a result of aggregating CXCR5-expressing *Irf4*$^{-/-}$ B cells (Fig. S3*B*, gate A). Mechanical disruption of the conjugates and reanalysis confirmed these conclusions (Fig. S3*C*) and excluded removal of CXCR5^{+} *Irf4*$^{-/-}$ T$_{FH}$ cells. As anticipated, we found a positive correlation for expression of CXCR5 and BCL-6 in *Irf4*$^{+/-}$ mice (Fig. 2*E*). Similar data were gained from PP of *Irf4*$^{-/-}$ mice (Fig. 3*C*, and Fig. S3 *D* and *E*). Taken together, these data strongly suggest that *Irf4*$^{-/-}$ T cells do not differentiate into T$_{FH}$ cells in vivo, but CXCR5 deficiency may explain lack of GC formation in *Irf4*$^{-/-}$ mice because of altered T-cell migration.

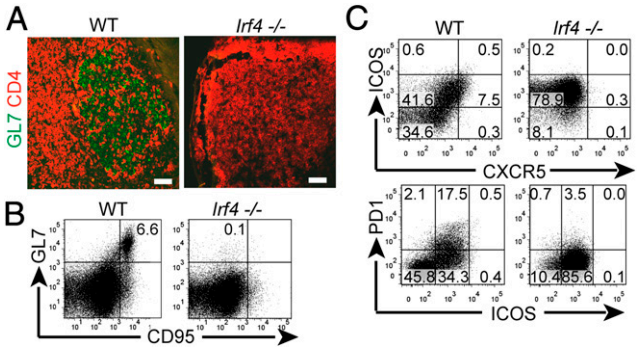

Fig. 3. Lack of T_{FH} cell differentiation in PP of naive *Irf4*$^{-/-}$ mice. (*A*–*C*) PP of naive mice were analyzed as described for Figs. 1 and 2.

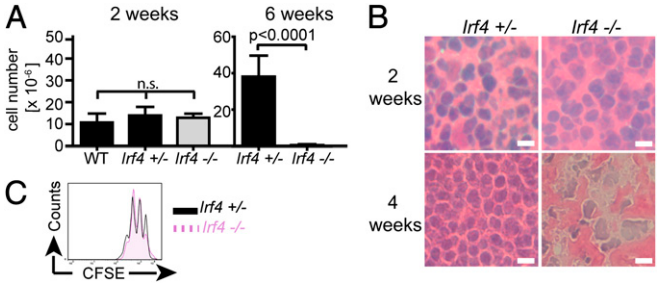

Fig. 4. Disappearance of lesion-draining LNs in *L. major* infected *Irf4*$^{-/-}$ mice. Mice of the indicated genotypes were infected with *L. major*. At the indicated time points, popliteal LNs were prepared and (*A*) cell numbers in single-cell suspensions counted (mean ± SD) or (*B*) tissue sections processed for HE staining and microscopy (40× magnification). (Scale bars, 10 μm.) (*C*) To control for proliferative capacity 2 wk after infection, cells were labeled with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), stimulated for 72 h with PMA/ionomycin, and stained for CD4. Data are representative of five (*A*) or three (*B* and *C*) different experiments, each with three mice per group and (*B* and *C*) show the results of one representative mouse per group.

To rule out a defect of *Irf4*$^{-/-}$ T cells in their receptor-triggered antigenic response as a trivial reason for T_{FH} cell deficiency, we restimulated LN cells of infected mice in vitro. In response to *Leishmania* antigens, *Irf4*$^{-/-}$ and *Irf4*$^{+/-}$ control CD4$^+$ cells secreted the precursor T-cell product IL-2 into their supernatants at the same order of magnitude (Fig. S4*A*). Similarly, the frequency of intracellularly stained IL-2–producing CD4$^+$ cells was similar between the two genotypes (Fig S4*B*).

Lack of *Irf4*$^{-/-}$ T_{FH} Cells Is Not Caused by a Cell Viability Problem. Previously, we reported on apoptotic death of *Irf4*$^{-/-}$ draining LN cells after about 6 wk of leishmaniasis (34). We therefore aimed to exclude that a cell viability problem hindered GC formation earlier after infection. In naive young *Irf4*$^{-/-}$ and *Irf4*$^{+/-}$ mice, the size of popliteal LNs was comparable (about 1×10^6 cells). Furthermore, the increase in LN size and histological appearance 2 wk after infection were similar (Fig. 4 *A* and *B*), but *Irf4*$^{-/-}$ and *Irf4*$^{+/-}$ CD4$^+$ LN T cells were comparably able to secrete IL-2 (Fig. S4) and to proliferate in response to phorbol 12-myristate 13-acetate (PMA) and ionomycin (Fig. 4*C*). These data confirm functional integrity of *Irf4*$^{-/-}$ LN CD4$^+$ T cells at the time point when the lack of GC formation was noted. In contrast, *Irf4*$^{-/-}$ LNs had almost totally disappeared 6 wk after infection (Fig. 4*A*), and severe damage in LN cell morphology was already visible 4 wk after infection (Fig. 4*B*). Cell death did not occur in other LNs of infected *Irf4*$^{-/-}$ mice. The divergence in LN cell viability of WT and *Irf4*$^{-/-}$ mice 6 but not 2 wk after infection is underscored by the compiled statistical significance of all mice tested (Fig. 4*A*). In conclusion, lack of *Irf4*$^{-/-}$ GC formation cannot be explained by disturbed cell viability.

Rescue of GC Formation by WT CD4$^+$ T Cells. Although a primary *Irf4*$^{-/-}$ T_{FH} cell defect was likely, a B-cell defect with altered T–B interactions and secondary T_{FH} cell deficiency remained possible. To directly prove a T-cell intrinsic T_{FH} promoting IRF4 activity, *Irf4*$^{-/-}$ mice were reconstituted intraperitonially with purified CD45 (Ly5.1$^+$) congenic CD4$^+$ cells from naive mice at the start of infection. Two weeks later—that is, at the maximum of GC formation in WT mice—transferred WT CD4$^+$ T cells had perfectly rescued the appearance of GCs and GL7$^+$ *Irf4*$^{-/-}$ B cells (Fig. 5*A*). Furthermore, the WT cells tended to accumulate close to the GC areas, but endogenous CD4$^+$ T cells mostly remained outside of them. By flow cytometry, WT CD4$^+$ T-cell transfer totally rescued the generation of *Irf4*$^{-/-}$ GL7$^+$CD95$^+$ GC B cells (Fig. 5*B*) and the compensatory up-regulation of ICOSL on *Irf4*$^{-/-}$ B cells was almost reverted (Fig. 5*C*). Because WT CD4$^+$ cells can induce GC formation and GC B-cell differentiation in *Irf4*$^{-/-}$ mice, their T_{FH} defect is caused by an intrinsic T- but not B-cell defect. Accordingly, B-cell–specific deficiency in IRF4 leads to disturbed plasma cell differentiation, but no change in GC formation (28). However, this plasma cell defect precludes demonstration of effects of the transferred WT CD4$^+$ T cells on antibody formation in our mice.

Rescue of LN Cell Survival by WT CD4$^+$ T Cells. Like GC formation, LN cell survival 6 wk after infection was rescued to a great extent by transferred CD4$^+$ cells and normal histological morphology was regained (Fig. 6 *A* and *B*). Most of the surviving LN cells did not express the Ly5.1 marker of the transferred WT cells (Fig. 6*C*) and Ly5.1-expressing cells were all CD4$^+$. Thus, LN cell viability was not secondary to outgrowth of WT CD4$^+$ cells. Instead, WT CD4$^+$ cells caused improved survival of endogenous *Irf4*$^{-/-}$ B and T cells. Of note, WT CD4$^+$ T cells were perfectly able to become ICOS^{hi} cells within *Irf4*$^{-/-}$ LNs (Fig. 6*C*). Thus, the defect in *Irf4*$^{-/-}$ T_{FH} cells is not caused by deficiencies of accessory cells (e.g., in antigen presentation or production of necessary cofactors). Furthermore, even the side-by-side presence of WT T_{FH} cells did not catalyze ICOS^{hi} expression, and thus T_{FH} cell differentiation in endogenous Ly5.1$^-$ *Irf4*$^{-/-}$ CD4$^+$ T cells (Fig. 6*C*).

Unlike T cells, transferred WT B cells neither protected LNs from cell death nor modified the effect of WT CD4$^+$ cells (Fig. 6*A*). As a positive control for their functionality, we took advantage of the unrelated *Irf4*$^{-/-}$ B-cell defect (28, 35), leading to drastically reduced serum IgM levels. Analysis of mouse sera after B-cell transfer revealed that the transferred WT B cells raised IgM in *Irf4*$^{-/-}$ mice almost to WT amounts (Fig. S5*A*).

Rescue of LN survival mirrored susceptibility toward *L. major*: transferred WT CD4$^+$, but not B cells, led to a healing phenotype, as measured by lesion size and parasite burden and compared with control *Irf4*$^{+/-}$ mice (Fig. S5 *B* and *C*). However, a high parasite load is probably not the main reason for ensuing cell death in *Irf4*$^{-/-}$ LN cells, because susceptible BALB/c or IRF1-deficient mice contain even more *Leishmania* (18), but keep the original size of the LN and develop a cheesy necrosis inside of it.

To formally link T_{FH} cells and protection from cell death, we transferred as few as 2×10^5 FACS-sorted (Fig. S6) WT T_{FH} cells from LNs and spleens of *L. major* infected Ly5.1$^+$ WT mice. For comparison, ICOS$^-$CXCR5$^-$ CD4$^+$ T cells from the same organs were transferred into different *Irf4*$^{-/-}$ mice. All mice were infected and the draining LNs analyzed 6 wk later. Transfer of T_{FH} cells, but not of ICOS$^-$CXCR5$^-$ CD4$^+$ T cells rescued endogenous LN cell viability, as seen from cell numbers and normal proliferative behavior (Fig. 6 *D* and *E*). Transferred T_{FH} cells also rescued

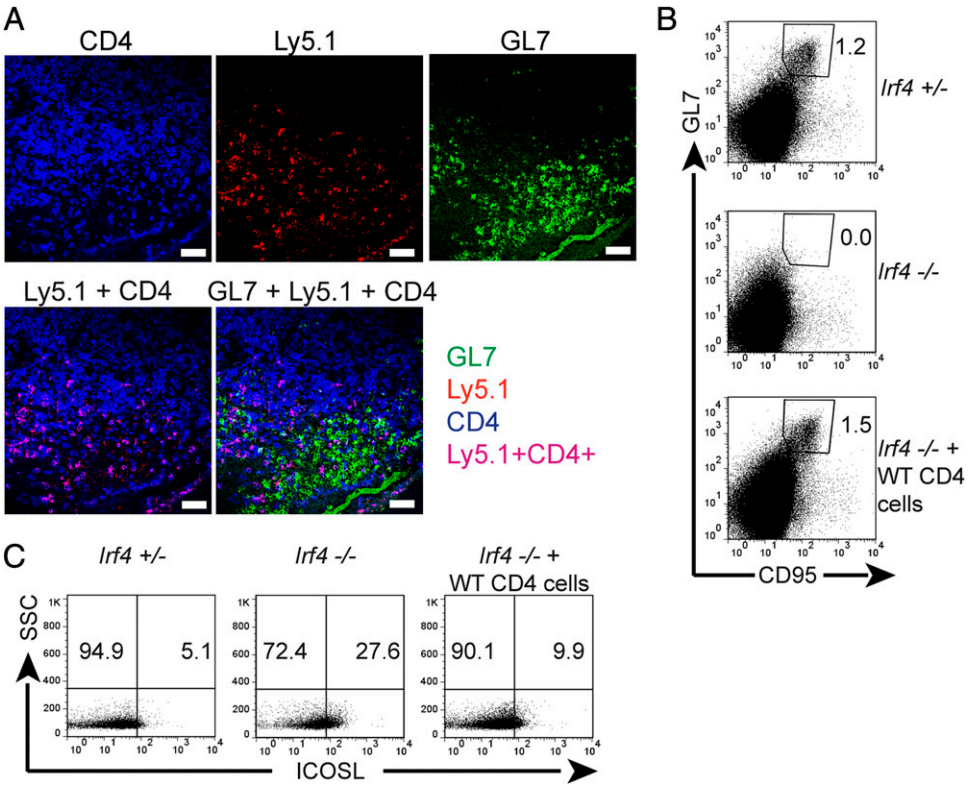

Fig. 5. Rescue of GC formation by WT CD4$^+$ T cells. *Irf4*$^{-/-}$ mice (*A*–*C*) or *Irf4*$^{+/-}$ mice (*B* and *C*) were infected with *L. major*. Where indicated, *Irf4*$^{-/-}$ mice received 8×10^6 Ly5.1 congenic WT CD4$^+$ cells by intraperitoneal adoptive transfer on the day of infection. Two weeks later, popliteal LNs were prepared. (*A*) Tissue sections were stained with antibodies to CD4 (blue), Ly5.1 (red), and GL7 (green) followed by fluorescence-microscopy (20× magnification). (Scale bars, 50 μm.) (*B* and *C*) Cells in suspension were stained for B220, GL7, ICOSL, and CD95 and analyzed (B220$^+$ gate) by flow cytometry. Numbers refer to percentages in the quadrant below the number (*C*) or within the indicated gate (*B*). SSC, side scatter. Data are from one representative mouse per group. Three (*A*) or two (*B* and *C*) different experiments, each with three mice per group, were performed with similar outcome.

a resistant phenotype during leishmaniasis (Fig. S5*D*), and ICOS$^-$CXCR5$^-$ CD4$^+$ T cells did not. Taken together, these data suggest a link between the T_{FH} cell defect and cell death in the draining *Irf4*$^{-/-}$ LNs during chronic leishmaniasis.

Role of IL-21. Next, we considered lack of a particular T_{FH} cell product as primary cause of LN cell death and missing *Irf4*$^{-/-}$ GC formation. An important candidate was IL-21, which is induced within T_{FH} cells via the ICOS–c-Maf axis (9, 36) and is required for GC B-cell differentiation (7–10). We considered IL-21 as well, because expression of ICOS and IL-21 by *Irf4*$^{-/-}$ cells is disturbed (Fig. 2 and ref. 21), and because IRF4 binds the ICOS promoter (25) and mediates cell responses to IL-21 together with STAT3 (37). To test for a role of IL-21, we compared the effects of adoptively transferred purified *Il21*$^{-/-}$ and WT CD4$^+$ T cells on GC formation and LN cell survival in *Irf4*$^{-/-}$ mice 2 wk after infection (Fig. 7).

Although transferred WT cells again perfectly rescued the appearance of GCs and GL7$^+$ cells (Fig. 7*A*), only few GL7$^+$ cells were detectable after transfer of *Il-21*$^{-/-}$ CD4$^+$ cells and were spread throughout the LN as in control *Irf4*$^{-/-}$ mice. Furthermore, transferred WT but not *Il-21*$^{-/-}$ CD4$^+$ cells rescued the frequency of GL7/CD95 coexpressing GC B cells (Fig. 7*B*), in comparison with *Irf4*$^{-/-}$ mice without cell transfer.

To analyze whether IL-21 was also involved in the rescue of LN cell survival by WT cells (Fig. 6), part of the mice was analyzed 6 instead of 2 wk after infection. Transfer of *Il-21*$^{-/-}$ CD4$^+$ cells created an intermediate phenotype compared with *Irf4*$^{-/-}$ mice receiving either no or WT CD4$^+$ T cells (Fig. 7*C*). Accumulation of all data from the different experiments revealed less potent effects of *Il-21*$^{-/-}$ than of WT CD4$^+$ cells with clear statistical difference. However, even if their effects on *Irf4*$^{-/-}$ LN cell survival were weaker, they still were demonstrable with high significance compared with *Irf4*$^{-/-}$ mice without cell transfer.

Discussion

In the past, IRF4 has been characterized as an important transcription factor for differentiation of Th2, Th9, and Th17 cells. In addition, aspects of Treg cell function entirely depend on IRF4 (20–23, 25–27). In the B-cell lineage, IRF4 is important for plasma cell differentiation and isotype switching (28, 29). Our results link these previous findings in B and T cells and show an additional important role of IRF4 for development of T_{FH} cells, which are mainly responsible for the intricate organization of T–B interactions and antibody maturation in vivo.

For our analysis, we used infection of mice with *L. major*, a model characterized by strong B- and T-cell interactions and LN hyperplasia (30). When analyzing LNs of WT and *Irf4*$^{-/-}$ mice at the height of GC formation in WT mice, a striking defect of *Irf4*$^{-/-}$ mice became apparent: despite normal structure of B- and T-cell areas, their LNs totally lacked GC formation and differentiation of GC B cells. These findings were confirmed in PPs of naive mice and after immunization with a peptide instead of *L. major* infection. In parallel, LN CD4$^+$ T cells expressed strongly reduced amounts of the T_{FH} cell-related (2, 11–13, 15, 16) molecules ICOS, IL-21, and BCL-6.

A remarkable result was obtained with respect to the T_{FH} marker CXCR5 in that LN cell suspensions contained conjugates of adherent B–T cells, which conferred the risk for misinterpreting FACS data on CXCR5-expression in CD4$^+$ T cells,

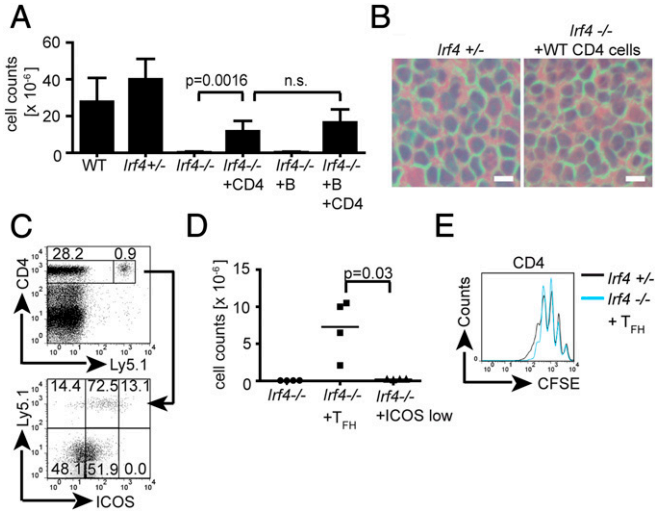

Fig. 6. Rescue of LN cell survival by WT CD4$^+$ cells. (*A*–*C*) Where indicated, *Irf4*$^{-/-}$ mice received 8 × 10^6 Ly5.1 congenic WT B or CD4$^+$ cells or both by intraperitoneal transfer, were infected, and analyzed 6 wk later in comparison with infected WT or *Irf4*$^{+/-}$ mice. (*A*) Popliteal LN cell numbers. (*B*) H&E staining (40× magnification) (Scale bars, 10 μm.) (*C*, *Upper*) total life cell gate. Numbers refer to the percentage of CD4$^+$Ly5.1$^-$ or CD4$^+$Ly5.1$^+$ among total cells. (*Lower*) Relative cell percentages among CD4$^+$Ly5.1$^+$ cells (*Upper* numbers) or CD4$^+$Ly5.1$^-$ cells (*Lower*) within the respective rectangles. (*D* and *E*) T_{FH} cells (CD4$^+$ICOS^{hi}CXCR5$^+$) or control CD4$^+$/ICOS$^-$/CXCR5$^-$ cells sorted (Fig. S6) from 2 wk-infected Ly5.1$^+$ WT mice were transferred intraperitoneally (2 × 10^5 per mouse) into *Irf4*$^{-/-}$ mice which, together with control mice, were infected with *L. major*. After 6 wk, cell numbers of the draining LNs were counted (*D*) and the proliferative capacity of the cells determined by CFSE dilution (*E*). Data are representative of three (*A*–*C*) or two (*D* and *E*) different experiments, each with three (*A*–*C*) or two (*D* and *E*) mice per group. (*D*) Accumulated data ± SD of all mice per group in the two experiments or data from one representative mouse (*E*).

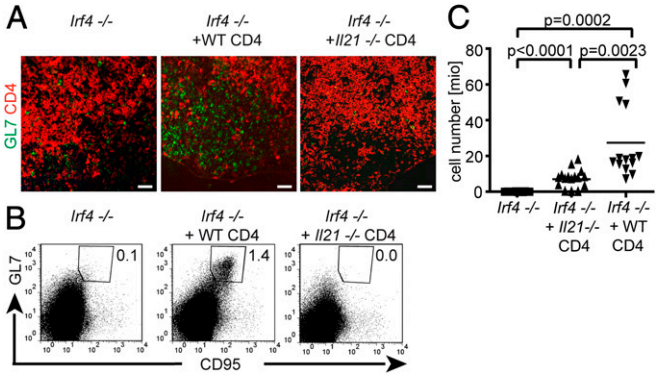

Fig. 7. Role of IL-21. *Irf4*$^{-/-}$ mice were infected with *L. major*. Where indicated, they also received 8 × 10^6 purified *Il21*$^{-/-}$ or WT CD4$^+$ cells by intraperitoneal adoptive transfer. Two weeks (*A* and *B*) or 6 wk (*C*) after infection, draining LNs were prepared (*B* and *C*) as cell suspensions. (*A*) Tissue sections stained with antibodies to CD4 (red) and GL7 (green) and analyzed by fluorescence-microscopy (20× magnification). (Scale bars, 50 μm.) (*B*) FACS analysis of gated B220$^+$ B cells stained for GL7 and CD95. Numbers indicate frequencies of B cells coexpressing GL7 and CD95. (*C*) Cell numbers within the draining LNs of individual mice with or without transfer of the indicated cells. Data are from one mouse representative for all experiments (*A* and *B*) or were compiled from the results of three individual experiments (*C*).

because of stronger CXCR5 expression on B cells. Similar B–T conjugates have recently been described and characterized for their B-cell part (5). The lower amounts of CXCR5 on single T cells correlated with high expression of ICOS and IL-21, indicating that CXCR5 can still be used to characterize T_{FH} cells. These findings were again confirmed in LNs of mice immunized with a peptide or in PPs from naive mice.

Integration of this information in the analysis of *Irf4*$^{-/-}$ T_{FH} cell development revealed that *Irf4*$^{-/-}$ LN CD4$^+$ cells were completely CXCR5$^-$. Importantly, this defect was only notable when the B–T conjugates were either out-gated or mechanically separated, because CXCR5 expression on *Irf4*$^{-/-}$ B cells was as high as in WT mice. Lack of CXCR5 on *Irf4*$^{-/-}$ CD4$^+$ but not B cells suggests a T-cell intrinsic differentiation defect that cannot solely be caused by binding of IRF4 to the *cxcr5* gene promoter. With respect to ICOS, its promoter has previously been linked to IRF4 (25). However, ICOS expression is not totally IRF4-dependent, because frequencies of *Irf4*$^{-/-}$ cells with intermediate ICOS expression were even increased (Figs. 2 and 3), perhaps in a feedback-loop triggered by deficient terminal T_{FH} cell differentiation. Lower ICOS expression may be one reason why *Irf4*$^{-/-}$ T cells produced almost no IL-21, because *Il-21* transcription is induced by an axis via ICOS and c-Maf (36). However, impaired IL-21 production may also mirror an intrinsic deficiency of *Irf4*$^{-/-}$ T cells, given that naive *Irf4*$^{-/-}$ CD4$^+$ T cells stimulated in vitro under Th17-inducing conditions also produced less IL-21 (21). In contrast to ICOS, the T_{FH} cell marker PD-1 was normally expressed in T cells of *L. major* infected *Irf4*$^{-/-}$ mice. Therefore, missing T-cell stimulation is unlikely to explain absent *Irf4*$^{-/-}$ T_{FH} cell development, a conclusion supported by normal IL-2 production in response to *Leishmania* antigens.

Importantly, missing *Irf4*$^{-/-}$ T_{FH} cells and GCs correlated with eventual total LN cell death after initial LN hyperplasia. We have previously described enhanced in vitro sensitivity of *Irf4*$^{-/-}$ CD4$^+$ cells to activation-induced cell death (34). Herein, we observed that death of *Irf4*$^{-/-}$ LN cells could be avoided by presence of WT CD4$^+$ but not B cells. Only few CD4$^+$ WT cells were required for protection, based on their low frequency within the surviving LN cells. LN cell survival was also saved by very few *Leishmania* primed WT T_{FH} cells, thus linking T_{FH} cells and protection from cell death. LN cell survival correlated with resistance to the parasite. Nevertheless, it is difficult to attribute this feature solely to the presence of T_{FH} cells, because *Irf4*$^{-/-}$ mice are also devoid of Th9 or Th17 cells (20, 26, 27). A direct causal link of parasite burden and LN cell survival is not likely, given that draining LNs of mice from other highly susceptible strains behave totally differently.

Of note, adoptive transfer of WT CD4$^+$ cells rescued not only LN cell survival, but also appearance of GCs and GC B cells with normalized expression of ICOSL in accordance with the feedback-loop between ICOS and ICOSL (33). These findings underline that the *Irf4*$^{-/-}$ T_{FH} cell defect is T-cell intrinsic and support a report on normal GC formation in mice with a B-cell–specific defect of IRF4 (28). Transfer of *Il-21*$^{-/-}$ CD4$^+$ T cells proved a central role for IL-21 during the rescue of GC formation. In contrast, IL-21 was necessary but not sufficient for protection from LN cell death, because *Il-21*$^{-/-}$ CD4$^+$ T cells supported cell survival only partially. LN cell survival during a chronic immune response is of central importance and may be regulated by independent effector molecules, such as IL-4, which is produced locally by T_{FH} cells (4, 5) and which (like IL-21) cannot be produced by *Irf4*$^{-/-}$ Th cells (22, 23).

As for the mechanism how IRF4 affects T_{FH} cell differentiation, we consider an important role to their key transcription factor BCL-6 (11–13), because its amounts are strongly reduced in *Irf4*$^{-/-}$ CD4$^+$ T cells. Apparently, this BCL-6–enhancing function of IRF4 is cell-type–specific, because IRF4 suppresses BCL-6 transcription in B cells (38). In addition, IRF4 physically binds to BCL-6 (39) and lack of this interaction may explain why *Irf4*$^{-/-}$ Th cells are totally unable to differentiate into T_{FH} cells,

although they still express some BCL-6. Possibly, the T_{FH} inducing capacity of BCL-6 requires its interaction with IRF4.

In conclusion, we demonstrate a decisive role of IRF4 for development of T_{FH} cells and, as a consequence, formation of GCs, differentiation of GC B cells, and survival of LN cells during an immune response. Together with the earlier findings that IRF4 also targets class-switching and terminal plasma cell differentiation, our study places IRF4 in the center of B–T cooperation during the formation of an adaptive immune response.

Materials and Methods

Mice and *L. major* Infection or Immunization. WT C57BL/6 mice, purchased from Charles River, CD45.1 congenic, *Il-21*$^{-/-}$ (from National Institutes of Health Mutant Mouse Regional Resource Centers), and *Irf4*$^{+/-}$ and *Irf4*$^{-/-}$ mice (bred in our own animal facilities) were used at 6–10 wk of age. Infection with promastigotes of the *L. major* strain MHOM/IL/81/FEBNI into the footpad, determination of lesion development or parasite burden, and leishmania-specific in vitro cell restimulation were performed as previously described (18, 34). Popliteal LNs or spleens were removed at the indicated timepoints for histology, qPCR, and FACS analysis, as detailed in *SI Materials and Methods*.

Total IgM levels were measured in a sandwich ELISA by coating with goat anti-mouse IgM followed by application of mouse sera and detection with AP-conjugated goat anti-mouse IgM (both reagents from Chemicon/Millipore). Immunization with MOG peptide and adjuvant was performed as previously described (20). All animal experiments were approved by "Regierungspräsidium Gießen" [permission number: V54-19c20-15(1) MR 20/6 Nr47/2009], the local institutional committee.

Statistics. For statistical analysis, we used an unpaired *t* test with Welch's correction.

ACKNOWLEDGMENTS. We thank Dr. Uli Steinhoff for helpful discussions. This work was supported by a Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz (LOEWE) grant Tumor and Inflammation (Germany), Behring-Röntgen-Stiftung, Deutsche Forschungsgemeinschaft (SFB TR22), and Deutscher Akademischer Austauschdienst, Germany.

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