European Journal of Immunology

Selective effects of NF-κB1 deficiency in CD4$^+$ T cells on Th2 and TFh induction by alum-precipitated protein vaccines

Karine Serre, Elodie Mohr*, Cécile Bénézech, Roger Bird, Mahmood Khan, Jorge H. Caamaño, Adam F. Cunningham and Ian C. M. MacLennan*

MRC Centre for Immune Regulation, the IBR, School of Immunity and Infection, University of Birmingham, Birmingham, UK

NF-κB1-dependent signaling directs the development of CD4$^+$ Th2 cells during allergic airway inflammation and protective responses to helminth infection. Here, we show that IL-4 and IL-13 production is NF-κB1-dependent in mouse OVA-specific CD4$^+$ (OTII) T cells responding to alum-precipitated OVA (alumOVA) immunization. More surprisingly, we found that NF-κB1 deficiency in OTII cells also selectively impairs their CXCR5 induction by alumOVA without affecting upregulation of BCL6, IL-21, OX40 and CXCR4 mRNA and PD-1 protein. This results in functional impairment of follicular helper T cells. Thus, fewer germinal center B cells develop in LN responses to alumOVA in T-cell-deficient mice reconstituted with NF-κB1$^{-/-}$ OTII cells as opposed to NF-κB1$^{+/+}$ OTII cells, while plasma cell numbers are comparable. Unlike CXCR5 induction in CD4$^+$ T cells, NF-κB1-deficient recirculating follicular B cells are shown to express normal levels of CXCR5. The selective effects of NF-κB1-deficiency on Th2 and follicular helper T cell induction do not appear to be due to altered expression of the Th2-associated transcription factors — GATA-3, c-Maf and Ikaros. Altogether, these results suggest that NF-κB1 regulates the expression of CXCR5 on CD4$^+$ T cells primed in vivo, and thus selectively controls the T-cell-dependent germinal center component of B-cell response to alumOVA.

Key words: Alum · CXCR5 · Follicular helper T cells · IL-4 · NF-κB1

Supporting Information available online

Introduction

Antigen-specific T-cell help for B cells is essential for both extrafollicular and follicular responses to most protein-based antigens. These antigens include alum-precipitated proteins, which are commonly used in vaccine formulations. Alum-precipitated OVA (alumOVA) causes OVA-specific CD4$^+$ T cells to develop into Th2 cells that help induce extrafollicular plasmablasts and GCs, and can direct Ig class switching to IgG1 [1–3]. Follicular helper T (TFh) cells are also produced in response to alumOVA and these are required for selection of B cells in GCs and for inducing the selected B cells to differentiate into plasma cells, memory B cells or centroblasts. TFh cells can also induce GC B cells to switch Ig class [4].

A proportion of both TFh cells and Th2 cells generated in the response to alumOVA produce IL-4. This Th2-cytokine, together

Correspondence: Dr. Karine Serre
e-mail: karine@kserre.net

*These authors contributed equally to this work.

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with IL-13 and IL-5, has pleiotropic roles and can provide protection against parasitic infections, but can also play a pathogenic role in atopic diseases [5]. The signaling pathway inducing IL-4 in this response in vivo remains unclear. This contrasts with the detailed knowledge of the IL-4 and STAT6-dependent pathway for Th2 cytokine induction in vitro when T cells are activated through the TCR in the presence of IL-4 [5]. The induction of Th2 cytokines in vivo in the absence of IL-4 or STAT6 signaling [2, 6–8] indicates that alternative pathways operate in vivo in Th2 induction.

In mammals, the NF-κB family is composed of five related transcription factors: NF-κB1 (p50), NF-κB2 (p52), RelA, c-Rel and RelB [9]. These combine with each other to form a range of heterodimers that have both overlapping and distinct functions. There is evidence that NF-κB1 is involved in the induction of Th2 responses in vivo and in Th2-associated protective and pathologic responses. Thus, NF-κB1-deficient mice show increased susceptibility to intestinal helminth infection and resistance to experimental allergic airway inflammation [10–14]. There is some uncertainty about the way this operates in these conditions. In responses of NF-κB1-deficient mice to alum-precipitated protein in vivo, IL-5 induction was severely reduced, while IL-4 levels were not compromised [13]. The cellular basis for this requires study for, at least in part, NF-κB1 may act through non-T cells in these responses. Thus, NF-κB1 in DCs is needed for optimal $CD4^+$ Th2 cell differentiation [14]. For these reasons this report specifically addresses the effect of NF-κB1 deficiency in transgenic OVA-specific naïve $CD4^+$ T cells (OTII cells) in responses to alumOVA in vivo. It shows there are distinct NF-κB1-dependent and NF-κB1-independent pathways to Th2 cytokine induction and describes selective roles for NF-κB1 on Th2 and TFh differentiation.

Results

Th2-cytokine induction is impaired in NF-κB1$^{-/-}$ OTII cell responses to alumOVA

First, we assessed the effect of NF-κB1 deficiency on Th2 and TFh cell induction by alumOVA. Naïve NF-κB1$^{+/+}$ OTII cells or NF-κB1$^{-/-}$ OTII cells were labeled with the vital fluorescent dye CFSE and transferred into groups of WT congenic C57BL/6 mice. NF-κB1 deficiency did not affect the survival of OTII cells over 4 days in non-immunized chimeras (Fig. 1A). Immunization 24 h after cell transfer with alumOVA in both rear footpads induced proliferation in both NF-κB1$^{+/+}$ and NF-κB1$^{-/-}$ OTII cells in the draining popliteal LN. This is shown by CFSE dilution in Fig. 1A and B. However, NF-κB1$^{-/-}$ OTII cells divided less frequently and were outnumbered 14-fold by NF-κB1$^{+/+}$OTII cells after 3 days. This is in line with the proposed roles for NF-κB1 both in proliferation and survival of T lymphocytes responding to antigen [15–17].

The Th2 cytokine response was evaluated 3 days after immunization. By this stage the NF-κB1$^{+/+}$ OTII cells' median mRNA level for IL-4 was 300 times and IL-13 was 170 times those of the largely non-responding endogenous $CD4^+$ T cells (Fig. 1C). By contrast, NF-κB1$^{-/-}$ OTII cells expressed a median of only 20 times the IL-4 mRNA of endogenous $CD4^+$ T cells and failed to induce IL-13 mRNA. We confirmed that IL-5 mRNA is not induced in $CD4^+$ T cells responding to alumOVA in LNs [18, 19]. The failure of NF-κB1−/− OTII to upregulate Th2 cytokines was selective as IL-2 mRNA (Fig. 1C), as well as the Th1-features — IFN-γ and T-bet – (Supporting Information Fig. 1A) were still induced at similar levels in both NF-κB1$^{+/+}$ and NF-κB1$^{-/-}$ OTII cells.

The role of NF-κB1 in TFh development was first assessed by quantifying mRNA for IL-21 and the receptor CXCR5. CXCR5 directs recirculating B cells and TFh cells to follicles by recognizing the chemokine CXCL13 secreted by follicular DCs and stromal cells [20, 21]. The median level of IL-21 mRNA in NF-κB1$^{-/-}$ OTII cells was only 2.5-fold below that in NF-κB1$^{+/+}$ OTII cells but 60 times that of largely non-responding endogenous $CD4^+$ T cells (Fig. 1C). By contrast, while CXCR5 mRNA was strongly induced in NF-κB1$^{+/+}$ OTII cells it was not significantly induced in NF-κB1$^{-/-}$ OTII cells (Fig. 1C). The changes in other mRNAs associated with TFh formation are not impaired — including upregulation of CXCR4 and OX40 as well as downregulation of CCR7 (Supporting Information Fig. 1A). There is also a defect in upregulating CXCR5 protein in NF-κB1$^{-/-}$ OTII cells, at 3 days post immunization, when responding OTII cells first colonize follicles [22] (Supporting Information Fig. 1B). By contrast, the upregulation of PD-1, another TFh marker [21], is not affected by this deficiency. Thus, we confirm that during in vivo responses to alumOVA, NF-κB1$^{-/-}$ OTII cells have selectively impaired early Th2-differentiation into IL-4/IL-13-expressing cells, and show partial failure of TFh maturation by not upregulating CXCR5. Further analysis of the effect of NF-κB1 deficiency in $CD4^+$ T cells on TFh development and follicular antibody responses is presented later in the results.

NF-κB1 is redundant for IL-4-directed Th2 polarization in vitro

Having confirmed a key role for NF-κB1 in Th2-cytokine induction by alumOVA in vivo, we assessed whether $CD4^+$ T cells require endogenous NF-κB1 for IL-4-guided production of Th2-cytokines in vitro. Previous in vitro studies report that NF-κB1$^{-/-}$ $CD4^+$ T cells cultured under Th2 polarizing conditions or WT $CD4^+$ T cells with the NF-κB1 inhibitor — SN50 — have markedly impaired IL-13 and IL-5 production, with a smaller effect on IL-4 induction [11, 13]. We cultured FACS-sorted polyclonal CD4+ T cells from WT and NF-κB1−/− mice with plate-bound anti-CD3 and CD28 in the presence of IL-4, neutralizing anti-IFN-γ and anti-IL-12. After 5 days NF-κB1$^{-/-}$ $CD4^+$ T cells had been induced to produce IL-4, IL-13 and IL-5 proteins at similar levels to those of WT $CD4^+$ T cells (Fig. 2A and top graph). Equally, IL-4, IL-13, IL-5 and IL-21 mRNA were upregulated independently of NF-κB1 in this in vitro response (Fig. 2B).

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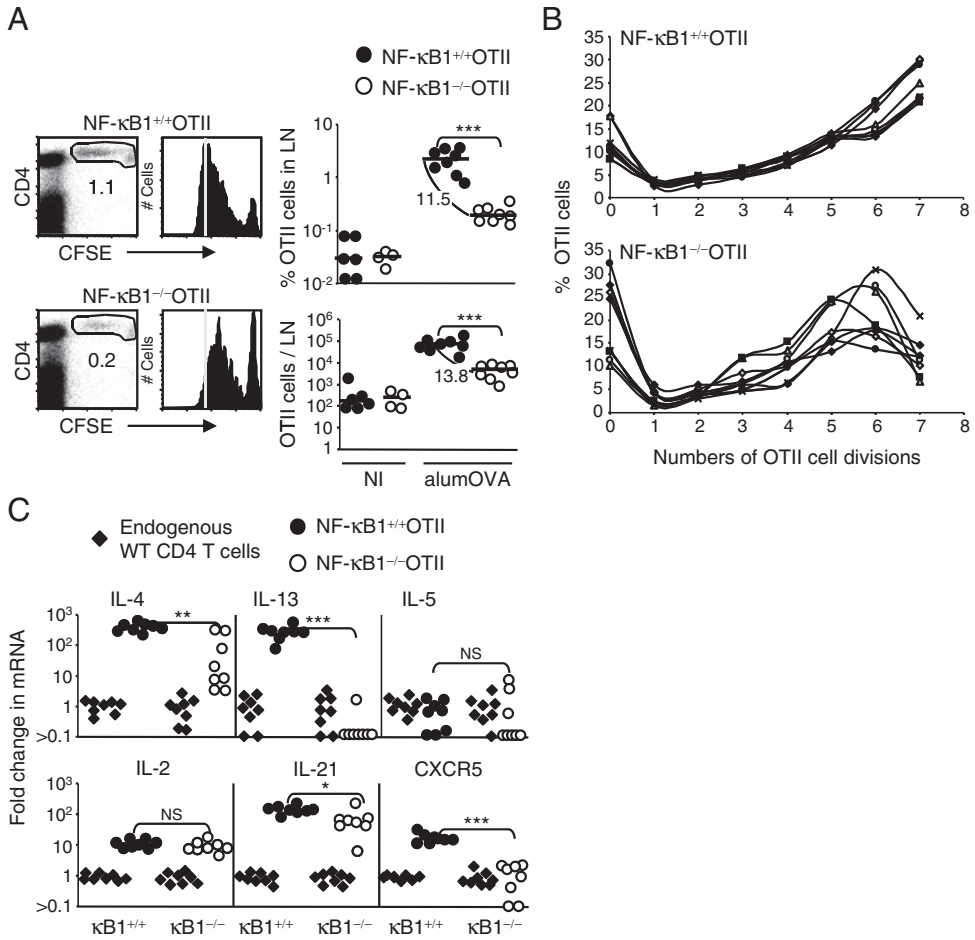

Figure 1. In vivo Th2 cytokine induction in CD4$^+$ T cells by alumOVA requires NF-κB1. (A) C57BL/6 mice received CFSE-labeled NF-κB1$^{+/+}$ OTII or NF-κB1$^{-/-}$ OTII cells and were then immunized with alumOVA in both footpads. Three days later, proliferation in the CFSE$^+$CD4$^+$ gated population of OTII cells from the draining popliteal LN was assessed by CFSE dilution. Non-immunized (NI) chimeric mice were used as controls. The percentages of draining LN cells, which are OTII cells (top graph), and the numbers of OTII cells in the LN (bottom graph) are shown for NF-κB1$^{+/+}$ chimeras (black circles) and NF-κB1$^{-/-}$ chimeras (open circles). Horizontal bars indicate median values. The numbers in the graphs indicate the fold differences between two immunized groups. (B) Graphs show the percentages of NF-κB1$^{+/+}$ OTII (upper graph) or NF-κB1$^{-/-}$ OTII (lower graph) cells as a function of the numbers of divisions accomplished. The results are from two independent experiments. Each line represents the results from one mouse. (C) In vivo-primed NF-κB1$^{+/+}$ OTII (black circles) or NF-κB1$^{-/-}$ OTII (open circles) cells in the CFSE$^+$CD4 T-cell gate (shown in A) were FACS-sorted. Largely non-activated endogenous CFSE$^-$CD4 cells served as control (black diamonds). The relative cytokine or chemokine receptor mRNA levels were determined by real-time RT-PCR relative to β2-microglobulin mRNA. Each symbol represents sorted cells pooled from the two popliteal LNs of one mouse. The data were normalized by setting the median of the results obtained for endogenous WT CD4$^+$ T cells as 1. Data are derived from two independent experiments with a total of eight mice in each group. Mann–Whitney two-tailed statistical differences between bracketed groups are indicated; NS = non significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Why are our results on the effects of NF-κB1 deficiency in vitro at variance with those of Das et al. [11]? A possible explanation is the role of NF-κB1 in CD4 T-cell proliferation and/or survival after in vitro stimulation through CD3 and CD28 [16, 17]. Thus, while we performed intracellular FACS staining on day 5 to detect cytokine production, Das et al. harvested the cultured cells at day 5 and then restimulated these for a further 3 days before measuring cytokine secretion by ELISA on day 8. This longer culture period may have resulted in many cells undergoing apoptosis. We found no obvious loss in NF-κB1$^{-/-}$ CD4$^+$ T cells compared with WT CD4$^+$ T cells during in vitro differentiation over 5 days (Fig. 2A bottom graph).

Th2 transcription factor expression in NF-κB1$^{-/-}$ OTII cells responding to alumOVA

To probe the selective impairment of Th2 and TFh development in NF-κB1$^{-/-}$ OTII cells we studied the requirement of NF-κB1 for the early expression of Th2- and TFh-associated transcription factors. This was done in NF-κB1$^{+/+}$ OTII and NF-κB1$^{-/-}$ OTII cells 3 days after immunization with alumOVA. While GATA-3 is essential for the differentiation and maintenance of Th2 cells [23], c-Maf directly regulates IL-4 production [24]. In addition, there is evidence that Ikaros regulates Th2-cytokines as well as the expression of GATA-3 and c-Maf mRNA [25]. As expected, NF-κB1 mRNA is not induced in

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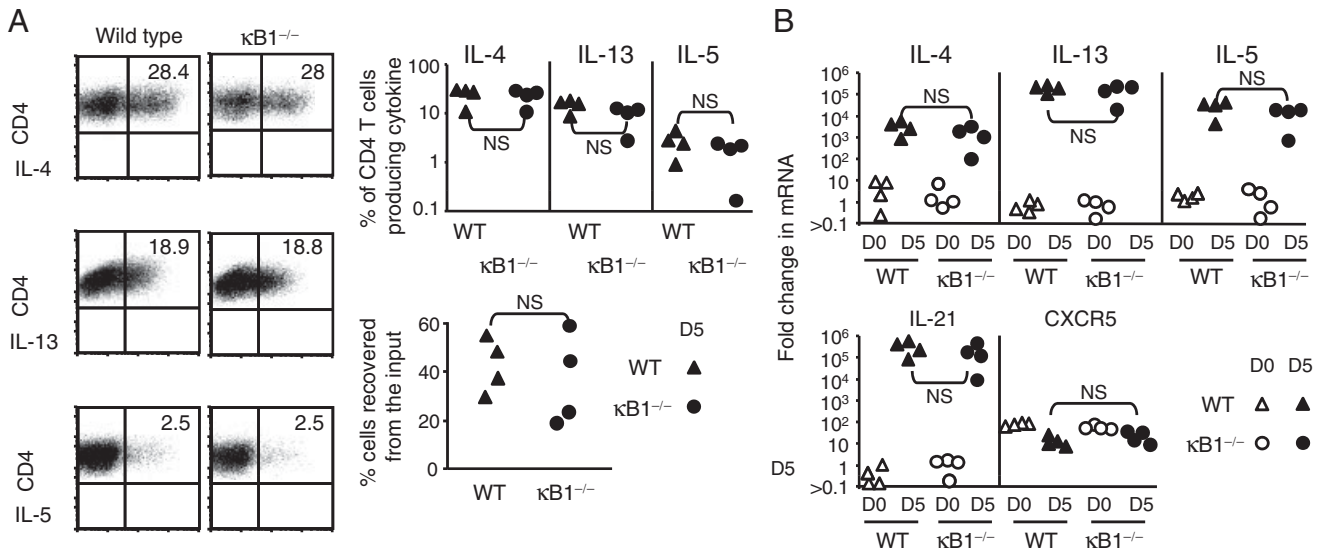

Figure 2. In vitro Th2 cytokine induction in CD4$^+$ T cells by IL-4-directed conditioning does not require NF-κB1. (A) FACS-sorted CD4$^+$ T cells from WT or NF-κB1$^{-/-}$ mice were cultured in vitro in Th2-skewing conditions. Dot plots show the proportions of cells induced to produce Th2-cytokines. The top graph shows a summary of the percentage of cytokine-producing CD4$^+$ T cells from WT (black triangles) or NF-κB1$^{-/-}$ (black circles) mice. The bottom graph shows the proportion of the starting number of WT CD4 cells (black triangles) or NF-κB1$^{-/-}$ CD4 cells (black circles) retrieved after 5 days of culture. (B) The graphs show expression of cytokine mRNA relative to β2-microglobulin mRNA after Th2 differentiation (black symbols D5) or freshly-isolated total LN cell suspensions (open symbols D0). Data in (A and B) are derived from two independent experiments with four donor mice in total. NS = non significant in Mann–Whitney two-tailed test between bracketed 5-day groups.

NF-κB1$^{-/-}$ OTII cells (Fig. 3A). There was a non-significant trend for GATA-3 to be lower, while c-Maf levels were two-fold lower ($p<0.05$) in the NF-κB1$^{-/-}$ OTII cells, but Ikaros mRNA induction was not obviously affected by the absence of NF-κB1.

The transcription factor BCL6 is required selectively for the generation of the TFh cells [26–28]. This distinguishes TFh from the other Th subsets — Th1, Th2 and Th17 — which respectively require transcriptional control from T-bet, GATA-3 and ROR-γT for their induction. BCL6 has been shown to repress the expression of many microRNAs predicted to control the TFh cell signature and in particular miR-17-92, which represses CXCR5 expression [26]. In striking contrast to the loss of CXCR5 upregulation (Fig. 1C), no major difference was seen in the levels of BCL6 mRNA induced in NF-κB1$^{+/+}$ compared with NF-κB1$^{-/-}$ OTII cells (Fig. 3A).

The transcription factor expression during Th2-cytokine induction in vivo differs from that in vitro

Transcription factor upregulation during the in vivo response to alumOVA described above (Fig. 3A) is strikingly more complex than that induced during the in vitro IL-4-directed Th2 cytokine induction (Fig. 3B). Only GATA-3 upregulation is comparable in the two responses, while the levels of NF-κB1, c-Maf, Ikaros and BCL6 are either not increased or fall during the in vitro response (Fig. 3B). This is in keeping with the present study showing that NF-κB1 is not needed for the in vitro induction of Th2 cytokines (Fig. 2). Finally, no difference was detected between WT and NF-κB1$^{-/-}$ CD4$^+$ T cells in the expression of transcription factors in the in vitro response (Fig. 3B).

NF-κB1 affects CXCR5 protein expression by TFh cells but not by follicular B cells

The finding that NF-κB1 is required for the upregulation of CXCR5 mRNA in OTII cells responding in vivo to alumOVA led us to test whether NF-κB1$^{-/-}$ OTII cells were impaired in their differentiation into TFh cells. To achieve this, CFSE-labeled CD45.2$^+$ NF-κB1$^{+/+}$ or NF-κB1$^{-/-}$ OTII cells were transferred into congenic CD45.1$^+$ WT recipients and the chimeras were immunized in both footpads with alumOVA. Seven days after immunization NF-κB1$^{+/+}$ or NF-κB1$^{-/-}$ OTII cells from the draining popliteal LN were analyzed for proliferation and phenotype. By this time multiple divisions in many OTII cells had caused loss of detectable CFSE content; consequently CD45 allotypes were used to discriminate between donor and host cells. Again both NF-κB1$^{+/+}$ and NF-κB1$^{-/-}$ OTII cells had divided although NF-κB1$^{-/-}$ OTII cells had consistently accomplished fewer rounds of division (Fig. 4A and B) and the median number of NF-κB1$^{+/+}$ OTII cells was 4.7 times that of NF-κB1$^{-/-}$ OTII cells (Fig. 4A).

While significant this difference is relatively modest compared to the marked reduction caused by NF-κB1 deficiency in the numbers of OTII cells acquiring TFh features. The median number of induced CXCR5$^+$PD-1$^+$ cells was 36 times greater for NF-κB1$^{+/+}$ OTII than for NF-κB1$^{-/-}$ OTII cells (Fig. 4C). Thus, the absence of NF-κB1 selectively impairs the capacity of CD4

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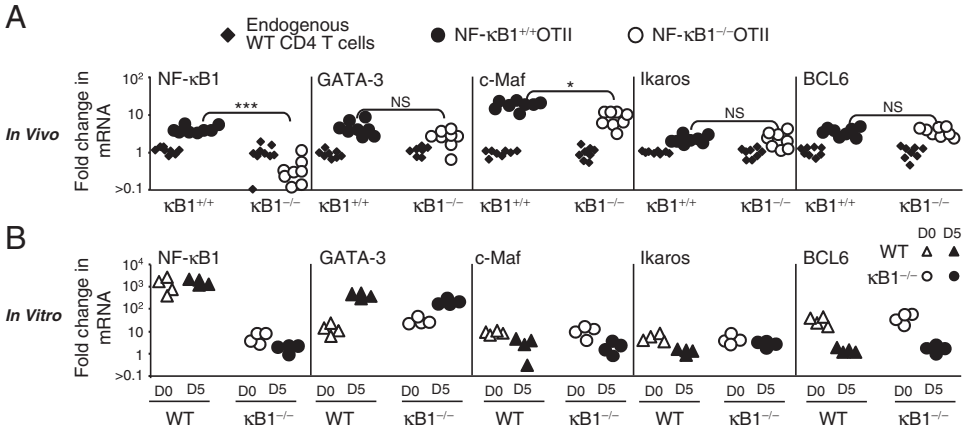

Figure 3. Th2 transcription factor expression in CD4$^+$ T cells after in vivo or in vitro Th2-differentiation. (A) The mice and LN suspensions are the same as those studied in Fig. 1. NF-κB1$^{+/+}$ OTII (black circles) or NF-κB1$^{-/-}$ OTII (open circles) cells in the CFSE$^+$ CD4 T-cell gate in Fig. 1A were FACS-sorted. Largely non-activated endogenous CFSE$^-$ CD4$^+$ T cells served as controls (black diamonds). The relative transcription factor mRNA levels were determined by real-time RT-PCR relative to β2-microglobulin mRNA. (B) The cell suspensions before and after Th2 polarization in vitro are the same as those studied in Fig. 2B. In vitro freshly isolated total LN cell suspensions served as control (open symbols D0). Mann–Whitney two-tailed statistical differences between bracketed groups are indicated; NS = non significant, *$p<0.05$, ***$p<0.001$.

T cells responding to alumOVA to differentiate into CXCR5$^+$ PD-1$^+$ TFh cells. This manifests as a functional defect identified by immunohistology on LNs of chimeras 7 days into a response to alumOVA. While in NF-κB1$^{+/+}$ OTII chimeras a median of 74% of CD3 T cells in secondary follicles were OTII cells in NF-κB1$^{-/-}$ OTII chimeras a median of 31% of CD3 T cells were OTII cells (Fig. 4D). Finally, we tested whether NF-κB1 deficiency causes reduced CXCR5 expression on follicular B cells. The data in Supporting Information Fig. 2 confirm that CXCR5 is equally expressed on WT and NF-κB1-deficient follicular B cells. This further emphasizes the selectivity of the effect of NF-κB1 deficiency on CXCR5 expression in TFh cells.

NF-κB1$^{-/-}$ OTII cells are partially defective in providing help to B cells in vivo

We next questioned the functional consequences of the T-cell-specific loss of NF-κB1 on their capacity to provide help to B cells in response to (4-hydroxy-3-nitrophenyl) acetyl conjugated to OVA (NP-OVA) as an alum precipitate (alum NP-OVA). This was assessed by quantifying the numbers of GC B cells and plasma cells at 9 days after immunizing OTII cell chimeras, a time when follicular and extrafollicular responses are fully established [3]. Endogenous OVA-specific CD4$^+$ T cells among normal polyclonal CD4$^+$ T cells can mask the direct effect of NF-κB1$^{-/-}$ OTII cells by inducing GC in response to alumOVA. Consequently, B-cell responses helped by NF-κB1$^{+/+}$ or NF-κB1$^{-/-}$ OTII cells were studied in TCRβδ-deficient mice, which do not have endogenous T cells.

First, the B220$^+$Fas$^+$GL7$^+$ GC B-cell response was assessed 9 days after immunization. This showed that NF-κB1$^{-/-}$ OTII cells had induced some B220$^+$Fas$^+$GL7$^+$ GC B cells, with a tenfold higher median percentage and 80-fold greater median absolute number of B220$^+$Fas$^+$GL7$^+$ GC B cells in the draining popliteal LN than in distant non-responding brachial LN (Fig. 5A left hand graphs). Nevertheless, median percentage of B220$^+$Fas$^+$GL7$^+$ GC B cells was a 20th and the median absolute number a 13th in TCRβδ-deficient chimeras created with NF-κB1$^{-/-}$ OTII cells compared with those receiving NF-κB1$^{+/+}$ OTII cells. Our previously published studies make it unlikely that the defect in the GC B-cell response 7 days after immunization depends on impaired IL-4-production by NF-κB1$^{-/-}$ OTII cells. These studies show that GCs are induced normally in mice that lack IL-4 signaling, although these GCs regress more quickly than in WT mice [2, 7]. Importantly, the differences in the percentages and numbers of GC B cells cannot be solely attributed to the percentages and total numbers of NF-κB1$^{+/+}$ or NF-κB1$^{-/-}$ OTII cells, for both cell types survived similarly in the TCRβδ-deficient recipient mice (Fig. 5B). Consistent with Fig. 4C, there are 30 times fewer CXCR5$^+$PD-1$^+$ TFh cells generated from NF-κB1$^{-/-}$ than NF-κB1$^{+/+}$ OTII cells (Fig. 5B). The effects of T-cell-specific loss of NF-κB1 on the B-cell response was selective to GC B cells since the numbers of CD138$^+$ antibody forming cells in the popliteal LN of chimeras created with NF-κB1$^{-/-}$ OTII cells were comparable to those in the NF-κB1$^{+/+}$ OTII cell chimeras (Fig. 5A right-hand graphs). This is consistent with a previous report that CXCR5-deficient T cells support early T–B cell collaboration and plasma cell formation but are impaired in supporting normal GC development [21]. In addition, we have shown that both NF-κB1$^{+/+}$ and NF-κB1$^{-/-}$ OTII cells induce class switch to IgG (Supporting Information Fig. 3A). In both the switching is largely to IgG1, which is typical for the response to alumOVA (Supporting Information Fig. 3B). Collectively these results indicate that NF-κB1 is important for the expression of CXCR5 on TFh cells primed in vivo, and the impairment of CXCR5 up-regulation selectively impairs the GC component of B-cell responses to alumOVA.

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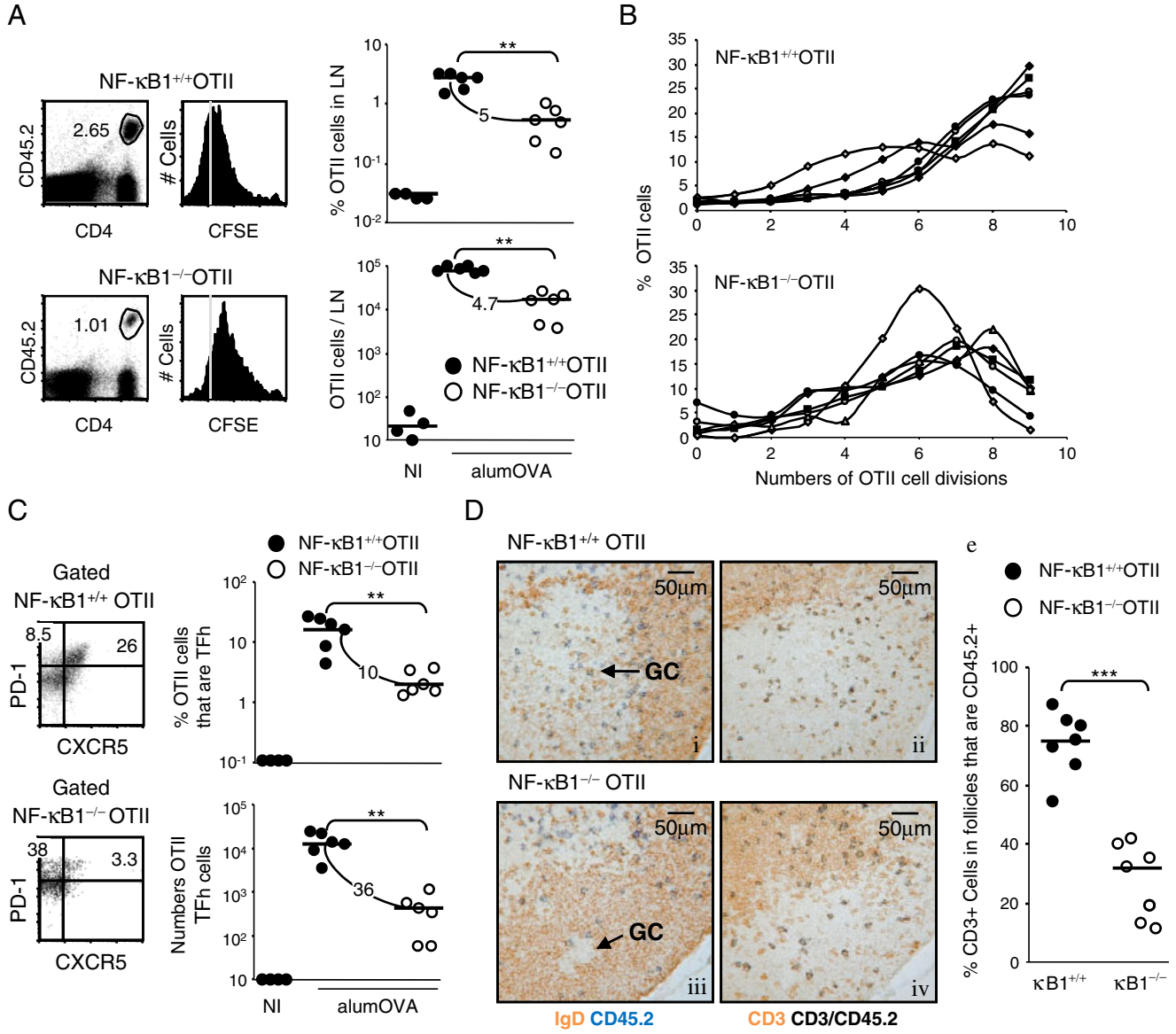

Figure 4. Selective defect in the ability of NF-κB1$^{-/-}$ OTII cells to differentiate into TFh cells in response to alumOVA. CD45.1^{+} congenic Boy/J mice received 4×10^{6} CFSE-labeled NF-κB1$^{+/+}$ OTII or NF-κB1$^{-/-}$ OTII cells. Groups of these chimeras were then immunized with alumOVA in both footpads, while a group of non-immunized (NI) NF-κB1$^{+/+}$ OTII cells chimeras served as controls. (A) Seven days after immunization the proportion and proliferation of NF-κB1$^{+/+}$ or NF-κB1$^{-/-}$ OTII cells was assessed in the draining popliteal LN, as CD4^{+}CD45.2^{+} cells. The percentages of draining LN cells that are OTII cells (top graph) and the numbers of OTII cells in the LN (bottom graph) are shown for NF-κB1$^{+/+}$ OTII cell chimeras (black circles) and NF-κB1$^{-/-}$ OTII cell chimeras (open circles). (B) Graphs show the percentages of NF-κB1$^{+/+}$ OTII (upper graph) or NF-κB1$^{-/-}$ OTII (lower graph) cells as a function of the numbers of divisions accomplished. Data are based on two independent experiments. Each line represents the results from one mouse. (C) The dot plots are gated on OTII cells and show the proportion of TFh cells assessed as CD4^{+}CD45.2^{+} CXCR5^{+}PD-1^{+} cells. The graphs show percentage of OTII cells that are TFh (top graph) or the number of TFh (bottom graph) in the draining LN of NF-κB1$^{+/+}$ OTII cell chimeras (black circles) or NF-κB1$^{-/-}$ OTII cell chimeras (open circles). The numbers in the graphs indicate the fold differences between two immunized groups. The data are representative of three independent experiments with a total of six mice in each group. (D) Photomicrographs of popliteal LN cells 7 days after alumOVA in the footpads. (i and ii) are adjacent sections of an NF-κB1$^{+/+}$ CD45.2^{+} OTII cell chimera. (iii and iv) are adjacent sections of an NF-κB1$^{-/-}$ CD45.2^{+} OTII cell chimera. In (i and iii) IgD is brown and CD45.2 blue, while in (ii and iv) CD3^{+}CD45.2^{-} cells are brown and CD3^{+}CD45.2^{+} (OTII) cells black. Magnification is indicated in each photomicrograph with a 50 μm scale bar. (v) Quantifies the proportion of CD3^{+} cells in follicles that were OTII cells. In (A, C and Dv) horizontal bars indicate median values. The data are representative of three independent experiments with a total of six mice for each group in (A and C) and seven mice in each group for (Dv). Mann–Whitney two-tailed statistical differences between bracketed groups are indicated; $^{**}p<0.01$, $^{***}p<0.001$.

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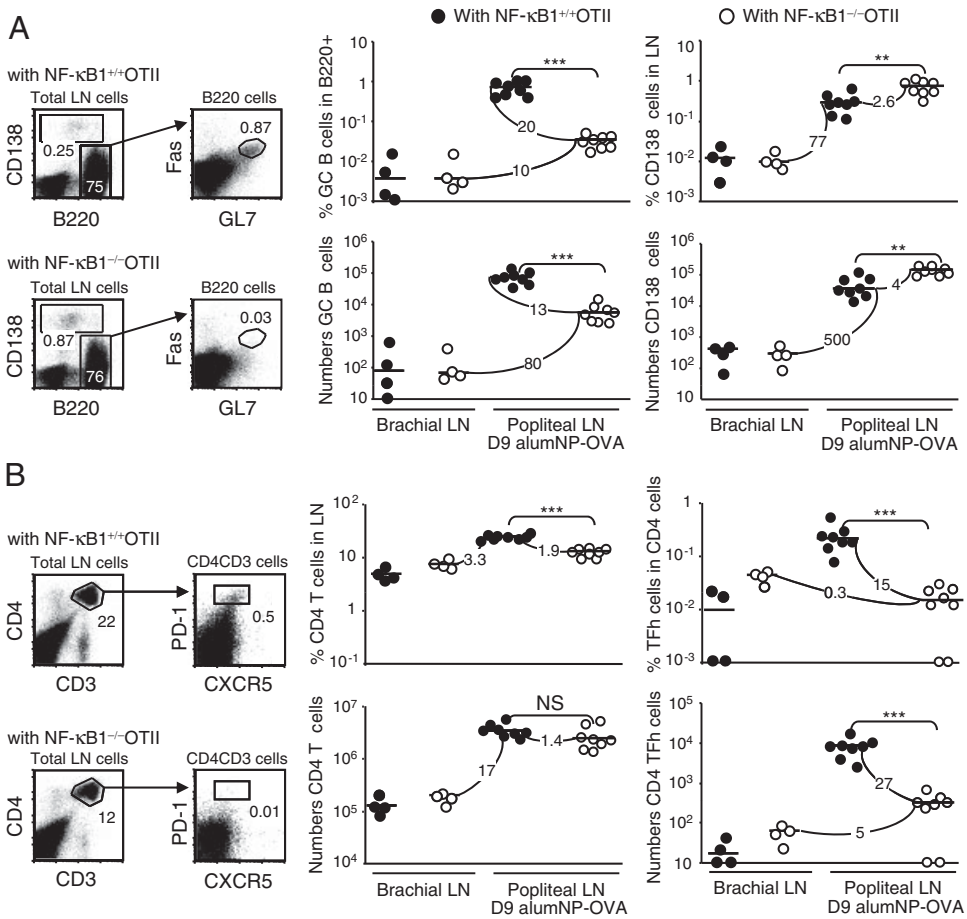

Figure 5. NF-κB1$^{-/-}$ OTII cells responding to alumOVA are associated with small numbers of GC B cells but normal numbers of plasma cells. TCRβδ-deficient congenic mice received 20×10^6 LN cells from NF-κB1$^{+/+}$ OTII or NF-κB1$^{-/-}$ OTII mice. Chimeras were immunized 1 day later with alum NP-OVA in both footpads. Nine days later, draining popliteal LN cell suspensions were stained to analyze T- and B-cell responses. (A) Analysis of B-cell response with CD138, B220, GL7 and Fas staining. Left hand side dot plots are of total LN suspensions while right hand side dot plots are gated on B220$^+$ LN cells. Graphs on the left show percentages (top) and numbers (bottom) of B220$^+$ cells that are GL7$^+$Fas$^+$. Graphs on the right show the percentages (top) and numbers (bottom) of LN cells that are CD138$^+$. Values for NF-κB1$^{+/+}$ OTII cell chimeras (black circles) and NF-κB1$^{-/-}$ OTII cell chimeras (open circles) are shown. The non-responding brachial LN of NF-κB1$^{+/+}$ OTII cell chimeras and NF-κB1$^{-/-}$ OTII cell chimeras were used as controls. (B) Analysis of T-cell response with CD4, CD3, PD-1 and CXCR5 staining. Left hand side dot plots are of total LN suspensions while right hand side dot plots are gated CD3$^+$CD4$^+$ LN cells. The left graphs show the percentages (top) and the number (bottom) of LN cells that are CD3$^+$CD4$^+$. The right graphs show the percentages (top) and the numbers (bottom) of CD3$^+$CD4$^+$ cells that are PD-1$^+$CXCR5$^+$. Controls from non-responding brachial nodes were used as in (A). The data are based on three independent experiments with four mice in total with both popliteal LNs analyzed separately and the two brachial LNs pooled. Horizontal bars indicate median values. The numbers in the graphs indicate the fold differences between two groups. Mann–Whitney two-tailed statistical differences between bracketed groups are indicated; NS = non significant, $^{**}p<0.01$, $^{***}p<0.001$.

Discussion

We show here that when CD4$^+$ T cells that lack NF-κB1 are primed with alum-precipitated protein in a NF-κB1-sufficient microenvironment they are impaired in the upregulation of the Th2-cytokines IL-4 and IL-13. In addition, their TFh cells have impaired CXCR5 expression resulting in significantly reduced GC responses. This indicates that there is a CD4 T-cell-intrinsic role for NF-κB1 in promoting the Th2/TFh differentiation program. The lack of CXCR5 is a selective defect, for upregulation of other TFh features – BCL6, IL-21, CXCR4, OX40 mRNA and PD-1 protein — in NF-κB1$^{-/-}$ OTII cells is not impaired.

Here, we observe defects in CXCR5 and IL-4 upregulation as early as 3 days after immunization. By this time some CD4$^+$ T cells have started to colonize the B-cell follicles, although GCs have not yet formed [22]. When during the first 3 days after immunization does signaling through NF-κB1 in CD4$^+$ T cells become important for TFh commitment? In T cells, NF-κB1 is activated by both TCR and CD28 engagement, through homodimerization or heterodimerization with RelA or cRel, and regulates IL-2 expression, cell cycle entry and survival of T cells [29, 30]. Our results show no difference in IL-2 mRNA upregulation in NF-κB1−/− compared with NF-κB1$^{+/+}$ OTII cells, although NF-κB1 upregulation starts when CD4$^+$ T cells first interact with DCs in the outer T cell zone. It has been shown that

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activation of NF-κB1 by OX40 contributes to CD4 T-cell expansion and survival [17]. We have previously shown that OX40 is upregulated on a proportion of OTII cells early in response to alumOVA, before they start to divide [19]. This is of interest, for several studies have reported that OX40 signaling also directs Th2 differentiation [31–35]. For instance, OX40 signaling increases GATA-3 and c-Maf in T cells cultured with DCs stimulated with thymic stromal lymphopoietin [36]. OX40 also plays a part in GC maintenance by triggering upregulation of CXCR5 [35, 37, 38]. It would be of interest to probe a possible role of NF-κB1 in the induction of CXCR5 by OX40.

Because each member of the NF-κB family, except for RelB, can form homodimers as well as heterodimers with one another, it is difficult to distinguish between a direct and indirect role for NF-κB1. Thus, NF-κB1(p50) [13] and c-Rel [12], which can combine to form heterodimers, are implicated in the pathogenesis of asthma. RelA(p65) also forms heterodimers with NF-κB1 and binds to the *Il-4* promoter [39]. The deficiency in Th2-cytokine induction in vivo might also reflect the lower levels of c-Maf mRNA found in primed NF-κB1$^{-/-}$ OTII cells.

During Th2 T-dependent antibody responses IL-4-producing CD4^{+} T cells first accumulate in the outer T zone close to the B-cell follicles and, later, to the GC [40–42]. Thus, CXCR5^{high}CCR7^{low} T cells have been found to express PD-1 strongly along with elevated IL-4 transcripts using the IL-4-GFP reporter mice [21]. This goes along with the observation that GCs are a major focus of IL-4 production during Th2 responses [40–42]. It is of particular interest that NF-κB1-deficient CD4^{+} T cells show a phenotype close to CXCR5-deficient CD4^{+} T cells [21]. Both cell types induce normal day 7 T-cell-mediated antibody responses but impaired GC responses, findings that correlate with reduced numbers of TFh cells reaching secondary B-cell follicles. Our results also show that both CXCR5 and IL-4 upregulation are impaired in NF-κB1−/− CD4^{+} T cells. It would be interesting to test if the reduced production of IL-4 is in part secondary to the defective expression of CXCR5 preventing primed T cells from reaching a specific microenvironment within GC that promotes IL-4 production by TFh cells.

Within the TFh cell population a GC-TFh subset has recently been identified based on the expression of CXCR5^{+}PD-1^{+}GL-7^{+} as the critical source of IL-4 in response to alum-precipitated proteins [43]. Surprisingly, this GC-TFh subset is not restricted to Th2 responses. LCMV infection induces a strongly Th1-biased response, but IL-4 expression still occurs within a population of GC CD4^{+} T cells expressing CXCR5^{+}PD-1^{+}GL-7^{+}. In addition, SLAM (Signaling Lymphocytic Activation Molecule, CD150), a surface receptor that uses SAP (SLAM-Associated Protein) signaling, is specifically required for IL-4 production by this GC TFh cell subset [43]. Intriguingly, SLAM/CD150 engagement increases nuclear NF-κB1 levels and IL-4 production in a SAP-dependent fashion [44]. Hence, NF-κB1 may also be a direct target of the CD150/SAP signaling pathway that leads to IL-4 production within GC. These studies collectively highlight the functional significance of NF-κB1 in CD4^{+} T cells during responses to alum-precipitated proteins.

Materials and methods

Mice

C57BL/6J mice were from HO Harlan OLAC (Bicester, UK). Congenic OTII mice, transgenic for αβTCR specific for 323–339 OVA-peptide in the context of H-2 I-A^{b} (Charles River, L'Arbresle, France), were crossed to NF-κB1$^{-/-}$ mice [45] that had been previously backcrossed on C57BL/6 for more than ten generations. TCRβδ-deficient mice and BoyJ (CD45.1 congenic background) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) and maintained in-house. All experiments with mice were conducted following local and Home Office regulations.

T-cell adoptive transfer and immunization

CD4^{+} T cells from LNs of NF-κB1$^{+/+}$ OTII or NF-κB1$^{-/-}$ OTII mice were purified using anti-CD4 MACS microbeads (Miltenyi Biotec, UK), labeled with CFSE (Cambridge Bioscience, UK); and 4×10^{6} cells were injected i.v. into WT congenic recipients. One day later these mice were immunized in both footpads with alumOVA or alumNP-OVA as in [3,18, 22].

Flow cytometry and FACS

Draining popliteal LNs were prepared as in [18, 22]. Antibodies against B220- PerCP-Cy5.5 (RA3-6B2), CD4-PerCP-Cy5.5 (RM4-5), CD45.2-PE (104), CD45.2-PE-Cy5.5 (104), CD138-PE (281-2), GL7-FITC (GL7), PD-1-PE (J43), biotinylated anti-Fas (Jo2), biotinylated anti-CXCR5 (2G8) and streptavidin-APCs were from PharMingen (BD Bioscience PharMingen, Oxford, UK) or e-Bioscience (Iceland, Ireland, UK). Intracellular FACS staining was performed using BD cytofix/cytoperm kit (Becton Dickinson, Oxford, UK). Anti-IL-4-APC (11B11), IL-5-APC (TRFK5), IL-13-PE (eBio13A) were from PharMingen. Goat biotinylated anti-mouse IgG (H + L) was from Southern Biotech. OTII cells were sorted by flow cytometry (MoFlo, Dako, UK), to purity >95%. Final analysis and graphical output used FlowJo software (Treestar, Costa Mesa, CA, USA).

In vitro Th2 polarization

CD4^{+} T cells, sorted by flow cytometry from WT and NF-κB1$^{-/-}$ mice, were incubated for 5 days in six microwells coated with anti-CD3 (145-2C11) at 5 μg/mL in complete RPMI medium containing anti-CD28 (37.51) (1 μg/mL), IL-4 (10 ng/mL), neutralizing anti-IL-12 (C17.8) (5 μg/mL) and anti-IFN-γ (X.MG1.2) (5 μg/mL). Restimulation was performed with coated anti-CD3 plus anti-CD28 for 5 h at 37°C. Cytokines were from PeproTech (PeproTech, London, UK) and antibodies from Insight Biotech (Insight Biotech, Wembley, UK).

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Immunohistochemical analysis

For in situ study of immune responses 5 μm cryostat sections were taken from snap-frozen LN for immunohistology as described in [3]. Sections were fixed in acetone at 4°C for 20 min and air dried. The staining to reveal IgD, CD3, IgG1, IgG2a have been described in [1, 2, 7]. OTII cells were revealed using CD45.2-biotinylated (104) (PharMingen) followed by streptavidinABComplex-alkaline phosphatase (Dako). Stained sections were mounted in Glycerol Gelatin (Sigma). The total numbers of $CD3^+CD45.2^-$ and $CD3^+CD45.2^+$ cells in the follicles in one section of each LN were enumerated at $\times 250$ magnification. The area of the follicle <63 μm, measured with an eyepiece graticule, from the outer edge of the $CD3^+$-cell-rich T cell zone was excluded from the counting. This prevents any T zone cells being counted.

Real-time semi-quantitative RT-PCR

Real-time semi-quantitative RT-PCR was performed as in [18, 19, 22]. TaqMan probes and primers were designed using Primer Express software (Applied Biosystems) and sequences are detailed in Supporting Information Table 1.

Statistical analysis

Mann–Whitney two-tailed non-parametric statistics were used; $p<0.05$ is considered significant and all the p-values are indicated on the figures.

Acknowledgements: This work was funded by a program grant from the MRC (ICMM), a Birmingham University Medical School scientific project committee grant and a publication data award (KS), a Wellcome Trust award (KS), and a BBSRC project grant (JHC).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviation: **alumOVA:** alum-precipitated OVA · **SAP:** SLAM-associated protein · **SLAM:** signaling lymphocytic activation molecule · **TFh:** follicular helper T

Full correspondence: Dr. Karine Serre, MRC Centre for Immune Regulation, IBR, School of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, UK
Fax: +44-1214143599
e-mail: karine@kserre.net

Current address: Elodie Mohr, Instituto Gulbenkian de Ciência, PT-2781-901 Oeiras, Portugal

Received: 3/10/2010
Revised: 11/2/2011
Accepted: 24/3/2011
Article accepted online: 6/4/2011

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European Journal of Immunology

Supporting Information

for

DOI 10.1002/eji.201041126

Selective effects of NF-κB1 deficiency in CD4^{+} T cells on Th2 and TFh induction by alum-precipitated protein vaccines

Karine Serre, Elodie Mohr, Cécile Bénézech, Roger Bird, Mahmood Khan, Jorge H. Caamaño, Adam F. Cunningham, and Ian C. M. MacLennan

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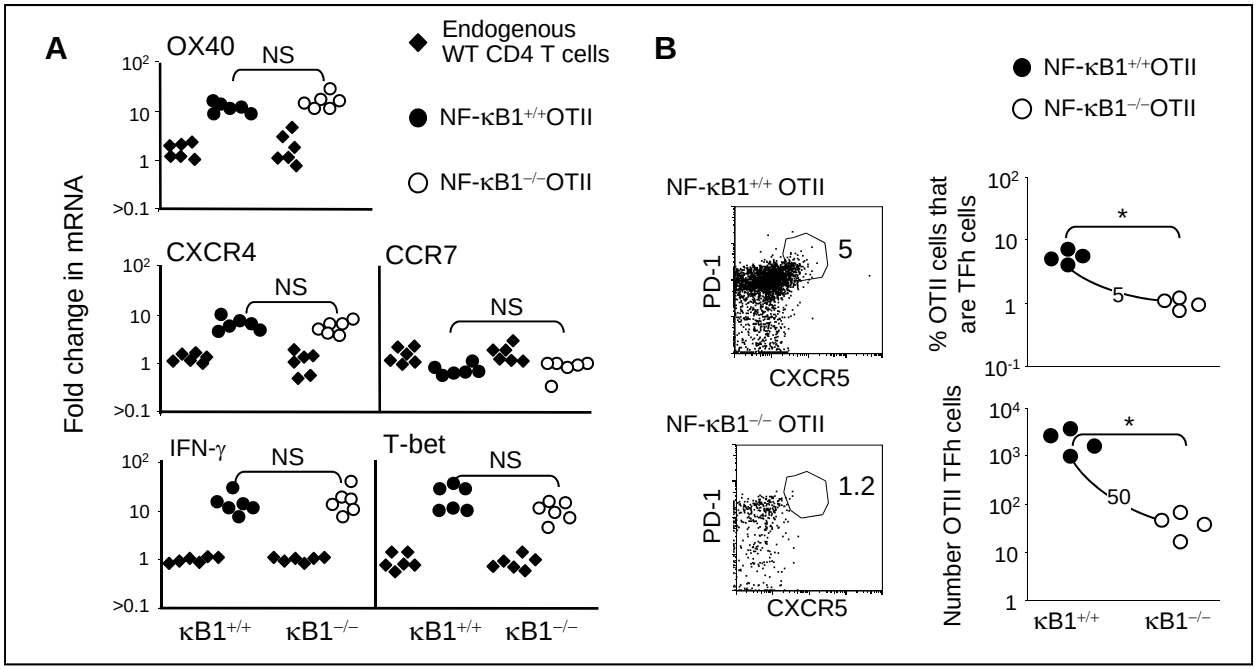
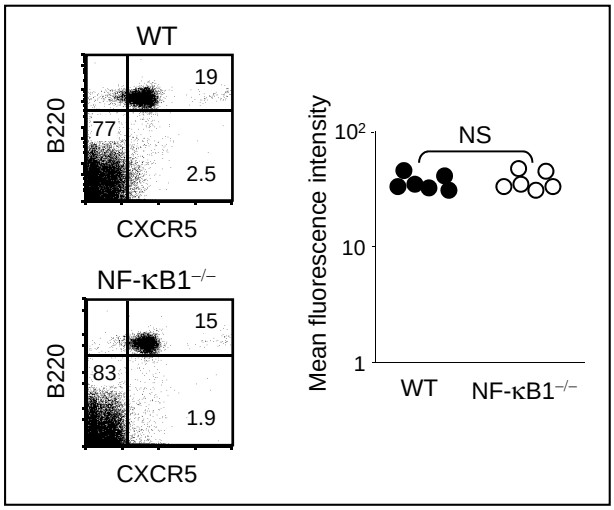


Supplemental Figure 1: Effects of NF-κB1 deficiency on OTII cells acquiring Th1, Th2 and TFh features in response to alumOVA *in vivo*

A) C57BL/6 mice received CFSE-labeled NF-κB1$^{+/+}$OTII cells or NF-κB1$^{-/-}$OTII cells and were then immunized with alumOVA in both footpads. Three days after immunization *in vivo*-primed NF-κB1$^{+/+}$ (black circles) or NF-κB1$^{-/-}$ (open circles) OTII cells in the CFSE^{+}CD4 T cell gate shown in Fig. 1A were FACS-sorted. Largely non-activated endogenous CFSE^{-}CD4 T cells from the same mice serve as control (black diamonds). The relative mRNA levels were determined by real time RT-PCR relative to β2-microglobulin mRNA. Each symbol represents sorted cells pooled from the two popliteal LN of one mouse. The data were normalized by setting the median of the results obtained for endogenous WT CD4 T cells as 1. Data are derived from 2 independent experiments with a total of 6 mice in each group. **B)** Total LN cell suspensions were harvested 72 hours after alumOVA and stained against CXCR5 and PD-1. CFSE^{+}CD4^{+}OTII cells were gated and the PD-1^{+}CXCR5^{+} TFh cells identified. The percent of OTII cells that were TFh cells and the number of TFh cells in the LN are shown in the graphs. As in (A) values for NF-κB1$^{+/+}$ OTII cells are shown in closed circles and those for NF-κB1$^{-/-}$ mice in open circles. The numbers in the graphs indicate the fold differences between 2 groups. Data are derived from 2 independent experiments with a total of 4 mice in each group. NS = non significant, * = $p<0.05$, using Mann-Whitney two-tailed statistics as indicated between bracketed groups.

It has been reported that the promoter of the *Cxcr5* gene contains NF-κB sites and that expression of CXCR5 is virtually absent in mice lacking both NF-κB1 and NF-κB2 [1]. By contrast, mice lacking NF-κB2 alone show regular levels of CXCR5, and mice with selective NF-κB1 deficiency were not assessed. We show here that this dependence on NF-κB1 for CXCR5 expression operates selectively in TFh cells, for brachial LN B220^{+} cells, from WT or NF-κB1$^{-/-}$ mice expressed similar levels of CXCR5. Most peripheral LN B cells from non-immunized mice are recirculating follicular B cells, which constitutively express CXCR5. (Supplemental Fig. 2).



Supplemental Figure 2: NF-κB1 deficiency does not affect the constitutive expression of CXCR5 on recirculating follicular B cells
Total cell suspension from brachial LN of non-immunized WT mice (black symbols) or NF-κB1$^{-/-}$ mice (open symbols) were stained for CXCR5 and B220 expression. Dot plots on the left show the levels of CXCR5 and B220 expression by cells from a single WT LN (top) and NF-κB1$^{-/-}$ LN (bottom). The graph shows mean fluorescence intensity of CXCR5 expression by the B220^{+} B cell population, where each symbol represents the value for a brachial LN from 1 mouse in two independent experiments. Mann-Whitney two-tailed statistical differences between bracketed groups are indicated; NS = non significant.

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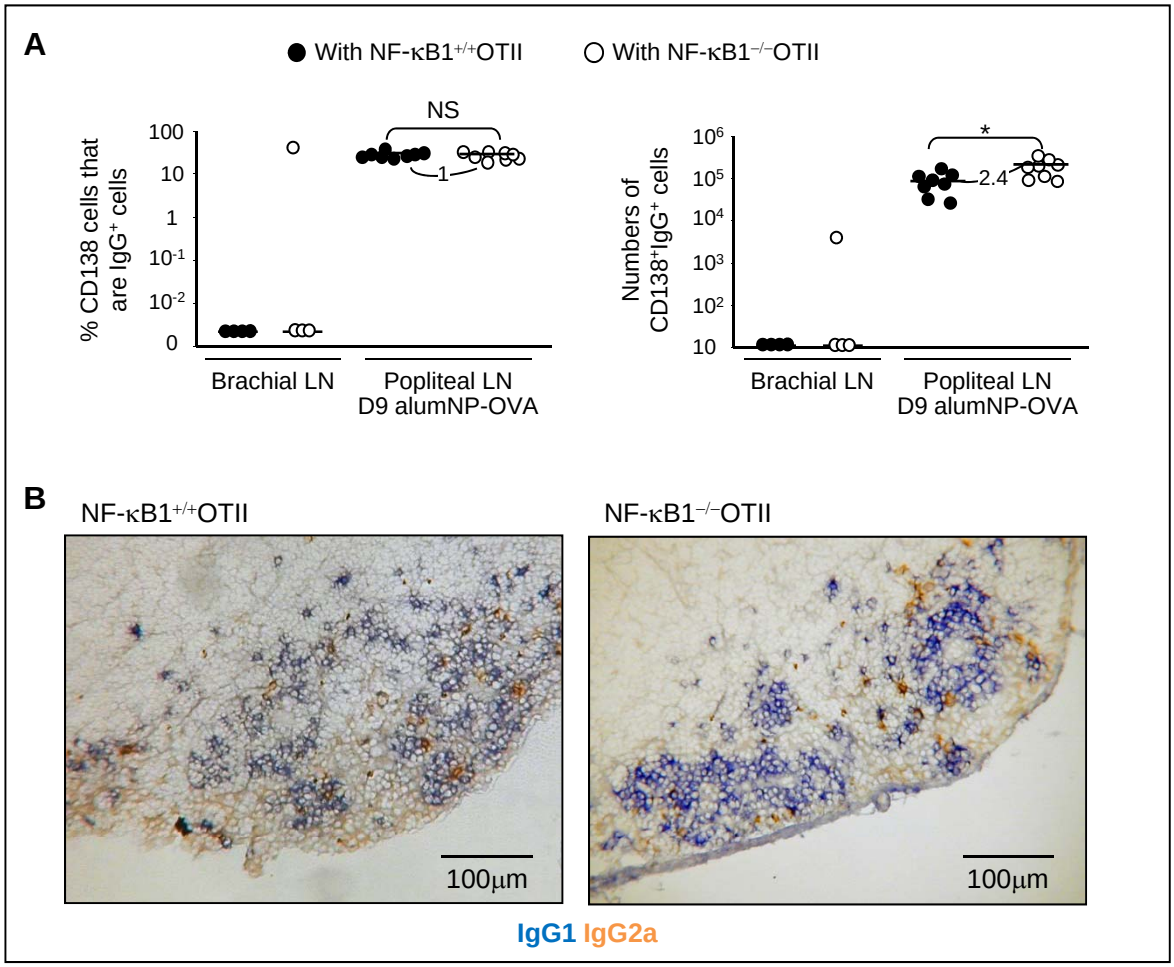

Supplemental Figure 3: NF-κB1$^{-/-}$ OTII cells responding to alumOVA induce IgG class switched plasma cells at comparable levels to those of NF-κB1$^{+/+}$ OTII cells

A) TCR βδ-deficient mice received 20 x 10^6 congenic LN cells from NF-κB1$^{+/+}$ OTII mice or NF-κB1$^{-/-}$ OTII mice. Chimeras were immunized one day later with alumNP-OVA in both footpads. Nine days later draining popliteal LN cell suspensions were stained for CD138, B220, and intracellular anti-IgG immunoglobulins. The mice and LN suspensions are similar as those studied in Fig. 5.

The graphs show percentages of CD138$^+$ cells that are IgG$^+$ (left graph) and the numbers CD138$^+$IgG$^+$ cells (right graph). The values for NF-κB1$^{+/+}$OTII cell chimeras are shown as black circles and NF-κB1$^{-/-}$ OTII cell chimeras as open circles. CD138$^+$ cells from brachial LN, which do not drain the site of immunization and hence are non-responding, were used as controls. The data are based on 3 independent experiments with 4 mice in total with both popliteal LN analyzed separately. Cells from the two brachial LN of each mouse were pooled. Horizontal bars indicate median values. The numbers in the graphs indicate the fold differences between the medians of the 2 immunized groups. Mann-Whitney two-tailed statistical differences between bracketed groups are indicated; NS = non significant, * = $p<0.05$. **B)** CD45.1$^+$ congenic Boy/J mice received 4 x 10^6 CFSE-labeled NF-κB1$^{+/+}$ OTII cells or NF-κB1$^{-/-}$ OTII cells. These chimeras were then immunized with alumOVA in both footpads. Photomicrographs show sections of popliteal LN from NF-κB1$^{+/+}$ OTII cell chimeras (left) and NF-κB1$^{-/-}$ OTII cell chimeras (right) taken 7 days after immunization. IgG1$^+$ plasma cells are stained blue while IgG2a$^+$ plasma cells are brown. The LN are similar as those studied in Fig. 4. Pictures are representative of 3 independent experiments with 7 mice in total.

	Primer Forward	Primer Reverse	Probe
β2 micro -globulin	CTGCAGAGTTAAGCATGCCAGTAT	ATCACATGTCTCGATCCCAGTAGA	CGAGCCCAAGACC
BCL6	CAGACGCACAGTGACAAACCA	ACTGCGCTCCACAAATGTTACA	CAGCCACAAGACTGTCCACACGGGT
c-Maf	ACCTCGGTCTTGCACTTTGC	TGCCTGGCTCTTATGGTTACTATTATT	CTCCGGATCCTTCGCGTGCGT
CCR7	TGGACCCAGGGAAACCCAGGA	CGAGCAGGCCCACGAAGCAG	Revealed using SYBR green
CXCR4	ATGGAACCGATCAGTGTGAGTA	TTGCCGACTATGCCAGTCAAG	Revealed using SYBR green
CXCR5	GCTCTGCACAAGATCAATTTCTACTG	CCGTGCAGGTGATGTGGAT	CCATCGTCCATGCTGTTCACGCC
GATA-3	CCACCCCATTACCACCTATCC	CACACACTCCCTGCCTTCTGT	TCGAGGCCCAAGGCACGATCC
IFN-γ	TCTTCTTGGATATCTGGAGGAACTG	GAGATAATCTGGCTCTGCAGGATT	TTCATGTCACCATCCTT
Ikaros	CGCCCCAGGATCATTCTTG	CTTGACCCTCATCGACATCCAT	CGGCGCACAAATCCACATAACCTGAA
IL-2	AGCAATATCAGAGTAACTGTTGTAAAACTAAAG	CCACCACAGTTGCTGACTCATC	TCTGACAACACATTTGAGTGCCAATTCGA
IL-4	GATCATCGGCATTTTGAACGA	AGGACGTTTGGCACATCCAT	TGCATGGCGTCCCTTCTCCTGTG
IL-5	AGAAATACATTGACCGCCAAAAAG	ACCAAGGAACTCTTGCAGGTAATC	CGTCCTCCGTCTCTCCTCGCCA
IL-13	TTGAGGAGCTGAGCAACATCAC	GCGGCCAGGTCCACACT	CAAGACCAGACTCCCCTGTGCAACG
IL-21	ACACCCAAAGAATTCCTAGAAAGACTAA	TGCATTCGTGAGCGTCTATAGTG	AGCATCTCTCCTAGAACACATAGGACCCGAAGAT
NF-κB1	ACATGGTGGTTGGCTTTGC	CCCTAATACACGCCTCTGTCATC	AACCTGGGAATACTTCA
OX40	GGGCAGGGAACACAGTCAAC	CAGAATTGCACACCTACTCAG	Revealed using SYBR green
T-bet	ATGCCAGGGAACCGCTTATA	AACTTCCTGGCGCATCCA	CCCAGACTCCCCCAACACCGGA

Supporting Information Table 1: Sequences for the primers and probes used in this study as indicated in the materials and methods in the section real-time semi-quantitative RT-PCR.