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IL-4 directs both CD4 and CD8 T cells to produce Th2 cytokines *in vitro*, but only CD4 T cells produce these cytokines in response to alum-precipitated protein *in vivo*

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ABSTRACT

While IL-4 directs CD4 T cells to produce Th2 cytokines (including IL-4, IL-13, IL-5) *in vitro* it has been shown that production of these cytokines can be induced *in vivo* in the absence of IL-4/IL-13/STAT-6 signaling. The present report shows that CD8 as well as CD4 T cells activated through their TCR, *in vitro* upregulate the Th2-features – IL-4, IL-13, IL-5, and GATA-3. However, *in vivo* while alum-precipitated antigen strongly and selectively induces these Th2-features in CD4 T cells, CD8 T cells mount a markedly different response to this antigen. This CD8 response is associated with strong proliferation and production of IFN-γ, but no Th2-features are induced. Alum-protein formulations are widely used in human vaccines and typically induce strong antibody responses characterized by the differentiation of IL-4-producing CD4 T cells and immunoglobulin class switching to IgG1. Nevertheless, the mechanism responsible for CD4 Th2 and follicular helper T cell commitment triggered by these alum-protein vaccines is still poorly understood. Analysis of the *in vivo* response to alum-precipitated protein shows that while subsets of CD4 T cells strongly upregulate Th2 and follicular helper T cell features including the surface markers OX40, CXCR5, PD-1, IL-17RB and the transcription factor c-Maf, CD8 T cells do not. These discrete differences between responding CD4 and CD8 T cells provide further insight into the differences between Th2 polarization of CD4 T cells directed by IL-4 *in vitro* and the induction of IL-4 production by CD4 T cells *in vivo* in response to alum-precipitated protein.

1. Introduction

CD4 T helper 2 (Th2) cells secrete IL-4, IL-5, IL-13 and orchestrate defence against extracellular pathogens. It is firmly established that *in vitro* IL-4 directs CD4 T cells that have been activated through their TCR to acquire Th2-features including the induction of IL-4 secretion. This *in vitro* model cannot explain fully Th2 differentiation *in vivo*, for several groups including our own have shown that the loss of IL-4 signaling at best has only a modest effect on the development of early Th2-features in response to various types of Th2 antigens including alum-precipitated proteins (Brewer et al., 1999, 1996; Cunningham et al., 2002, 2004b; van Panhuys et al., 2008). These studies show IL-4 independence by using mice deficient in IL-4 and/or IL-13, or IL-4Rα chain, or the transcription factor STAT-6. For example CD4 T cells in these mice upregulate IL-4 mRNA to equivalent levels as WT mice and still promote B cells selectively to produce γ1 and ε germline transcripts. This indicates that mechanisms, which differ from the well-explored pathway for *in vitro* induction of Th2-features, exist and contribute to Th2 differentiation *in vivo*. To date the molecular basis of the IL-4/STAT-6-independent pathway for Th2-cytokine induction has remained obscure.

Alum-precipitated protein antigen is a good model to study Th2 immune responses *in vivo*. It triggers the production of Th2 cytokines (IL-4, IL-13), Th2-associated transcription factors (GATA-3, c-Maf, NIP45), and switching to IgG1 and IgE (Brewer et al., 1999, 1996; Cunningham et al., 2002, 2004a; Serre et al., 2008). In addition alum-precipitated proteins induce strong germinal center reactions and with these induce follicular helper T (TFh) cells, another CD4 T cell subset that has been found to produce IL-4. Although insight into alum's mode of action is incomplete it has proven adjuvant efficacy in many vaccine formulations (Brewer, 2006; Lambrecht et al., 2009; McKee et al., 2007). Surprisingly its

Abbreviations: Ag, antigen; alumOVA, alum-precipitated ovalbumin; TFh cells, follicular helper CD4 T cells; LN, lymph node; OVA, ovalbumin; PD-1, programmed cell death gene-1; Th cells, Th cellsT helper cells.

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adjuvanticity and the induction of most of the Th2-features are Toll-like receptor (TLR)- and TLR signaling-independent (Eisenbarth et al., 2008; Gavin et al., 2006; Piggott et al., 2005; Sun et al., 2003). Alum-precipitated proteins can activate caspase-1 and induce proinflammatory cytokines such as IL-1β, IL-18 (Li et al., 2007) and IL-33 (Li et al., 2008). It has also been shown that some of alum's effects are mediated through the inflammasome NLRP3 (nucleotide-binding domain leucine-rich repeat containing family, pyrin domain containing 3) (Eisenbarth et al., 2008; Li et al., 2008), possibly in part via the induction of uric acid (Kool et al., 2008). Nevertheless, these mechanisms do not fully explain the way alum promotes strong Th2-directed antibody responses. This study aims to obtain insight into the diversification of CD4 and CD8 T cells in response to alum-precipitated protein-containing antigens.

Although our knowledge of the diversity in cytokine production by CD8 T cells lags behind CD4 T cells, they too have been reported to fall into two subpopulations based on cytokine secretion so that T cytotoxic type 1 (Tc1) CD8 T cells secrete IFN-γ, whereas Tc2 secrete IL-4 (Carter and Dutton, 1996; Cerwenka et al., 1998; Croft et al., 1994; Noble et al., 1995; Sad et al., 1995). There is evidence that Tc2 can be induced *in vitro* by TCR ligation in the presence of IL-4 (Croft et al., 1994; Noble et al., 1995; Sad et al., 1995), but we questioned whether Tc2 polarization would be achieved *in vivo* in response to primary immunization with alum-precipitated protein. By addressing this question we have obtained further insight into the way early Th2/TFh-features are acquired by CD4 T cells *in vivo* in response to alum-precipitated protein. The approach has been to compare the polarization of transgenic naïve ovalbumin-specific CD4 (OTII) and CD8 (OTI) T cells. This has been done both in their response to TCR ligation in the presence of IL-4 *in vitro*, as well as during their response to alum-precipitated ovalbumin (alumOVA) *in vivo*. There is remarkable qualitative homology between the responses of CD4 and CD8 T cells after *in vitro* polarization in the presence of IL-4. By contrast, *in vivo*, although both CD4 and CD8 OVA-specific T cells proliferate in response to alumOVA the acquisition of Th2/TFh-features, such as IL-4 production and, IL-13 and IL-21 mRNA upregulation, as well as CXCR5 and PD-1 cell surface expression, is exclusively confined to CD4 T cells. These results suggest that there is a correlation between the differentiation of Th2 and TFh CD4 cells. This paper provides further insight into the *in vivo* pathway for Th2/TFh cytokine induction by analyzing differences in these CD8 and CD4 T cell that respond to alum-precipitated OVA. Whether IL-4 production is a cause or a consequence of differentiation into TFh cells and depends on specific signals delivered by the follicular or germinal center microenvironment still remains to be elucidated.

2. Materials and methods

2.1. Mice

Wild-type C57BL/6J mice were from HO Harlan OLAC Ltd. (Bicester, UK). OTII mice are transgenic for αβTCR specific for 323-339 OVA-peptide in the context of H-2 I-A^b. OTI mice are transgenic for αβTCR specific for SIINFEKL OVA-peptide in the context of H-$2K^b$. Both OTI and OTII strains were from Charles River (L'Arbresle, France), and were crossed to CD45.1$^+$ C57BL/6J congenic mice (The Jackson Laboratory, Bar Harbor, Maine, USA). All animals were maintained under standard animal house conditions in accordance with local and UK Home Office regulations.

2.2. T cell purification and adoptive transfer

CD4 T cells from lymph node (LN) of OTII mice were purified using anti-CD4 MACS microbeads and CD8 T cells from LN of OTI mice were purified using anti-CD8 MACS microbeads (Miltenyi Biotec Ltd., Bisley, UK). No difference in OTI or OTII cell activation or proliferation has been observed when these were negatively purified (Serre et al., 2006) or positively selected with the MACS microbeads (Serre et al., 2009, 2008). OTI and OTII cells were labeled with CFSE (Cambridge Bioscience, Cambridge, UK) and were injected i.v. at 2×10^6 cells per congenic CD45.2$^+$ recipient mouse. In some experiments OTI and OTII cells were mixed at a ratio 1 to 2 before CFSE labeling and transfer into recipient mice. Mice were immunized the following day.

2.3. Antigen and immunization

Endotoxin-free OVA – EndoGrade Ovalbumin (Profos AG, Regensburg, Germany) – was mixed with 9% aluminum potassium sulfate (A7167 Sigma–Aldrich, Dorset, UK) solution then, after adjusting to pH7, the mix was left to precipitate in the dark for 30 min. Ten micrograms of OVA precipitated with alum in a final volume of 10 μl was injected subcutaneously into the plantar surface of both footpads.

2.4. Flow cytometry, T cell analysis and FACS-sorting

Cell suspensions were made from the two popliteal LN of individual mice and these were resuspended in FACS buffer for analysis (PBS, 5 mM EDTA, 0.5% FCS). No pools were made between mice and the results from each individual mouse are shown. Staining was performed at 4 °C for 30 min in FACS buffer. Anti-CD45.1-PE (A20), CD4-PerCP-Cy5.5 (RM4-5), CD8α-PerCP-Cy5.5 (53-6.7), biotinylated anti-CD69 (H1.2F3), CXCR5 (2G8), Vα2 (B20.1), OX40 (OX-86), PD-1 (J43) and streptavidin-APC were from PharMingen or e-Biosciences. Cell phenotype was assessed either on a FACScalibur (Becton Dickinson, Oxford, UK) or a Cyan (Dako, Ely, UK). CD45.1$^+$CD8$^+$ OTI and CD45.1$^+$CD4$^+$ OTII cells were sorted by flow cytometry (MoFlo, Dako, Ely, UK). The purity of MoFlo-sorted cells was routinely >90%. Final analysis and graphical output were performed using FlowJo software (Treestar, Costa Mesa, CA, USA).

2.5. In vitro T cell polarization

Total LN OTI cells or OTII cells were incubated at 5×10^6 cells/ml in 6 well plates with 1 μM free SIINFEKL (Alta Bioscience, University of Birmingham, UK) for CD8 T cell stimulation, or 323-339 OVA-peptide (Alta Bioscience) for CD4 T cell stimulation in complete medium. To induce Th2-cytokine production, IL-4 (10 ng/ml) and anti-IL-12 (C17.8) (5 μg/ml) plus anti-IFN-γ (X.MG1.2) (5 μg/ml) were included in cultures. Cells were incubated for 6 days. Restimulation prior to cytokine detection was performed by culturing the cells in anti-CD3 (145-2C11) coated (5 μg/ml) 6 well plates with soluble anti-CD28 (37.51) (1 μg/ml) for 5 h at 37 °C. Cytokines were from PeproTech (PeproTech, London, UK) and antibodies from Insight Biotech (Insight Biotech, Wembley, UK).

2.6. Ex vivo restimulation and intracellular cytokine staining

Day 7 after immunization popliteal LN cells were incubated at 10×10^6 cells/ml in 24 well plates with 10 μM free SIINFEKL or 323-339 OVA-peptide for 5 h prior to cytokine detection. Intracellular FACS staining was performed using cytofix/cytoperm kit (Becton Dickinson, Oxford, UK) according to manufacturer's instructions. Anti-IL-4-APC (11B11), IL-5-APC (TRFK5), IFN-γ-PE (X.MG1.2) and the isotype control (R3-34) conjugated either to phycoerythrin (PE) or allophycocyanin (APC) were from PharMingen (BD Bioscience PharMingen, Oxford, UK) or Insight Biotech (Insight Biotech, Wembley, UK).

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.

	Primer forward	Primer reverse	Probe
β2-microglobulin	CTGCAGAGTTAAGCATGCCAGTAT	ATCACATGTCTCGATCCCAGTAGA	CGAGCCCAAGACC
c-Maf	ACCTCGGTCTTGCACTTTGC	TGCCTGGCTCTTATGGTTACTATTATT	CTCCGGATCCTTCGCGTGCGT
CXCR5	GCTCTGCACAAGATCAATTTCTACTG	CCGTGCAGGTGATGTGGAT	CCATCGTCCATGCTGTTCACGCC
GATA-3	CCACCCCATTACCACCTATCC	CACACACTCCCTGCCTTCTGT	TCGAGGCCCAAGGCACGATCC
IL-4	GATCATCGGCATTTTGAACGA	AGGACGTTTGGCACATCCAT	TGCATGGCGTCCCTTCTCCTGTG
IL-5	AGAAATACATTGACCGCCAAAAAG	ACCAAGGAACTCTTGCAGGTAATC	CGTCCTCCGTCTCTCCTCGCCA
IL-13	TTGAGGAGCTGAGCAACATCAC	GCGGCCAGGTCCACACT	CAAGACCAGACTCCCCTGTGCAACG
IL-21	ACACCCAAAGAATTCCTAGAAAGACTAA	TGCATTCGTGAGCGTCTATAGTG	AGCATCTCTCCTAGAACACATAGGACCCGAAGAT
IL-25	AGGATGGCCCCCTCAACA	GGCATCGAGCGTGGTACAG	AGTCCCTGTCCAACTCATAGCTCCAAGGA
IL-25	Applied Biosystem TaqMan gene expression assay: Mm00499822_m1		
IL-17RB	AATATGAATGAGGACAGCCCTTCT	CAACCATCTTCTCGTTCTTTTTACAAG	CTCGCCAGGCTGCCTAAACCACGTAAT
OX40	GGGCAGGGAACACAGTCAAC	CAGAATTGCACACCTACTCAG	Revealed using SYBR green

2.7. Real time semi-quantitative RT-PCR

Cell suspension mRNA was prepared using RNeasy columns from Qiagen. Reverse transcription was performed with random oligonucleotides (Promega, Southampton, UK) using MMLV reverse transcriptase (Promega) for 1 h at 42 °C. When possible, relative quantification of target cDNA species to β2-microglobulin messenger was carried out in a duplex PCR (Applied Biosystems, Warrington, UK). As a prerequisite, the amplification efficiency was confirmed for each combination for the two sets of primers/probes. When not compatible, PCRs were run individually. Probes for target genes were detected via a 5′ label with FAM, while probe for β2-microglobulin was 5′ labeled with NED. TaqMan probes and primers were designed using Primer Express software (Applied Biosystems) and sequences are detailed in Table 1. Primers and probes were from Applied Biosystems or Eurogentec. The detection of OX40 messenger was carried out using iQ SYBR Green Supermix (Bio-Rad, Hemel Hempstead, UK). Standard reaction conditions for the TaqMan PCR were used on the ABI 7900. Relative quantification of signal was achieved by setting thresholds within the logarithmic phase of the PCR for β2-microglobulin, and the target 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 β2-microglobulin. The relative amount was calculated as $2^{-\Delta C_T}$.

2.8. Statistics

Statistical analysis was performed using a two-tailed non-parametric Mann–Whitney test. Values of $p < 0.05$ were considered significant. All p-values are indicated on the figures.

3. Results

3.1. Both CD4 and CD8 T cells are polarized to produce Th2 cytokines by IL-4 in vitro while CD4, but not CD8 T cells, produce these cytokines in vivo in response to alumOVA

OVA-specific CD8 (OTI) and OVA-specific CD4 (OTII) T cells were activated *in vitro* using Th2-polarizing conditions. LN cells from OTI or OTII mice were cultured with the OVA-peptide their T cells respectively recognize. Selective Th2-polarization was achieved in both CD8 and CD4 T cells by adding IL-4 with anti-IL-12 and anti-IFN-γ (Fig. 1A). After 6 days of culture there were many similarities between the responses of the two cell types and the main difference in IL-4 cytokine production was quantitative. Thus medians of 20% of OTII cells and of 2% of OTI cells differentiated into IL-4-producing cells (Fig. 1A). Surprisingly IL-5 protein was induced in a median of 1.4% of OTI cells but was never detected in OTII cells. In parallel experiments we questioned whether CD8 T cells are also able to acquire Th2-features in response to alumOVA *in vivo*. The responding cells were analyzed for cytokine production after *ex vivo* restimulation. CD4 T cells were induced to produce Th2 cytokines by this antigen. A subset of 2% of OTII cells differentiated into IL-4-producing effector cells in response to alumOVA (Fig. 1B). On the other hand, neither IL-4 nor IL-5 was induced *in vivo* in OTI cells responding to alumOVA in LN. This contrasts with the IL-4-directed IL-4-induction in CD8 T cells observed *in vitro*. The OTI cells did respond to alumOVA by proliferating and producing IFN-γ — a classical feature of Th1 immune responses.

Next we compared the cytokine mRNA expression by real time RT-PCR of OTI and OTII cells in both settings. To do so, after *in vivo* priming OTI and OTII cells were FACS-sorted as $CD8^+CD45.1^+$ cells and $CD4^+CD45.1^+$ cells, respectively, and gene expression was assessed. The qualitative difference in IL-4 protein induction between OTI and OTII cells stimulated *in vitro* was confirmed at the IL-4 mRNA level with a 400 fold increase in OTI cells compared with a 10,000 fold increase in OTII cells (Fig. 2A). There was comparable and strong induction of IL-13 and IL-5 mRNA in both OTI and OTII cells. By contrast, priming with alumOVA *in vivo* only induced IL-4 and IL-13 mRNA in OTII cells (Fig. 2B). Of note neither IL-5 protein nor IL-5 mRNA is induced in alumOVA-responding OTII cells in LN. In addition there was strong induction in OTII cells of IL-21 mRNA – a cytokine associated with TFh cell differentiation, germinal center formation and B cell responses – in both the *in vivo* and *in vitro* responses. By contrast there was little or no induction of IL-21 mRNA in OTI cells.

Finally, in the real time RT-PCR studies of the LN response to alumOVA chimeras were constructed by transfer of OTI or OTII cells or OTI with OTII cells (Fig. 2B). The results from the mixed OTI + OTII chimeras indicate that there was no clear cross effect between the CD4 and CD8 T cells in relation to the induction of IL-4, IL-13 or IL-21 mRNA.

Taken together these data indicate that CD8 T cells mimic the response of CD4 T cells to activation through the TCR *in vitro* in the presence of IL-4. By contrast, the acquisition of Th2-features *in vivo* in response to alumOVA is confined to CD4 T cells although this antigen induces CD8 T cells to differentiate into IFN-γ-producing cells. Thus, *in vivo* alumOVA induces CD8 T cells to acquire Th1 as opposed to Th2-associated features. These marked differences between the *in vivo* and *in vitro* induction of Th2-cytokine production confirm that the *in vitro* model of Th2 polarization directed by IL-4 at best only partially reflects the signaling pathways involved in Th2 polarization *in vivo*. With this in mind we next set to identify differences in the induction of signaling pathways and transcription regulators in OTI and OTII cells during the *in vitro* and *in vivo* responses. The aim of these studies was to obtain clues about molecules involved in Th2-differentiation signaling during the response to alumOVA *in vivo*.

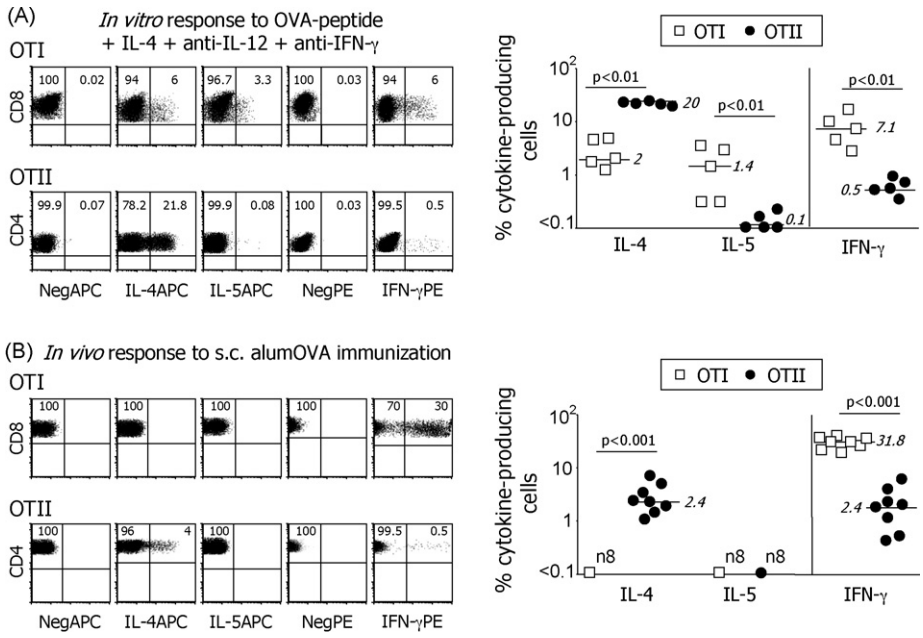

Fig. 1. Th2 cytokines are induced in OTI and OTII cells by OVA-peptide with IL-4 *in vitro*, but only in OTII cells responding to alumOVA *in vivo*. (A) LN cell suspensions from OTI or OTII mice were cultured for 6 days with OVA-peptide (SIINFEKL or 323-339, respectively), IL-4, anti-IL-12 and anti-IFN-γ. The cultured cells were then restimulated in anti-CD3-coated culture wells with soluble anti-CD28 for 5 h before testing for cytokine production by intracellular flow cytometry. Dot plots (left) show gated CD8$^+$ OTI cells (top row) or CD4$^+$ OTII cells (bottom row). The graph (right) shows the proportion of cytokine-producing OTI cells (open squares) or OTII cells (closed circles) in five independent experiments. (B) CD45.2$^+$ C57BL6 mice received congenic CD45.1$^+$ OTI cells or CD45.1$^+$ OTII cells. One day later the chimeras were immunized with alumOVA in both footpads. After 7 days draining popliteal LN cell suspensions were restimulated with OVA-peptide (SIINFEKL or 323-339) and assessed as in (A). Dot plots (left) are gated on OTI cells (CD45.1$^+$/CD8$^+$) (top row) or OTII cells (CD45.1$^+$/CD4$^+$) (bottom row). The graph (right) shows the percentage of cytokine-producing OTI or OTII cells from the popliteal LN of eight mice in two independent experiments. Parallel studies (not shown) indicate that <0.1% OTI and OTII cells from non-immunized chimeric mice produce any of these cytokines. Allophycocyanin (APC) or Phycoerythrin (PE) anti-cytokine or isotype control (Neg) antibodies were used. n8 indicates the number of observations where <0.1% cells contained the cytokine. Horizontal lines and italicized numbers indicate the medians of groups. Two-tailed Mann–Whitney non-parametric statistical differences between OTI cell and OTII cell values are shown in the graphs.

3.2. Differential expression of transcription factors associated with Th2 response

The Th2-master transcription factor GATA-3 (Pai et al., 2004) can be expressed in CD4 as well as CD8 T cells (Chen et al., 2005). After *in vitro* culture with IL-4, median GATA-3 expression was upregulated by around 30 fold in both OTI and OTII cells from the level in naïve cells (Fig. 3A). The baseline GATA-3 mRNA levels were about three fold higher in naïve CD4 than CD8 T cells isolated from chimeric non-immunized mice (Fig. 3B). GATA-3 levels doubled in both OTI and OTII cells in the response to alumOVA *in vivo* but, were significantly expressed at higher levels in OTII cells (Fig. 3B). The presence of OTI cells in the responding node did not alter the GATA-3 response in OTII cells in the same node (Fig. 3B). The transcription regulator c-Maf has also been implicated in the regulation of IL-4 production (Ho et al., 1998; Kim et al., 1999). During *in vitro* polarization with IL-4 the level of c-Maf expression did not significantly change compared to naïve cells in both OTI and OTII cells (Fig. 3A). On the other hand c-Maf was exclusively upregulated by around 30 fold only in OTII cells responding to alumOVA *in vivo*, but not in OTI cells (Fig. 3B). In addition, c-Maf upregulation was not affected by the presence of OTI cells (Fig. 3B).

3.3. Differential expression of the cell surface receptors OX40 and IL-17RB during Th2 response in vivo

The selective expression of OX40 and IL-17RB may be involved in the mechanisms underlying the disparity in the acquisition of Th2-features of the OTI and OTII cell responses described above (Akiba et al., 2000; Angkasekwinai et al., 2007; Ballantyne et al., 2007; Fallon et al., 2006; Flynn et al., 1998; Ishii et al., 2003; Jember et al., 2001; Linton et al., 2003; Owyang et al., 2006; Salek-Ardakani et al., 2003; Tamachi et al., 2006; Wang et al., 2007). As previously reported (Kim et al., 2003), the costimulatory molecule OX40 was found to be upregulated *in vitro* during CD4 T cell polarization directed by IL-4, and a similar upregulation occurred in OTI cells cultured in this way (Fig. 3A). By contrast, expression of OX40 during the response *in vivo* to alumOVA was restricted to OTII cells (Fig. 3B). Additionally the presence of OTI cells did not alter this upregulation of OX40 in OTII cells.

In previous *in vivo* studies we have shown that the cytokine receptor IL-17RB – also known as IL-25R – is selectively expressed in OTII cells responding to alumOVA, but not in OTII cells responding to a Th1-inducer – OVA-expressing live attenuated *Salmonella* (Serre et al., 2008). A functional significance of this finding was implied by IL-25 major role during Th2 responses (Fort et al., 2001). In the present studies no induction of IL-17RB mRNA was found in OTI or OTII cells during *in vitro* polarization with IL-4 (Fig. 3A). This rules out a sole signaling-dependency on TCR and IL-4 to induce IL-17RB expression. By contrast, we confirm that mRNA specific for IL-17RB is strongly induced *in vivo* in alumOVA-responding OTII cells, but not OTI cells responding to the same antigen (Fig. 3B). As described above for other Th2-features the presence of OTI cells does not alter the upregulation of IL-17RB in OTII cells (Fig. 3B).

Lastly we looked at CXCR5 expression. This is a marker for TFh cells that promotes their migration into B cell follicles, a process essential for germinal centers to be productive (Vinuesa et al., 2005). CXCR5 mRNA is not induced after *in vitro* stimulation with IL-4 (Fig. 3A), but we confirm that OTII cells responding to alumOVA upregulate CXCR5 mRNA. Surprisingly, as CD8 T cells do not normally enter follicles (MacLennan, 1994), we found that CXCR5 mRNA levels were also upregulated in OTI cells although to significantly lower levels than in OTII cells (Fig. 3B).

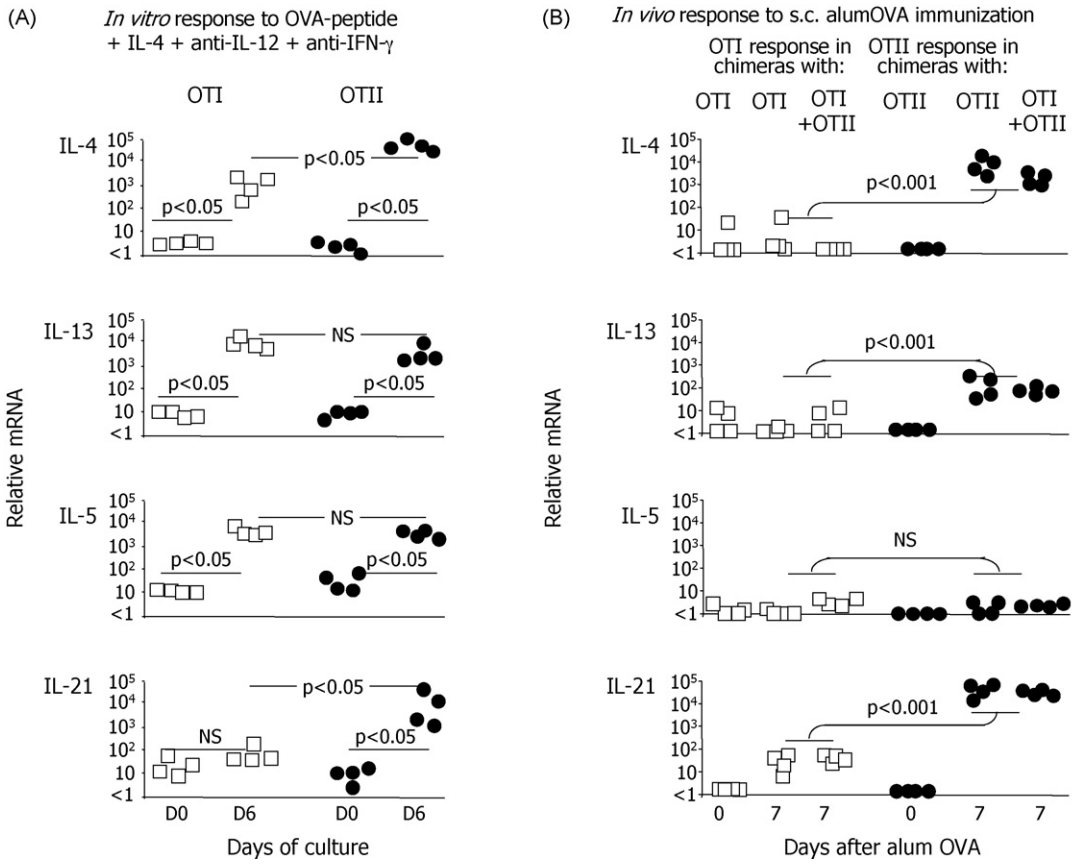

Fig. 2. Th2-cytokine mRNAs are induced in OTI and OTII cells by OVA-peptide with IL-4 *in vitro*, but only in OTII cells responding to alumOVA *in vivo*. (A) LN cell suspensions from OTI (open squares) or OTII (closed circles) mice were cultured for 6 days with OVA-peptide (SIINFEKL or 323-339, respectively), IL-4, anti-IL-12 and anti-IFN-γ. The cultured cells were then restimulated in anti-CD3-coated culture wells with soluble anti-CD28 for 5 h. They were then analyzed for Th2 and TFh cytokine mRNA expression by real time RT-PCR. Data are derived from four independent experiments. (B) CD45.2$^+$ C57BL6 mice received congenic CD45.1$^+$ OTI cells or CD45.1$^+$ OTII cells or both OTI and OTII cells. One day later the chimeras were immunized with alumOVA in both footpads. After 7 days OTI cells (CD45.1$^+$/CD8$^+$) or OTII cells (CD45.1$^+$/CD4$^+$) were FACS-sorted from draining popliteal LN cell suspensions. Cytokine mRNA expression was assessed in the sorted cells by real time RT-PCR. Data are derived from two independent experiments each with four mice in each group. Statistical differences between groups indicated in the graph were assessed by the two-tailed Mann–Whitney non-parametric test.

3.4. CXCR5, OX40 and PD-1 protein expression are specifically induced in CD4 T cells, but not CD8 T cells, responding to alumOVA

The unexpected finding that CXCR5 mRNA is upregulated in OTI CD8 T cells responding *in vivo* to alumOVA led us to test whether this upregulation was translated into CXCR5 cell surface protein. In addition, acquisition of another TFh marker – programmed cell death gene-1 (PD-1) (Haynes et al., 2007) – was assessed. To do this, mixed chimeras constructed with OTI and OTII cells were immunized with alumOVA and the cell surface expression of CXCR5, OX40 and PD-1 was assessed by flow cytometry. In addition the proportion of OTI and OTII cells responding and the extent of the response was monitored both by assessing CD69 expression, and cell proliferation detected by CFSE dilution (Fig. 4). Before immunization the OTI and OTII cells present a homogeneous phenotype characteristic of naïve T cells with no sign of activation or proliferation, as they are CFSE^{high} CD69$^-$ and do not express CXCR5, OX40 (Fig. 4A) or PD-1 (not shown). By 24 h after immunization CD69 is upregulated in almost all OTII cells and about half the OTI cells. CFSE dilution shows that both OTI and OTII cells have simultaneously started to divide between 24 h and 48 h after immunization and by 48 h both cell types have divided up to 4 times. At this stage of the response the doubling time of the proliferating OTI and OTII cells is about 6 h, for by 72 h both cells have undergone 7–8 divisions. Thus, OTI and OTII cells have similar induction and rate of proliferation in response to alumOVA *in vivo*, suggesting that this form of antigen is efficiently presented by both MHC class I as well as MHC class II molecules.

Contrary to the mRNA results, it is mainly the proliferating OTII cells that start to express CXCR5 protein from 48 h to 72 h. This correlates with the onset of OTII cell migration into B follicles during the third day after immunization with alumOVA (Ansel et al., 1999; Garside et al., 1998; Serre et al., 2009). The differential CXCR5 expression is maintained for at least a week, with 60% of the OTII cells being CXCR5$^+$ against some 10% of the OTI cells (Fig. 4B). The differential expression of OX40 mRNA by OTII cells in this response is reflected by OX40 protein expression on some 30% of OTII cells at 24 h and 60% by 72 h, while only a small sub-fraction of OTI cells express this protein (Fig. 4A). The differential OX40 expression is maintained for at least a week, with 60% of the OTII cells being OX40$^+$ against about 10% of the OTI cells (Fig. 4B). Interestingly, 7 days after immunization the expression of PD-1 a marker for TFh cells (Haynes et al., 2007) was exclusively restricted to a subset of some 20% OTII cells but not expressed by OTI cells (Fig. 4C). Finally, the expression of the cytokine receptor IL-17RB could not be followed as we were unable to obtain a reliable antibody to detect this IL-25 receptor.

4. Discussion

The study shows that the Th2 cytokines – IL-4, IL-13, IL-5 – as well as the transcription factor GATA-3 are induced *in vitro* in both OTI and OTII cells when stimulated with OVA-peptide (SIINFEKL

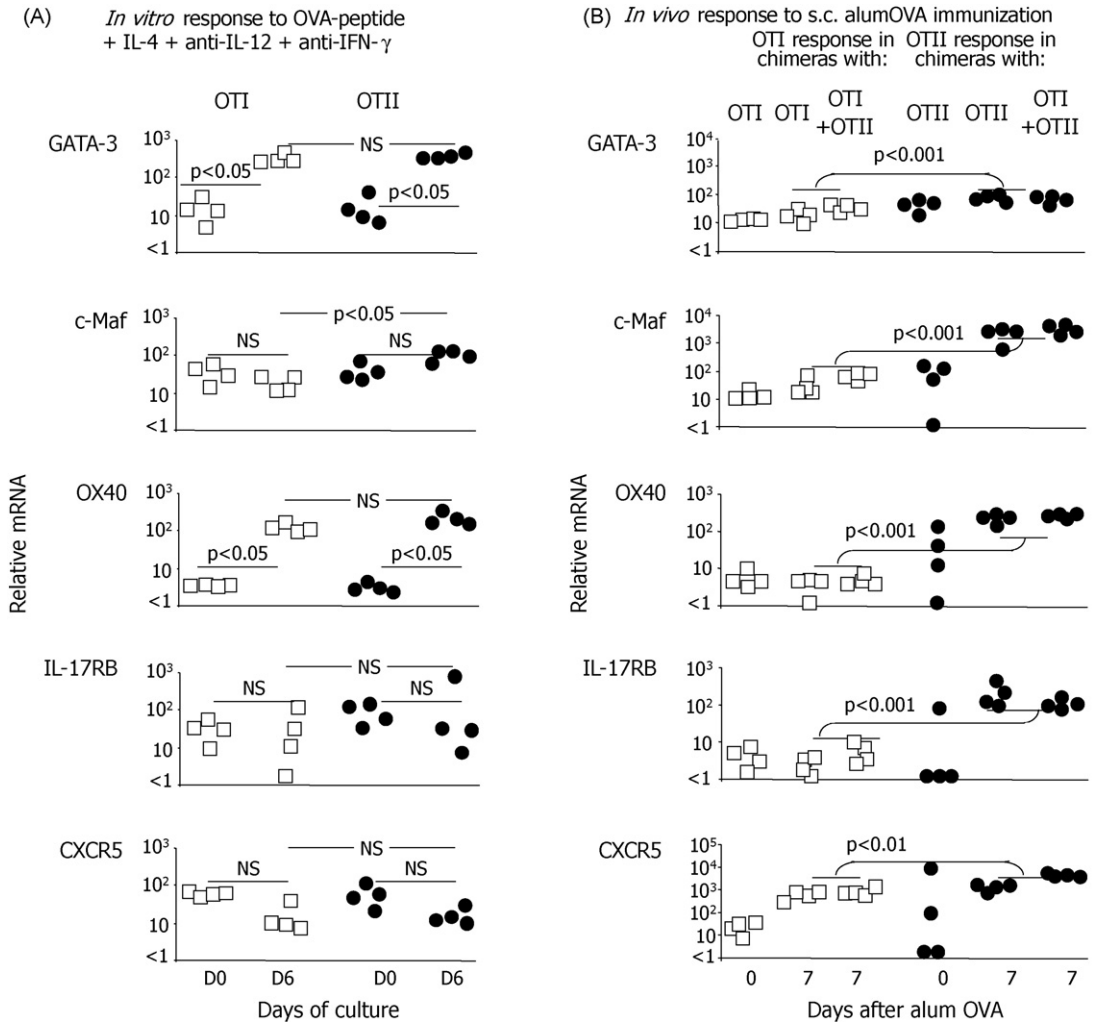

Fig. 3. Differences in transcription factor and cell surface molecule mRNA levels between OTI and OTII cells responding in the *in vitro* or *in vivo* Th2 cytokine-inducing responses. (A) LN cell suspensions from OTI mice (open squares) or OTII mice (closed circles) were cultured for 6 days with OVA-peptide (SIINFEKL or 323-339, respectively), IL-4, anti-IL-12 and anti-IFN-γ. The cultured cells were then restimulated in anti-CD3-coated culture wells with soluble anti-CD28 for 5 h. The mRNA expression was then assessed by real time RT-PCR for the transcription factors: GATA-3 and c-Maf and the cell surface molecules OX40, IL-17RB and CXCR5. These data are derived from two independent experiments each with four mice in each group. (B) mRNA expression was also assessed by real time RT-PCR in LN suspensions from the chimeric mice described in Fig. 2B. The mRNA levels are shown for sorted OTI cells' or OTII cells' from the chimeras constructed with OTI cells only, or OTII cells only, or both OTI and OTII cells 7 days' responding to alumOVA. These mRNA levels are compared to levels in OTI or OTII cells from non-immunized chimeras.

or 323-339, respectively) in the presence of IL-4. Although the relative levels of IL-4 expression by responding OTI cells may differ from those induced in OTII cells, in qualitative terms the responses of these two cell types to culture with IL-4 are similar for all other Th2-features. This contrasts with failure of OTI cells responding to alumOVA *in vivo*, to mimic the Th2 response of OTII cells responding to this antigen. These results further confirm that the *in vitro* models of Th2 polarization using IL-4 provide at best a poor reflection of Th2 responses *in vivo* in terms of the diversity of activated T cells produced and the proportion of cells expressing different cytokines. The proliferative response and IFN-γ production induced in CD8 T cells by alum-precipitated protein clearly shows that this form of exogenous antigen is being taken up, processed and effectively presented in association with the MHC class I molecules, a process termed cross-presentation. This is of interest for, despite the poor reported ability of alum-protein vaccines to induce cytotoxic CD8 T cell responses (Bomford, 1980; Garulli et al., 2008), OTI cells clearly respond to alum-precipitated proteins. This is consistent with previous reports which show that endogenous CD8 T cells proliferate (McKee et al., 2009) as well as develop cytotoxicity (Dillon et al., 1992) in response to alum-precipitated antigen. In addition, a previous report on a response to OVA-expressing *Listeria monocytogenes* described differences in the proliferative properties of OTI and OTII T cells (Foulds et al., 2002). By contrast, in our study both the induction and rate of proliferation of OTI and OTII cells responding to alumOVA were similar.

Although the early induction of IL-4 production in CD4 T cells responding to alum-precipitated protein *in vivo* is independent of IL-4 and STAT-6 signaling (Brewer et al., 1999, 1996; Cunningham et al., 2002, 2004b; van Panhuys et al., 2008) the molecular basis of these Th2 immune responses remains poorly understood. Previous results showed that CD4 T cells responding to alumOVA significantly upregulate IL-17RB by 3 days after immunization (Serre et al., 2008), while the same cells do not upregulate this IL-25 receptor in response to the Th1-inducer — OVA-expressing *Salmonella*. These findings indicate that the induction of IL-17RB expression is a selective feature of CD4 T cells making a Th2 response *in vivo*. This indication of selectivity is confirmed in the present study, where the IL-17RB mRNA expression induced in OTII cells responding to alumOVA was not seen in the OTI cells responding within the same microenvironment in the same LN to the same antigen. In addition neither OTI nor OTII cells upregulated this receptor

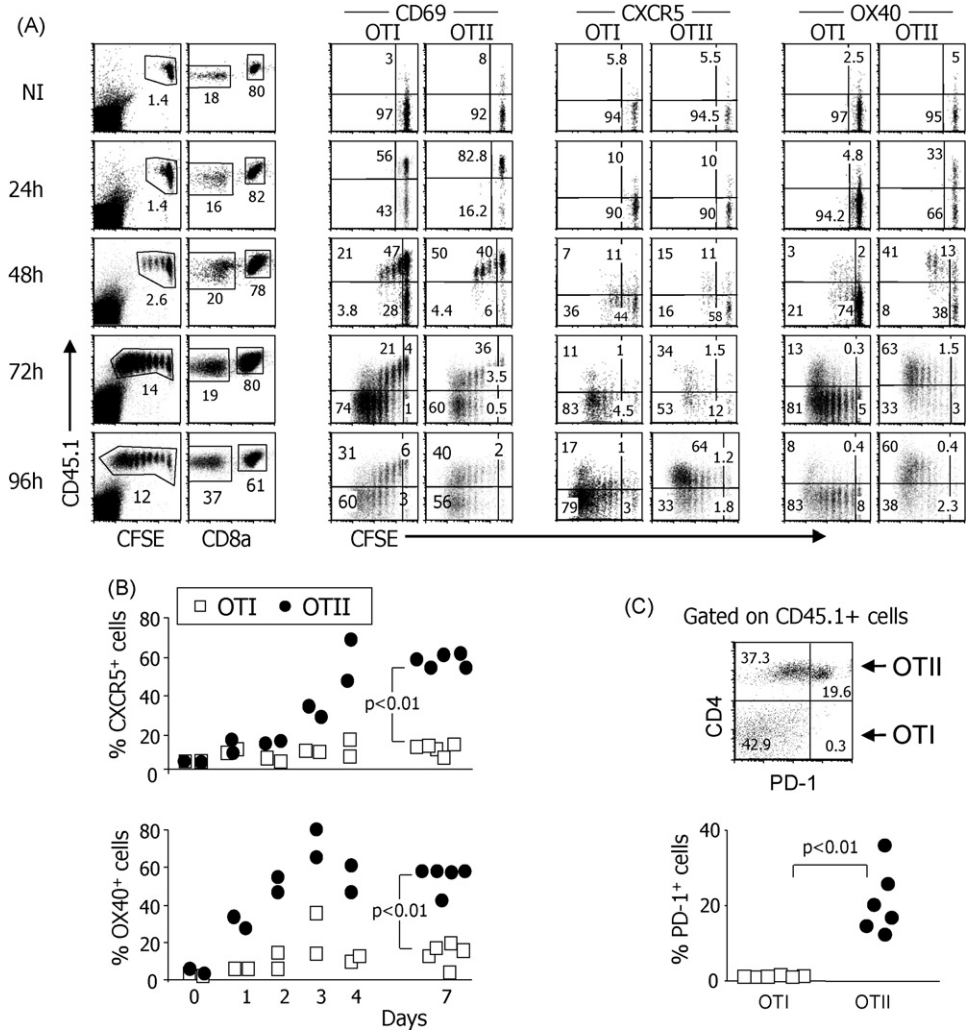

Fig. 4. Phenotypic changes related to proliferation among OTI and OTII cells responding to alumOVA in the same popliteal draining LN. CD45.1$^+$ OTI cells plus CD45.1$^+$ OTII cells, were CFSE-labeled and co-injected into congenic CD45.2$^+$ C57BL6 mice. One day later they were immunized with alumOVA in both footpads. (A) Representative flow cytometric dot plots at various times post-immunization. The plots in the left hand column show analysis of the total LN cells with a gate round the CFSE$^+$ CD45.1$^+$ donor cells. This donor cell gate is resolved in next column of plots into CD8α^+ OTI cells and CD8α^- OTII cells. In the next three paired columns of dot plots CD69, CXCR5, and OX40 expression in relation to cell division assessed by CFSE dilution is shown for OTI cells (left) and OTII cells (right). (B) Graphs show the percentage OTI and OTII cells expressing CXCR5 or OX40 at different times after immunization. Each symbol represents OTI or OTII cells from the two popliteal LN of 1 mouse. Data are derived from two independent experiments for days 0–4 and three independent experiments for day 7. (C) PD-1 expression in OTI and OTII cells 7 days after immunization assessed by flow cytometry in six mice in two independent experiments.

under *in vitro* polarizing conditions. The receptor IL-17RB binds to IL-17B and IL-17E (IL-25). IL-25 has been found to play major roles during Th2 responses and, for instance, IL-25 injection or systemic over-expression in transgenic mice provokes the upregulation of Th2-features or proallergic pathologies (Angkasekwinai et al., 2007; Fallon et al., 2006; Kim et al., 2002; Pan et al., 2001). As previously reported (Wang et al., 2007), it likely that IL-17RB engagement is involved in the maturation rather than primary induction of Th2 response to alumOVA as this receptor is itself induced in OTII cells during the response. We have been unable to detect IL-25 mRNA in naïve or immunized LN cell suspensions in the present study (data not shown). Nevertheless, innate immune cells (Angkasekwinai et al., 2007; Ikeda et al., 2003; Wang et al., 2007) have been reported to produce this cytokine. It will be of interest to see if the production of IL-25, for instance in dendritic cells, relies on a signaling pathway dependent on the inflammasome (Eisenbarth et al., 2008; Kool et al., 2008; Li et al., 2008).

OX40 appears on the surface of CD4 T cells, but not CD8 T cells, 24 h after immunization. The effects of signaling through OX40 are still only partially defined. In different circumstances it can amplify both IFN-γ and IL-4 production (De Smedt et al., 2002; Rogers and Croft, 2000). Nevertheless, several studies have reported that OX40 signaling directs Th2 differentiation (Akiba et al., 2000; Flynn et al., 1998; Ishii et al., 2003; Jember et al., 2001; Linton et al., 2003; Salek-Ardakani et al., 2003). In addition, OX40 plays a part in germinal center maintenance by triggering upregulation of CXCR5, a key event enabling a subset of responding CD4 T cells to migrate into follicles (Brocker et al., 1999; Flynn et al., 1998; Walker et al., 1999). The reduced expression of CXCR5 protein on the surface of CD8 T cells responding to alumOVA may in part be due to their failure to upregulate OX40. This raises the question of whether there is a correlation between TFh cell differentiation and IL-4 expression. CXCR5^{high}CCR7^{low} T cells have been found to have elevated IL-4 and PD-1 mRNA expression (Haynes et al., 2007). In addition, recent studies have reported that IL-4 secretion, is first restricted to CD4 T cells in the outer T zone close to the B cell follicles and, later, to the germinal centers (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). This compartmentalization of IL-4 production during CD4 T cell responses may affect the CD8 T cell response, as these OX40$^-$CXCR5$^-$PD-1$^-$ CD8 T cells are excluded from folli-

cles (MacLennan, 1994) and hence the focus of IL-4 production in the responding node. This is consistent with the acquisition of specific TFh markers, including IL-21 (Chtanova et al., 2004; Spolski and Leonard, 2009), c-Maf (Bauquet et al., 2009; Pot et al., 2009), CXCR5 (Brocker et al., 1999; Flynn et al., 1998; Haynes et al., 2007; Walker et al., 1999) and PD-1 (Haynes et al., 2007; King and Mohrs, 2009; Yu et al., 2009; Zaretsky et al., 2009), being exclusively associated with responding OTII cells, but not with OTI cells. Further studies are warranted to define the role of these molecules during induction of IL-4 in CD4 T cells *in vivo*. In this respect, it is interesting to note that extensive heterogeneity develops in CD4 T cells as they respond to alum-precipitated protein antigens. Only a small proportion of the responding cells produce Th2 (IL-4 and IL-13)- and TFh (IL-21)-cytokines (Serre et al., 2009). Although some of the responding cells produce these cytokines simultaneously others show specialization by only producing one of the cytokines. In addition, as mentioned above, a substantial proportion of responding CD4 T cells migrate to the follicles while others remain in the T zone. Yet others leave the node as memory or effector cells (Serre et al., 2009). This functional heterogeneity within the primed CD4 T cell population responding to alum OVA is only starting to be fully appreciated and needs to be correlated with the expression of GATA-3, c-Maf, OX40, CXCR5, PD-1 and IL-17RB at the single cell level. This may give insight into (i) the specific phenotype of IL-4 and/or IL-21-producing CD4 effector T cells and (ii) the signal(s) required to induce individual or simultaneous cytokines. In this way the current study provides an approach for further studies elucidating the role of these molecules in the induction of Th2- and TFh-features in response to alum-protein vaccines.

Finally, emergence of "Th2-refractory" IFN-γ-producing OTI cells during response to alum-precipitated proteins may have some implication for vaccine designs. Even though the CD8 T cells do not seem to modify the features of the CD4 T cell response studied here, we have found, in data to be published elsewhere, that they have an important impact on the B cell response to the Th2-antigen. During priming with alumOVA, B cells essentially switch to IgG1 (Mohr et al., 2009). However, in the presence of OTI cells the antibody response acquires more Th1-features and B cells switched to a variety of IgG1-, IgG2a- and IgG2b-producers (Mohr et al., unpublished data). This indicates that the production of IFN-γ by CD8 T cells responding to alumOVA has physiological consequences within the overall immune response to alum-precipitated antigen.

Conflict of interest

The authors have no conflicting financial interests.

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