Pinpointing IL-4-independent acquisition and IL-4-influenced maintenance of Th2 activity by CD4 T cells

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Naive CD4 T cells develop Th2 activity early in primary responses to alum-precipitated proteins by producing IL-4 mRNA and inducing B cells to produce γ1 and ε switch transcripts. Both IL-4-dependent and IL-4-independent pathways for IL-4 induction are recognized, but their relative contribution to the different phases of primary Th2 responses *in vivo* is uncertain. We show the primary induction of IL-4 synthesis in lymph nodes responding to alum-precipitated protein is overwhelmingly in antigen-specific CD4 T cells and is unimpaired in IL-4R$\alpha^{-/-}$ mice, which can produce but do not respond to IL-4 and IL-13. Ig class-switching in extra-follicular responses, reflecting Th2 activity, is also unimpaired in these mice. By contrast, 7 days after immunization — when T cells are selecting B cells in germinal centers and T cell priming has occurred — non-responsiveness to IL-4 is associated with smaller germinal centers, increased levels of T-bet and γ2a switch transcripts and reduced γ1 and ε transcripts. These data indicate that Th2 characteristics acquired during T cell priming and the initial CD4 T cell interaction with B cells are largely IL-4-independent, whereas IL-4 production induced during priming has a significant role in maintaining the Th2 phenotype as T cells select B cells in germinal centers.

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1 Introduction

It is firmly established that *in vitro* IL-4 directs mouse CD4 T cells, activated through their TCR, to acquire the Th2 characteristics of secreting IL-4 and inducing B cells to switch to produce IgG1 and IgE [1–3]. This differentiation is achieved by signaling through the IL-4R, STAT6 and GATA-3 [4, 5]. At the same time IL-4 suppresses the development of Th1 characteristics, including the expression of IFN-γ and the IL-12R [6]. It also inhibits B cells switching to γ2a under the influence of Gram-negative bacterial LPS or CD40 ligation, while it enhances switching to IgG1 and IgE [7, 8].

Despite these data pointing to a key role of IL-4 in the induction of Th2 responses, studies of mice deficient in IL-4 and IL-13, in IL-4Rα or in STAT6 all indicate that Th2 activities can also develop in the absence of either IL-4 or IL-13 signaling [9–17]. Mice double-deficient in IL-4 and IL-13 mount an extra-follicular LN antibody response to Th2 antigens. They produce very similar numbers of IgG1-secreting plasma cells in the medullary cords as congenic wild-type mice do [18]. Nevertheless the poor IgE and to a lesser extent IgG1 levels seen in late primary and secondary antibody responses of IL-4-deficient mice indicate a positive role for IL-4 in the response *in vivo* [19, 20]. The main object of the studies reported here is to assess the relative importance of the IL-4-dependent and IL-4-independent mechanisms in the primary induction of Th2 activity. This should facilitate subsequent studies to unravel the as yet obscure signaling pathways responsible for inducing Th2 activity in the absence of IL-4 or IL-13.

Responses in popliteal LN to alum-precipitated protein are used in these studies. These LN give a strong selective development of Th2 activity with consistent follicular and extra-follicular antibody responses [18, 21, 22].

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Abbreviations: CFSE: Carboxyfluorescein diacetate succinimidyl ester **CGG:** Chicken gamma globulin **NP:** (4-hydroxy-3-nitrophenyl)-acetyl

Quantification of cellular and molecular changes in this model is assisted by low background levels of antibody production in these LN in specific pathogen free mice. In addition background levels of mRNA for IL-4 and the Th1 inducer T-bet are low, as are levels of γ1 and ε switch transcripts [18]. The primary T cell and B cell interactions that induce extra-follicular antibody responses occur between 48 h after immunization and day 5, when antibody production results in clearance of free antigen that is available for uptake by B cells and dendritic cells. At this stage antigen becomes localized on follicular dendritic cells and is available for uptake by germinal center B cells and their selection by follicular CD4 T cells [23]. Plasmablast growth in the extra-follicular response draws to a close by day 8 and at that stage is no longer dependent on T cell signals [24]. By contrast, from around day 6 of the response B cell selection by cognate interaction with T cells occurs in germinal centers [25]. Without this selection by T cells, the B cell population in the germinal centers collapses by mass apoptosis around 5 days after the initial recruitment of the B cells into the follicular response [26].

This timing means that changes associated with CD4 T cell priming should be identifiable at day 3, whereas those associated with B cell selection by T cells in follicles will be apparent at day 7 into the response. The present study uses wild-type mice and congenic mice deficient in IL-4Rα [27] to study the IL-4-dependent and IL-4-independent processes in primary LN responses *in vivo*. IL-4Rα deficiency allows IL-4 production, but deprives B and T lymphocytes of the capacity to receive signals either from IL-4 or IL-13, for both operate through this receptor. By comparing the popliteal LN response at day 3 with that at day 7, the relative contribution of IL-4 to naive CD4 T cell priming, initial cognate interaction of primed T cells with B cells that have taken up antigen and CD4 T cell selection of germinal center B cells was assessed.

2 Results

2.1 A major IL-4 response is induced in antigen-specific CD4 T cells as they are primed to alum-precipitated OVA

Initial studies were carried out to determine whether the upregulation of IL-4 synthesis that occurs on the third day of primary immune responses to alum-precipitated proteins is associated with the responding CD4 T cells [21]. To test this we assessed the relative amounts of IL-4 mRNA produced in different cell populations in popliteal LN responses to alum-precipitated OVA plus killed *Bordetella pertussis* bacteria. Previous studies have shown that although *B. pertussis* induces a Th1 response against itself, it enhances — but does not deviate — the Th2 response to alum-precipitated protein given at the same time [21].

To increase the number of responsive naive CD4 T cells, transgenic OVA-peptide-specific CD4 T cells from OT-II mice [28] were transferred into congenic recipients. CD4 T cells were positively selected from non-immunized OT-II mouse LN cells, giving a yield of $>$90% purity. The sorted cells were labeled with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) to allow proliferation to be assessed by dilution of the dye. They were then transferred into congenic wild-type littermates and one day later the chimeras were immunized in the feet. By day 3, CFSE dilution showed the OT-II T cells had completed up to six rounds of division (Fig. 1A). The popliteal LN cells from the chimeras were then separated into three cell fractions by fluorescence-activated cell sorting: non-CD4 cells; host CD4 cells; and OT-II CD4 cells (Fig. 1A).

IL-4 expression is regulated at the level of transcription and as such detection of IL-4 message is likely to reflect the level of IL-4 protein produced [4]. In the cell fractions sorted at 3 days after immunization, the OVA-specific OT-II cells had a median IL-4 mRNA level around 200-fold higher than that of the non-CD4 population and nearly 16-fold higher than the median level in host CD4 T cells (Fig. 1B). At this time-point, the median levels of IL-4 message among endogenous CD4 T cells were approximately 12-fold higher than the non-CD4 cells, indicating a significant host T cell response to the OVA by this stage. The majority of IFN-γ mRNA in the LN 3 days after immunization was associated with non-CD4 cells (Fig. 1B). Both host and transferred CD4 T cells from the popliteal LN of non-immunized mice had very low levels of IL-4 mRNA. In addition, CFSE-labeled OT-II cells were not induced to proliferate in response to immunization with heat-killed *B. pertussis* and alum without OVA (data not shown).

2.2 Induction of IL-4 synthesis and CD4 T cell proliferation during priming are independent of IL-4 or IL-13 signaling

IL-4R$\alpha^{-/-}$ mice and congenic wild-type mice were used to assess the role of IL-4 and IL-13 signaling in the primary induction of IL-4 synthesis in naive CD4 T cells by alum-precipitated protein. The IL-4R$\alpha^{-/-}$ mice can produce both these cytokines but their cells are unable to respond to either IL-4 or IL-13 [14].

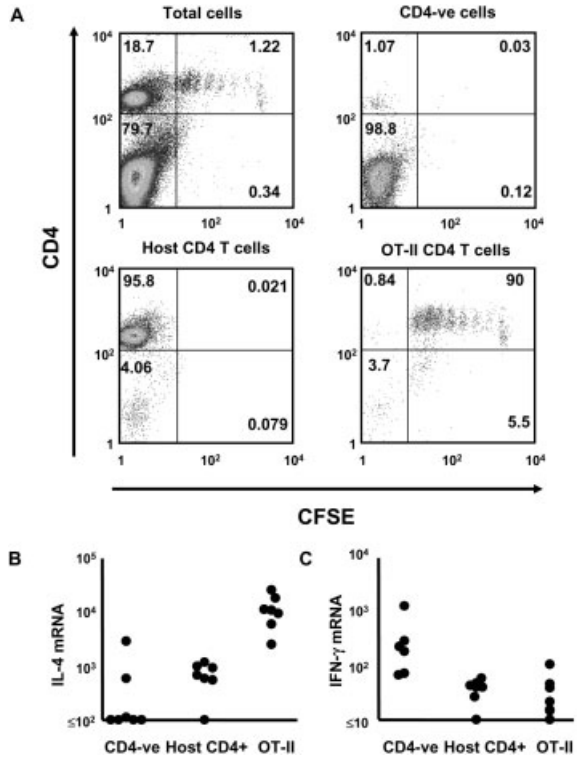

Fig. 1. Antigen-activated CD4 T cells are responsible for the IL-4 production induced during T cell priming to alum-precipitated OVA. The OVA-specific transgenic CD4 T cells (OT-II cells) were labeled with CFSE and transferred into congenic wild-type recipients. The following day the chimeric mice were immunized with alum-precipitated OVA plus killed *B. pertussis* and the response in the draining popliteal LN was assessed 3 days later. (A) Cells from the draining LN were analyzed by flow cytometry (top left panel) and the cells in the common small and large lymphocyte gate were sorted by flow cytometry into three fractions: $CD4^-$ cells (top right panel), host CD4 T cells, *i.e.* $CD4^+CFSE^-CD45.1^-$ cells (bottom left panel) and donor OT-II cells *i.e.* $CD4^+CFSE^+CD45.1^+$ cells (bottom right panel). (B) Levels of IL-4 mRNA (left) and IFN-γ mRNA (right) in the three fractions. Fewer than 0.1% of the donor CD4 T cells at 3 days post-immunization expressed NK1.1 (these data and the CD45.1 expression data are not shown).

Preliminary experiments were carried out to confirm that lymphocytes from IL-4Rα$^{-/-}$ mice do not respond to IL-4. The model selected to assess this was the switching to IgG1 that is induced by IL-4 in LPS-activated B cells. Splenocytes from IL-4Rα-deficient and congenic wild-type controls were cultured with LPS, with or without IL-4. The low-level γ1 switch transcript production induced by LPS alone is unaffected by loss of IL-4Rα activity. By contrast, the 50-fold augmentation of γ1 switch transcript levels that is induced by IL-4 is totally lost in splenocytes from the deficient mice (data not shown). In addition, IL-4 can induce suppression of the low-level γ2a switch transcript production that is induced by LPS; this suppression is also lost in the IL-4Rα-deficient mice.

After confirming the loss of responsiveness to IL-4 of the IL-4Rα-deficient mice, we used a different system in which we immunized groups of these mice and congenic wild-type controls in the foot with an alum-precipitated conjugate of (4-hydroxy-3-nitrophenyl)-acetyl (NP) and chicken gamma globulin (CGG) plus killed *B. pertussis.* The characteristic increase in size of the draining popliteal LN was unimpaired 3 days into the response in the IL-4Rα-deficient mice (Fig. 2A). By day 7 the responding LN in the deficient mice were significantly smaller than those of the wild-type mice ($p<0.05$). The association of this with reduced germinal center responses in the deficient mice is considered in section 2.3 (Fig. 3B). The levels of IL-4 mRNA induced in the responses of wild-type and IL-4Rα-deficient mice were similar in the two groups of mice both at day 3 and day 7 of the response (Fig. 2B). Thus neither IL-4 nor IL-13 had an obvious role in the primary induction of IL-4 production in this response.

The experiments depicted in Fig. 1B showed that among the LN leukocyte suspension, antigen-specific CD4 T cells were overwhelmingly responsible for the IL-4 response. In Fig. 2B, comparisons of the induction of IL-4 mRNA in wild-type and IL-4Rα-deficient mice were studied in LN sections, which might have included IL-4-producing stromal elements that might not have been isolated in the leukocyte suspensions studied in the experiments shown in Fig. 1. To test for this possibility, a further group of IL-4Rα-deficient mice were immunized as before with alum-precipitated NP–CGG plus killed *B. pertussis* in both feet. The IL-4 mRNA levels in leukocyte suspensions from the left popliteal LN were compared with those in homogenates of the right draining LN. This showed no significant difference between the levels of IL-4 mRNA in the leukocyte suspensions (mean units relative to β-actin 0.46; SE=0.09, n=6) and whole LN homogenates (mean 0.50; SE=0.11, n=6). It follows that the IL-4 induction in the IL-4Rα-deficient mice could not be attributed simply to a population of cells that was not recovered in the LN leukocyte preparation.

Finally the T cell proliferative response in the T zone at this stage was assessed and found to be similar in the wild-type and IL-4Rα-deficient mice (Fig. 2C).

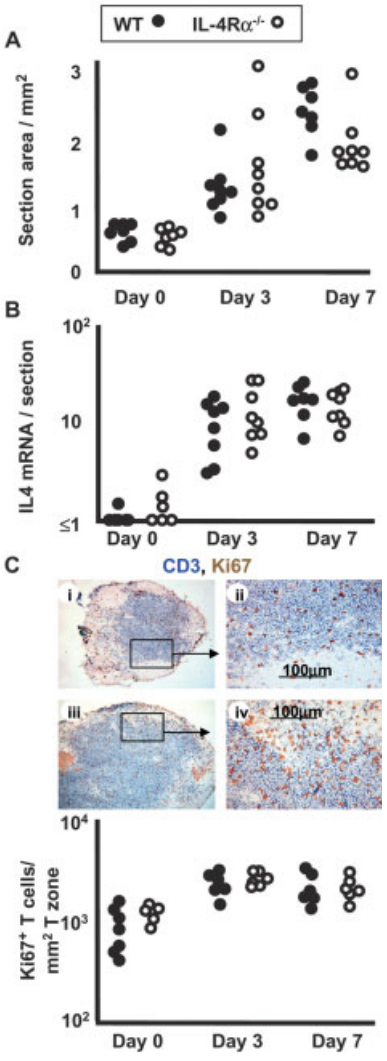

Fig. 2. IL-4Rα-deficiency impairs neither the induction of IL-4 synthesis nor T cell proliferation in the T zone. Responses were studied in popliteal LN draining the site of immunization with NP–CGG and heat-killed *B. pertussis* in wild-type mice ([WT] closed circles) and IL-4Rα$^{-/-}$ mice (open circles). (A) Shows the effect of IL-4Rα-deficiency on LN size as assessed by LN section area. (B) Shows the induction of IL-4 mRNA in the LN from the two groups of mice. (C) Shows quantification of T cell proliferation in the T zone as assessed by Ki67-expressing CD3 cells. Ki67 is a marker of proliferation that is expressed in all stages of the cell cycle, but is not expressed in cells in G0 [55]. The photomicrographs show LN sections from IL-4Rα-deficient mice before immunization (i) and 3 days after immunization (iii). High-power views of the boxed areas of (i) and (iii) are shown in (ii) and (iv). Ki67 staining of nuclei of cells in cell cycle is brown; T cells are stained blue on the basis of CD3 expression.

2.3 Absence of IL-4 or IL-13 signaling reduces the follicular antibody response but has little effect on the extra-follicular antibody response

We have previously reported that combined IL-4 and IL-13 deficiency has little effect on the size of the extra-follicular antibody response or switching in this response [18]. As expected, a similar result was achieved when these responses in IL-4Rα-deficient mice were compared to those of wild-type mice. The levels of γ1 and ε switch transcripts are marginally reduced at day 3 — when T cell interaction with B cells is occurring in the T zone (Fig. 3A). They are more affected by day 7 — when B cell switching is being induced in follicles during the selection of germinal center B cells, although there is still some induction of switch transcripts in the IL-4Rα-deficient mice at this time. The proportions of extra-follicular plasma cells producing IgG1 or IgG2a were similar in wild-type and IL-4Rα-deficient mice (Fig. 3B).

The smaller germinal center size in these mice (Fig. 3B) is likely to be associated with fewer B cells being selected by interaction with T cells, and this reduction in cell number will be responsible in part for the reduction in γ1 and

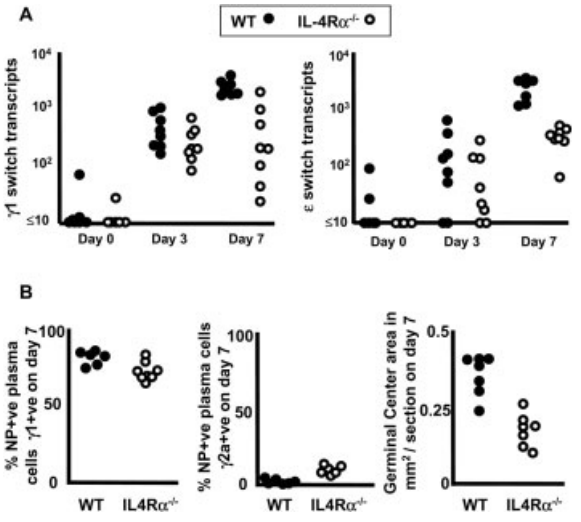

Fig. 3. Th2 activity of primed T cells that drive the extra-follicular response to alum-precipitated hapten–protein develops normally in IL-4Rα-deficient mice. This is shown by the day 3 levels of γ1 and ε switch transcripts (A) and the proportions of hapten-specific plasma cells that have switched to IgG1 or IgG2a that are seen in medullary cords on day 7 (B). However, γ1 and ε switch transcript levels are reduced at day 7 in IL-4Rα-deficient mice when CD4 T cell interactions with germinal center B cells are occurring in follicles. The reduced size of germinal centers at day 7 associated with IL-4Rα-deficiency is also shown in (B).

ε switch transcripts induced in the deficient mice. The effects of IL-4Rα deficiency on follicular and extrafollicular responses are similar to those of IL-4 plus IL-13 deficiency. This excludes a possible role for the intergenic region between the IL-4 and IL-13 genes, which was deleted in the double-cytokine-deficient mice. A potential role for these peri-cytokine regions has been reported [29].

2.4 Suppression of Th1 activity, which occurs in wild-type mice after IL-4 synthesis is induced, is impaired in IL-4α-deficient mice

By day 7 of the LN response to alum-precipitated NP-CGG plus *B. pertussis*, there is evidence of increased Th1 activity in the IL-4Rα-deficient mice as assessed by the expression of mRNA for the Th1 regulator T-bet [30] as well as the induction of γ2a switch transcript production (Fig. 4). By contrast, there were no clear differences in the production of IL-12 in the deficient mice. The effect on T-bet expression postdates the primary interaction between naive CD4 T cells and dendritic cells, for at day 3 of the response — when the effects of primary cognate interaction between these cells is seen — the induction of this indicator of Th1 activity is not enhanced in the IL-4Rα-deficient group of mice.

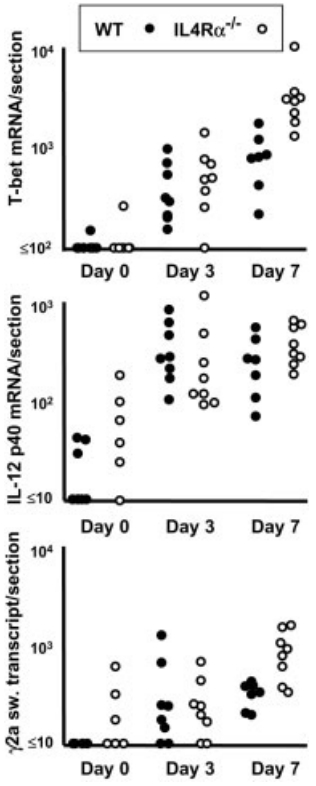

Fig. 4. Augmentation of Th1 activity in IL-4Rα-deficient mice that is seen at the time germinal center B cells are being selected by CD4 T cells. The amount of mRNA/section from wild-type mice (closed circles) and IL-4Rα$^{-/-}$ mice (open circles) are shown. A failure to recognize IL-4 is associated with augmented expression of Th1-directing transcription factor T-bet (top panel) by day 7 post-immunization. The trend to a slight increase in IL-12 p40 expression is not significant (central panel) but by day 7 there is a significant increase in the levels of γ2a switch transcript produced in the IL-4Rα$^{-/-}$ mice (bottom panel).

3 Discussion

There is intense interest, for therapeutic purposes, in identifying mechanisms that will modify the balance between the Th1 and Th2 pathways of differentiation. The main focus has been on the IFN-γ and IL-4 signaling pathways. These remain legitimate and important targets, but the present study shows that the induction of IL-4 synthesis is an event parallel to Th2 priming. Thus IL-4 is not primarily responsible either for Th2 induction, or for selective IL-4 production in LN responses to alum-precipitated protein. The role of IL-4 appears to be to confirm the Th2 phenotype, to promote expansion of Th2 cell numbers and to suppress the emergence of the Th1 phenotype. Consequently the mechanism of initial selective IL-4 induction dictates the pattern of the later primary and memory response, but not the initial direction of T cell help.

Classically, *in vitro*, strong IL-4 expression can be induced in freshly isolated naive T cells by TCR signaling in the presence of IL-4 [3, 31, 32]. It has been reported that during the first 48 h after T cell priming, T cells remain uncommitted to their final Th fate as they can synthesize both IL-4 and IFN-γ [31, 33]. *In vitro*, the cross-conversion of Th1 or Th2 clones to the other phenotype can be achieved for some 3 weeks after the clonal phenotype has emerged [34, 35]. The early production of IL-4 or IFN-γ can exhibit different kinetics and is closely linked to the cell cycle during Th differentiation [36–38]. By contrast, at day 3 in the present study OT-II cells showed selective production of IL-4 mRNA without up-regulating IFN-γ message in response to OVA.

It has been repeatedly proposed that polarization to the Th2 phenotype *in vivo* is attributable to exogenous IL-4. There are a number of sources for exogenous cytokine. In agreement with others [9–13, 39–41], we find that the primary induction of IL-4 is in antigen-specific CD4 T cells. NK1.1 cells have been proposed as an important source of IL-4 in this context [42]. IL-4 could be derived from T cells responding to other antigens, and mast cells could provide a source of IL-4 [43]. These sources of IL-4

are likely to be variable and this variability counters the finding that Th2 responses can be rapidly and consistently induced in the popliteal LN of mice bred and maintained in pathogen free conditions as well as in mice that have had a heavy antigenic load. Radbruch and colleagues [44] investigated the origin of the IL-4 generated in the response to NP–OVA. They showed that after transfer of wild-type CD4 cells into IL-4-deficient mice, normal or near-normal Th2 antibody responses were achieved, but the possibility that IL-4 was produced by previously-primed donor CD4 T cells was not excluded. The critical feature of the present study is that the primary induction of IL-4 synthesis in naive T cells *in vivo* requires exogenous sources of neither IL-4 nor IL-13 and that the levels of IL-4 mRNA produced in these mice are equivalent to those induced in wild-type mice.

In addition, autocrine potentiation of IL-4 or IL-13 production is not required, as IL-4Rα-deficient T cells do not respond to these cytokines. As IL-4 cannot be responsible for its own induction in these mice, there must be alternative mechanisms for the induction of the Th2 phenotype. Induction of IL-4 by IL-4 via STAT6 and GATA-3-dependent chromatin remodeling of the IL-4 locus is well documented [45–47]. GATA-3 appears to be crucial, as it can also induce the IL-4-promoting transcription factor c-maf [15]. IL-4 can be produced in primary responses in an IL-4- and STAT6-independent, but GATA-3-dependent manner [14–16]. Our data point to the primary direction of differentiation being dictated during the interaction of naive CD4 T cells and dendritic cells. The way the dendritic cell signals the selective induction of IL-4 synthesis is likely to reflect innate and other influences during the initial encounter of the dendritic cell with antigen. The finding of IL-4 induction without IL-4 signaling strongly supports an instructive model of T cell activation [35].

Several factors may play a role in the way Th1 or Th2 lineage decisions are made. These include the effects of antigen dose, TCR affinity/avidity and dendritic cell subset [48–53]. In this study these were not varied and their influence was not appraised. Although novel cell surface molecules may be involved in selective early IL-4 induction it could be that the initial selective response reflects variation in signaling through the molecules known to have a key role in CD4 T cell priming, for instance CD28 and TCR. The way CD86, CD80 and class II MHC molecules are brought to the surface following innate activation of the dendritic cell and the way in which these CD28/CTLA4 ligands associate or fail to associate with class-II–peptide complexes may differ between antigens that induce Th1 responses and those that evoke Th2 activity. Although the early Th lineage can be manipulated, for instance by ectopic expression of STAT6 [46], under normal circumstances the final lineage instruction is already dictated during priming. The next challenge in understanding Th1 and Th2 differentiation is at the level of the signals delivered to naive T cells by dendritic cells during priming.

4 Materials and methods

4.1 Mice and immunizations

Specific pathogen free mice were used throughout. IL-4Rα-deficient mice were on a BALB/c background [27] and OT-II mice with an $\alpha\beta$ TCR specific for OVA were on a C57BL/6 background [28]. Wild-type mice used were matched for age, sex and genetic background. OT-II mice were obtained from Charles River (Wilmington, MA, USA).

Alum-precipitated NP–CGG was prepared as described previously [22]. Mice were injected in hind footpads with 20 μg alum-precipitated NP–CGG, or NP–OVA plus 5×10^8 heat-killed *B. pertussis* (Evans Medical, Liverpool, GB).

4.2 Tissue preparation

After CO_2-mediated asphyxia of mice, popliteal LN draining the sites of immunization were removed. Frozen LN were sectioned as described previously [18, 21]. Five-μm sections were cut for immunohistology and 2×25-mm sections for mRNA extraction. Glass-mounted sections were air-dried for an hour, fixed in acetone (20 min, 4°C) and air dried.

4.3 Immunohistological reagents, staining and analysis

NP, IgG1 and IgG2a staining were detected as described previously [18, 21]. Primary rat anti-mouse-IgG1 or anti-IgG2a that had bound specifically to mouse Ig in sections were detected using horseradish peroxidase (HRP)-conjugated rabbit anti-rat-Ig (Dako, High Wycombe, GB). IgD was recognized using a polyclonal sheep anti-mouse-IgD (The Binding Site, Birmingham, GB), and the bound anti-IgD was detected with a polyclonal HRP-linked donkey anti-sheep Ig (The Binding Site). Ki67 was detected using rabbit anti-mouse-Ki67 (a gift from Johannes Gerdes, Borstel, Germany), then swine anti-rabbit-Ig (Dako) was attached to the bound anti-Ki67 and, to this, HRP linked to rabbit anti-HRP complexes (Dako) were bound. HRP labeling was detected using hydrogen peroxide substrate and 3,3' diaminobenzidine tetrahydrochloride solution [22]. NP-binding cells were detected using NP conjugated to sheep IgG fraction. Biotinylated donkey anti-goat-Ig antibodies (Dako), which bind sheep Ig, were used as a conjugate to detect bound NP–sheep-Ig. After washing, StreptABComplex linked to alkaline phosphatase (Dako) was added to sections with biotin-conjugated antibodies. CD3 was detected using rat anti-mouse-CD3 (Serotec, Oxford, GB), followed with bioti-

nylated rabbit anti-rat-Ig (Dako), and StreptABComplex linked to alkaline phosphatase (as above). Alkaline phosphatase activity was detected using naphthol AS-MX phosphate and fast-blue salt with levamisole [22]. The surface area of LN was determined using the point counting technique of Weible [54]. Germinal centers were identified as areas localized within follicles that were IgD^-.

4.4 *In vitro* stimulation of splenocytes

Splenocytes were obtained using a 70 mm nylon cell strainer (Becton Dickinson, Oxford, GB), and red cells were removed by brief exposure to 0.83% ammonium chloride. After washing, the leukocytes were suspended in RPMI-1640 containing 5% FCS, 50 μM 2-mercaptoethanol and antibiotics. Cells were then cultured in 6-well plates at 4×10^6 cells per well for 72 h. Splenocytes were stimulated with 2 μg/ml of LPS (Sigma, Poole, GB) alone, 2 μg/ml of LPS with 10 ng/ml of IL-4 (Peprotech, London, GB), or 2 μg/ml of LPS with 2 ng/ml IFN-γ (Peprotech).

4.5 Adoptive transfer and sorting of OT-II cells after immunization

T cells were purified from OT-II mice using anti-CD4 MACS microbeads (Miltenyi Biotec Ltd, Bisley, GB) and labeled with CFSE (Cambridge Bioscience, Cambridge, GB); 2×10^6 of these cells were injected i.v. and the mice were immunized the following day. After 3 days, popliteal LN were harvested and cell suspensions were incubated with collagenase type II (Lorne Laboratories Ltd, Reading, GB) and DNase (Sigma). Cells were labeled with PE–CD4 (Becton Dickinson). Labeled cells were analyzed and sorted by flow cytometry (FACSvantage Becton Dickinson) as follows: OT-II cells ($CD4^+CFSE^+$); endogenous $CD4^+$ cells in the $CD4^+CFSE^-$ fraction; and other endogenous cells in the $CD4^-CFSE^-$ fraction. Analysis was performed using FlowJo software (Treestar, Costa Mesa, CA, USA) and cDNA was generated from each fraction. NK1.1 cells were stained with biotin–DX5 antibody and streptavidin–allophycocyanin (Becton Dickinson).

4.6 Reverse transcription of mRNA and its relative quantitation by PCR

The cDNA was prepared as described previously [18]. RNA from LN sections and cell suspensions was extracted using RNAzol B (Biogenesis, Poole, GB). The RNA pellet was resuspended in 10 mM Tris / 0.1 M EDTA buffer (pH 8.0) containing 1 μg of oligo-$dT_{12\text{-}18}$ (Amersham Pharmacia Biotech, High Chalfont, GB) and denatured at 70°C for 10 min. RNA was reverse transcribed using MMLV reverse transcriptase (Invitrogen, Paisley, GB) at 42°C for 60 min. Relative quantitation of specific cDNA species to β-actin message was carried out in a multiplex PCR on the ABI 7700 (Applied Biosystems, Warrington, GB) as previously described [18].

Probes for cytokines and switch transcripts were detected via a 5' label with FAM (Applied Biosystems), whereas probes for β-actin were 5' labeled with VIC (Applied Biosystems). Sequences for β-actin, γ1 switch transcripts, γ2a switch transcripts, IL-4, IL-12 p40 and IFN-γ have all been published previously [18]. Sequences for T-bet were: forward CCAAAGGATTCCGGGAGAA, reverse CCCCCAAGCAGTTGACAGTT and probe CTCGTATCAACAGATGCGTACATGGACTCAAA. Reaction tubes contained Universal PCR Master Mix (Applied Biosystems), β-actin-specific primers and probe, test-gene-specific primers and probe, and cDNA template. Reaction conditions were the standard conditions for the TaqMan PCR with 60°C annealing temperature but with 45 PCR cycles.

Quantitative PCR works by relating the number of amplicons generated for a target gene to the number of amplicons generated for a reference gene. This is measured by the Taq-dependent digestion of a probe that anneals to the target amplicon between the 5' and 3' amplifying primers. The digestion of the probe leads to the release of a fluorescent dye from its quencher and this fluorescence is measured. Relative quantification of signal per cell was achieved by setting thresholds within the logarithmic phase of the PCR for β-actin and the test gene and determining the cycle number at which the threshold was reached (C_T). The C_T for the target gene was subtracted from the C_T for β-actin. The relative amount was calculated as $2^{\Delta CT}$. To obtain the mRNA per LN section, the relative amount was multiplied by the section area (in mm^2).

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