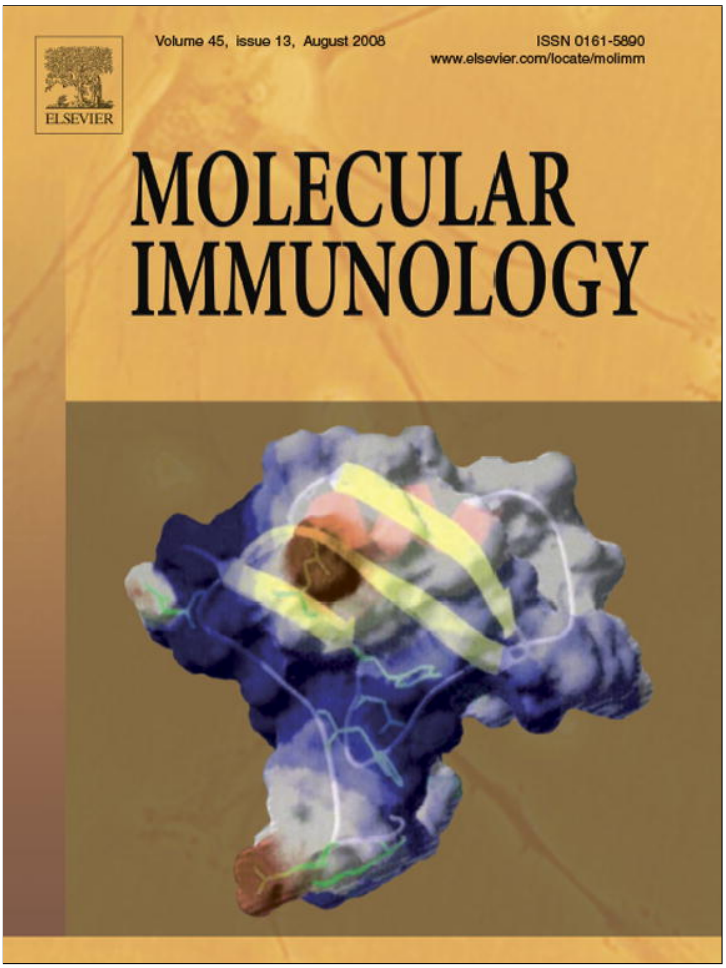

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Molecular differences between the divergent responses of ovalbumin-specific CD4 T cells to alum-precipitated ovalbumin compared to ovalbumin expressed by *Salmonella*

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ABSTRACT

CD4 T helper (Th) cell differentiation defined by *in vitro* cytokine-directed culture systems leaves major gaps in our knowledge of the mechanisms driving divergent Th differentiation. This is evident from our analysis of the response of mouse ovalbumin-specific CD4 T cells to different forms of ovalbumin that induce markedly distinct responses *in vivo*. We show that live attenuated ovalbumin-expressing *Salmonella* (SalOVA) induce Th1-associated T-bet and IFN-γ. Conversely, alum-precipitated ovalbumin (alumOVA) induces the Th2-associated GATA-3 and IL-4. The early diversity occurring within these CD4 T cells isolated 3 days after immunization was assessed using real-time RT-PCR microfluidic cards designed with 384 selected genes. The technique was validated both at the population and single cell levels at different stages of the responses, showing β2-microglobulin to be a more stably expressed reference mRNA than either β-actin or 18S RNA. SalOVA was then shown selectively to induce the OVA-specific CD4 T cells to produce many chemokines and pro-inflammatory cytokines, contrasting with alumOVA-induced cells that only produced a few Th2-associated cytokines. Several cytokines and features associated with follicular helper functions were induced in the OVA-specific CD4 T cells by both antigens. Finally, IL-17RB is strongly associated with OVA-specific CD4 T cells responding to alumOVA, suggesting that alum may promote Th2 immune response through a role for the IL-25/IL-17RB pathway.

1. Introduction

Appropriate immune responses against pathogens rely upon the development of different subsets of CD4 T helper (Th) cells that display specific functions. The nomenclature of CD4 Th cells, including Th1, Th2, Th3, Th17, Treg, Tr1 and follicular helper cells, mainly relates to the selective production of cytokines (Reinhardt et al., 2006). Generally Th1 CD4 T cells secrete IFN-γ and protect against intracellular pathogens, while Th2 cells secrete IL-4 and combat extracellular infections. Our knowledge of Th commitment has been extensively provided by *in vitro* systems. This is achieved by subjecting T cells to TCR/CD28 stimulation in the presence of cytokines (O'Garra, 1998). IL-12 drives Th1 differentiation (Openshaw et al., 1995) whereas IL-4 induces Th2 cells (Hsieh et al., 1992). Although *in vitro* IL-4 initiates Th2 differentiation, *in vivo* IL-4 and IL-4-signaling are dispensable for the induction of many Th2-features (Cunningham et al., 2002, 2004b; Finkelman et al., 2000; Jankovic et al., 2000; Noben-Trauth et al., 1997; Ouyang et al., 2000). Indeed, various types of immunization inducing Th2 responses, such as alum-precipitated protein or *Nippostrongylus brasiliensis*, or ovalbumin-induced allergic lung pathologies, have been reported to induce IL-4 producing CD4 T cells efficiently in IL-4/IL-13- or IL-4Rα-deficient mice (Cunningham et al., 2002, 2004b; Voehringer et al., 2006). This cautions that CD4 T cell polarization may be more heterogeneous *in vivo*, where a range of signals are delivered by dynamic cellular and molecular interactions within specific microenvironments (Bajenoff et al., 2006, 2003; Katakai et al., 2004). Therefore the early gene expression profile of T cells committed towards Th1 or Th2 *in vivo* may give insight into signaling

Abbreviations: Ag, antigen; SalOVA, live attenuated *Salmonella typhimurium* engineered to express an ovalbumin transgene; alumOVA, alum-precipitated ovalbumin; LN, lymph node.

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pathways involved in this polarization. To study the requirements during Th differentiation *in vivo*, we transferred naïve ovalbumin (OVA)-specific transgenic CD4 T (OTII) cells into normal mice that were then immunized with specific forms of OVA driving either a Th1 or a Th2 type of immune response. The response in the draining lymph node (LN) was followed after immunizing subcutaneously, either with a Th1 inducer – live attenuated *Salmonella typhimurium* engineered to express an OVA transgene (SalOVA), or Th2 inducer – alum-precipitated OVA (alumOVA).

The resolution of primary *Salmonella enterica* serovar typhimurium infection depends on the appropriate induction of CD4 Th1 cells (Hess et al., 1996; Ravindran et al., 2005). Protective roles have been shown for IFN-γ, TNF-α, IL-1α, IL-12, IL-15, and IL-18, whereas IL-4 and IL-10 have been reported to inhibit host defenses against *Salmonella* (Eckmann and Kagnoff, 2001). T-bet, a key transcription factor for IFN-γ induction, is required for resistance to *Salmonella* infection (Ravindran et al., 2005). In addition, attenuated *Salmonella* induce a T-dependent antibody response with switching to IgG2a (IgG2c in C57Bl/6 mice) (Cunningham et al., 2007, 2004a; McSorley and Jenkins, 2000). By contrast, the use of alum-precipitated proteins induces Th2 type of immune responses (Brewer et al., 1999). Although alum is one of the most widely used adjuvant in human vaccines, the mechanism by which it promotes very strong antibody- and Th2-immune responses remains unclear. It promotes the production of cytokines (IL-4), Th2-associated transcription factors (GATA-3, c-maf, NIP45), and switching to IgG1 and IgE (Cunningham et al., 2002, 2004a). GATA-3, a key transcription factor for IL-4 production, is important for the development and maintenance of Th2-responses (Pai et al., 2004; Zhu et al., 2004). We have used the fundamentally different T and B cell outcomes of these immunization protocols to analyze the divergent differentiation of OTII cells in responses to SalOVA and alumOVA.

In this study, after characterizing the classical Th1 and Th2 T cell features of the two responses, a more extensive search for differences and similarities in effector functions that develop alongside IFN-γ- or IL-4-producing OTII cells was assessed. We performed real time RT-PCR using low-density microfluidic cards designed with 384 individual gene expression assays selected for their roles in different biological processes relevant to T cell differentiation and function (Chtanova et al., 2001, 2005, 2004; Rogge et al., 2000). This report validates the use of this approach and shows that gene expression profile, assessed 3 days after challenge, shows multiple consistent differences and similarities between SalOVA- and alumOVA-responding OTII cells.

2. Materials and methods

2.1. Mice

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

2.2. T cell purification and adoptive transfer

CD4 T cells from LN of OTII mice were purified using anti-CD4 MACS microbeads (Miltenyi Biotec Ltd., Bisley, U.K.). Five 10^6 T cells were labeled with CFSE (Cambridge Bioscience, Cambridge, U.K.) and injected i.v. into recipients. Mice were immunized the following day.

2.3. Immunization, Ag and ovalbumin-expressing Salmonella

OVA (Pierce) is mixed with 9% aluminium potassium sulfate (A7167 Sigma) solution then, after the pH is adjusted to pH7, the mix is let 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 footpad of the rear feet. *Salmonella enterica* serovar typhimurium aroA-strain SL3261 (Hoiseth and Stocker, 1981) expressing OVA were generated as follows. The ova gene was amplified from the pcDNA3-OVA vector (gift from Dr. Martin Zenke, Aachen University Medical School, Germany) (Diebold et al., 2001) and cloned into pCR2.1TOPO (Invitrogen). The gene was then ligated into a modified pet22b+ (Novagen, Merck Biosciences, U.K.) that allows constitutive gene expression by replacement of the T7 promoter region and lac operator by an adapted tac promoter using GeneEditor site-directed mutagenesis system (Promega). The resulting plasmid pettacOVA was then electroporated into *Salmonella*. OVA protein expression was confirmed by Western blotting. The stability of the plasmid was tested by showing that 35 days after i.p. infection >90% of the recoverable bacteria still expressed the plasmid. Further tests confirmed that plasmid containing organisms colonized the host as well as non-modified *Salmonella*. The bacterial concentration was quantified at OD_{600} using growth curves. Bacteria growing in mid-log range were used for studies of the immune response to SalOVA. 10^6 live organisms were injected subcutaneously in the rear footpads in 10 μl.

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

Popliteal LN were digested for 20 min at room temperature with collagenase type II (1 mg/ml) (Lorne Laboratories Ltd, Reading, U.K.) and DNase I (0.15 mg/ml) (Sigma), followed by 5 min treatment with 10 mM EDTA (Sigma). Anti-CD45.1-PE (A20), CD4-PerCP-Cy5.5 (RM4-5), biotinylated anti-CD69 (H1.2F3), CD62L (MEL-14), CD44 (IM7), and streptavidin-APC were from PharMingen. Populations or single cells were sorted by flow cytometry (MoFlo, DakoCytomation, U.K.). Sorted cells purity was assessed on a FACScalibur (Becton Dickinson). Final analysis and graphical output were performed using FlowJo software (Treestar, Costa Mesa, CA).

2.5. Intracellular cytokine staining

Day 6 after immunization popliteal LN cells were incubated at 5×10^6 cells/ml with or without 10 μM free 323–339 OVA-peptide for 6 h prior to cytokine detection. Intracellular FACS staining was performed using Cytofix/cytoperm kit (Becton Dickinson). Anti-IL-4-APC (11B11), IFN-γ-biotin (X.MG1.2), and streptavidin-APC were from PharMingen.

2.6. Real-time semi-quantitative RT-PCR

Cell suspension mRNA was prepared using RNeasy columns from Qiagen. Reverse transcription was performed with random oligonucleotides (Promega) using MMLV reverse transcriptase (Invitrogen) for 1 h at 42 °C. Relative quantification of specific cDNA species to β-actin or β2-microglobulin messenger was carried out in a duplex PCR using TaqMan chemistry (Applied Biosystems, Warrington, U.K.). As a prerequisite, the amplification efficiency was confirmed for each combination for the two sets of primers/probes. Probes for cytokines and transcription factors were detected via a 5′ label with FAM, while probe for β-actin or β2-microglobulin were 5′ labeled with VIC or NED, respectively. TaqMan probes and primers were designed using Primer Express software (Applied Biosystems) and sequences are detailed below.

Single cells were collected in 384 well plates containing 2 μl of cell-to-signal lysis buffer (Ambion, U.K.). mRNA were specifically reverse-transcribed and the PCR performed by adding 8 μl of a mix containing the Quantitect enzyme RT-PCR master mix (Qiagen Quantitect Probe RT-PCR kit), primers and probes.

Standard reaction conditions for the TaqMan RT-PCR or PCR were used on the ABI 7900.

2.7. Sequences for primers and probes

β-actin: forward, CGTGAAAAGATGACCCAGATCA; reverse, TGGTACGACCAG-AGGCATACAG; probe, TCAACACCCCAGCCAT-GTACGTAGCC;
β2-microglobulin: forward, CTGCAGAGTTAAGCATGCCAGTAT; reverse, ATCACA-TGTCTCGATCCCAGTAGA; probe, CGAGCCCAA-GACC;
GATA-3: forward, CCACCCCATTACCACCTATCC; reverse, CACACACTCCCT-GCCTTCTGT; probe, TCGAGGCCCAAGGCAC-GATCC;
IFN-γ: forward, TCTTCTTGGATATCTGGAGGAACTG; reverse, GAGATAATC-TGGCTCTGCAGGATT; probe, TTCATGTCACCATCCTT;
IL-4: forward, GATCATCGGCATTTTGAACGA; reverse, AGGACGTTTGGCAC-ATCCAT; probe, TGCATGGCGTCCCTTCTC-CTGTG;
IL-13: forward, TTGAGGAGCTGAGCAACATCAC; reverse, GCG-GCCAGGTCCACACT; probe, CAAGACCAGACTCCCCTGTGCAACG;
T-bet: forward, ATGCCAGGGAACCGCTTATA; reverse, AACTTCCTG-GCGCATCCA; probe, CCCAGACTCCCCCAACACCGGA.

Primers and probes were from Applied Biosystems or Eurogentec.

2.8. Microfluidic cards real-time semi-quantitative RT-PCR and analysis

The microfluidic cards were designed using commercial TaqMan® Low Density Array (Applied Biosystems). TaqMan® Gene Expression Assays were loaded into the array's 384-well cards by the manufacturer. The list of all 384 genes spotted onto the microfluidic cards has been deposited in GEO database (Platform GPL5073). mRNA was prepared using RNeasy columns from Qiagen in a final volume of 100 μl to which 660 μl of a mix containing the Quantitect enzyme RT-PCR master mix was added. 95 μl of this mix was loaded onto each of the 8 channels of the 384-well cards. Reverse transcription and real-time PCR were performed sequentially onto the microfluidic cards. Standard reaction conditions for the TaqMan RT-PCR or PCR were used on the ABI 7900.

mRNA transcript level of each gene was analyzed using Applied Biosystem's SDS software by setting thresholds determining the cycle number at which the threshold was reached (C_t). For a given gene, the threshold value was kept constant in all 9 samples. The C_t of the β2-microglobulin was subtracted from the C_t of the target gene and the relative amount was calculated as $2^{-\Delta C_t}$. These data are deposited in GEO database under GSE7481 access number (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rdgvfckuwcuaqxk&acc=GSE7481). Genes for which the assay failed or was below the detection level ($C_t > 40$) were given a fixed value (0 for $2^{-\Delta C_t}$ and −15.5 for $-\Delta C_t$) to reflect the absence of expression. Genes that were not expressed or had 4 or less values shared out within the three conditions (naïve, SalOVA, alumOVA) were removed from the analysis. Relative amounts from the 324 remaining genes were subjected to three entries (naïve, SalOVA, alumOVA) multi-class significance analysis of microarrays applying a 0.5% false discovery rate. Statistical significance of differentially expressed genes was determined using TIGR MeV3.1 software (Saeed et al., 2003). This approach retained 248 genes (65%). Only genes for which difference between naïve and activated cells was superior to 2 were kept in the analysis. The remaining 194 genes were classified by functional family for analysis. In order to facilitate the viewing of the results, the $-\Delta C_t$ values were submitted to a clustering analysis. The variation of each gene expression is displayed with a mean-centered normalization. Genes that had a 2–5-fold or more than 5-fold differences between SalOVA- and alumOVA-responding OTII cells were highlighted using turquoise or blue boxes/text in the corresponding figure.

3. Results

3.1. Generation of IFN-γ- or IL-4-producing OTII cells in vivo

To monitor T cell differentiation, CFSE-labeled CD45.1$^+$ OTII cells were transferred into congenic wild-type CD45.2$^+$ recipients. The following day, mice were immunized s.c. in footpads, either with SalOVA or alumOVA. Six days after immunization, OTII cells were isolated from the draining popliteal LN by FACS sorting (Fig. 1A). The sorted cells' expression of mRNA that characterize Th1 (IFN-γ and T-bet) or Th2 (IL-4, IL-13 and GATA-3) commitment was assessed by real time RT-PCR (Fig. 1A). When responding to SalOVA OTII cells displayed median IFN-γ and T-bet mRNA levels 200- and 500-fold higher, respectively, as compared to OTII cells responding to alumOVA. Conversely, median IL-4, IL-13 and GATA-3 mRNA levels were, respectively, 300-, 60- and 10-fold higher in OTII cells responding to alumOVA compared to those responding to SalOVA. Intracellular cytokine staining shows that the mRNA is effectively translated into proteins (Fig. 1B, dot plots). A median of 20% of OTII cells from 6 SalOVA-immunized mice produced IFN-γ compared with a 4% median from 6 mice immunized with alumOVA (Fig. 1B). This confirms previous observations that few IFN-γ-producing cells are induced by immunization with alumOVA (Smith et al., 2004). Histograms in Fig. 1B also show that the amount of IFN-γ produced per cell is higher after SalOVA immunization than after alumOVA. By contrast, IL-4-production is restricted to mice immunized with alumOVA (2% median from 6 mice). CFSE dilution shows that, although immunization had induced most OTII cells in both responses to proliferate, only a small proportion of the responding cells, respectively, produced IFN-γ or IL-4 (Fig. 1B).

3.2. Further characterization of OTII cells polarized in vivo 3 days after immunization

Before immunization transferred OTII cells present a homogeneous phenotype, characteristic of naïve CD4 T cells (non-divided, CD69$^-$, CD62L$^+$, CD44$^-$) (Fig. 2A). In contrast, 72 h after immunization most OTII cells have undergone between 5 and 7 divisions. At this stage, most cells express CD44, but there is heterogeneity in CD69 and CD62L expressions. The total number of OTII cells recovered 3 days after alumOVA was always greater than at the same stage after SalOVA. These differences may be attributable to the amount of OVA injected for mice received 10 μg of OVA when immunized with alumOVA, while OVA contained in the injected SalOVA is estimated at 0.14 μg and represents about 1/1000 of the total *Salmonella* proteins.

3.3. Assessment of the reproducibility of the results obtained by dedicated real-time semi-quantitative RT-PCR microfluidic cards

Studies of gene expression by OTII cells recruited into the response to either SalOVA or alumOVA were carried out by sorting, 3 days after immunization, OTII cells that had completed

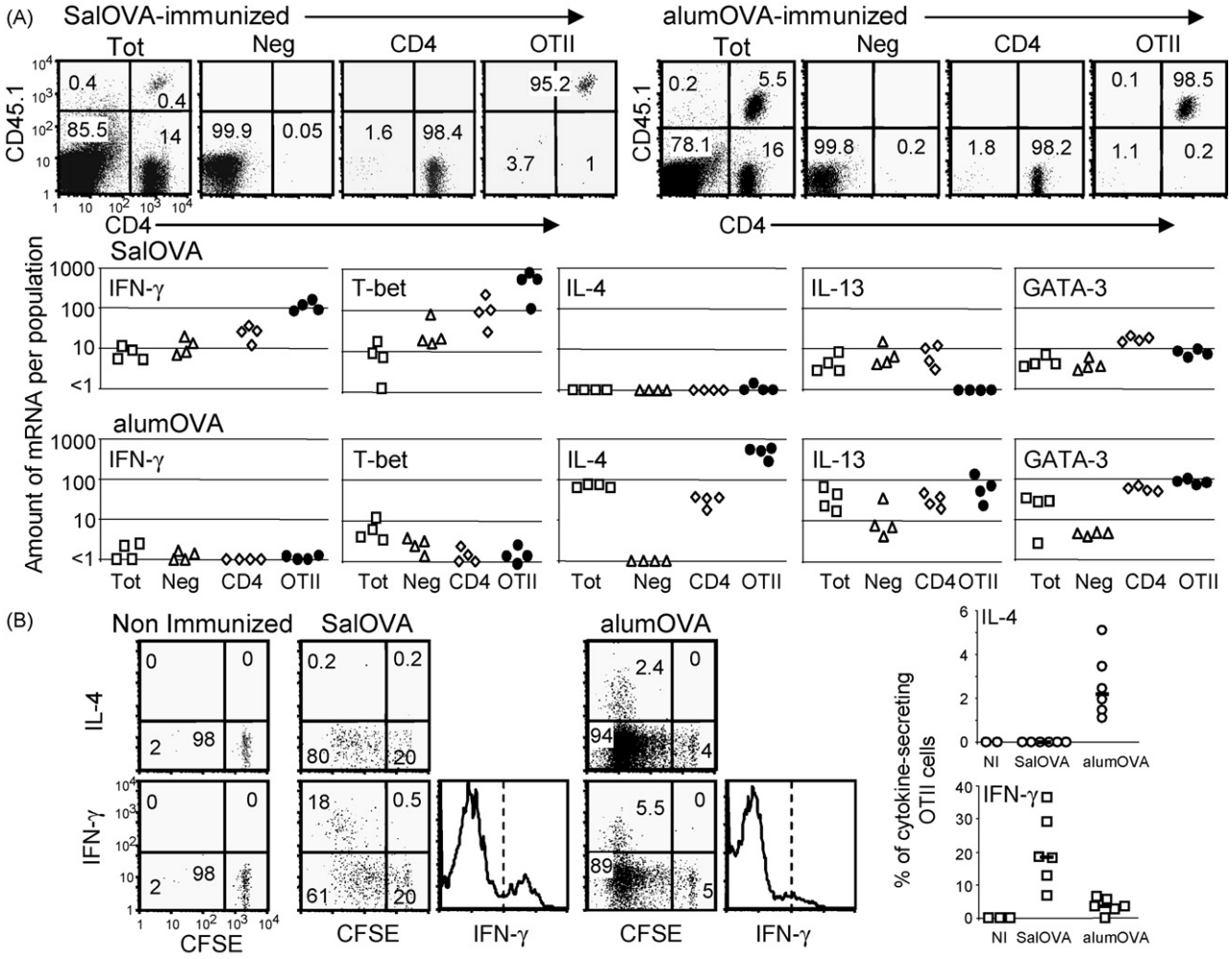

Fig. 1. Rapid and selective induction of IFN-γ- or IL-4-production by OTII cells *in vivo*. One day after receiving CD45.1+ OTII cells congenic CD45.2+ B6 mice were immunized either with SalOVA or alumOVA in the footpads. (A) Six days later popliteal LN cells were taken and total LN cells (Tot) were sorted on the basis of CD4 and CD45.1 expression into: CD4−/CD45.1− (Neg), CD4+/CD45.1− (CD4), CD4+/CD45.1+ (OTII) fractions. cDNA was prepared from each of these populations and the graphs show IFN-γ, T-bet, IL-4, IL-13, and GATA-3 mRNA levels, assessed by real time PCR. Each symbol represents total or sorted cells pooled from the two popliteal LN per mouse. The data are representative of two independent experiments. (B) LN cell suspensions from non-immunized mice or mice 6 days after immunization with SalOVA or alumOVA were incubated *in vitro* for 6 h with OVA-peptide to re-stimulate the OTII cells. Dot plots and histograms are gated on OTII cells. Plots (right) show the proportion of cells producing IFN-γ or IL-4 in the responding OTII population. Each symbol represents the percentage OTII cells producing IL-4 or IFN-γ from an independent experiment. Parallel studies (not shown) indicate that non-modified *Salmonella* does not induce OTII cell activation.

at least one cell cycle (Fig. 2B). Naïve OTII cells were also isolated from peripheral LN of non-immunized chimeric mice. The mRNA extracted from each of the populations was loaded onto the microfluidic cards. The numbers of cells obtained for each population of OTII cells analyzed are shown in Table 1.

The mRNA transcript levels of the different genes were assessed as described in Section 2. Not surprisingly activated OTII cells expressed 20% more of the 384 genes represented on the microfluidic cards than the naïve population. The percentages of reproducibly expressed genes in OTII cells from naïve, SalOVA-activated and alumOVA-activated LN, were, respectively, 88.2% (−/+2.2), 93.4% (−/+3) and 94.9% (−/+0.8) (Fig. 3A). The correlation coefficients, respectively, scoring 0.977 (−/+0.011), 0.985 (−/+0.010) and 0.978 (−/+0.017) for the naïve, SalOVA- and alumOVA-activated populations show a high level of reproducibility (Fig. 3A). As expected, the greatest variability was observed for genes displaying low level of expression and was more pronounced for samples prepared from the lowest number of cells (naïve and SalOVA) (Fig. 3A). Conversely, significant differences were observed when values obtained for each gene from naïve OTII cells were compared to those of activated OTII cells (Fig. 3B). In this case, the overall correlation coefficient scored 0.60 (−/+0.04). Finally, differences were observed when values obtained for each gene from SalOVA-responding OTII cells were compared to those of alumOVA-responding OTII cells (Fig. 3B), the overall correlation coefficient scored 0.976 (−/+0.02). Taken together these results show that the microfluidic cards are a reliable tool to compare gene expression in different populations.

Table 1
Numbers of cells used for each population in microfluidic card experiments

OTII	Naïve	SalOVA	AlumOVA
1	234,000	92,000	523,000
2	234,000	120,000	490,000
3	92,000	160,000	475,000

3.4. Comparison of endogenous reference genes for expression profiling of SalOVA- and alumOVA-activated OTII cells

Analysis of variation of gene expression in differentiating populations relies upon the comparison with housekeeping genes that

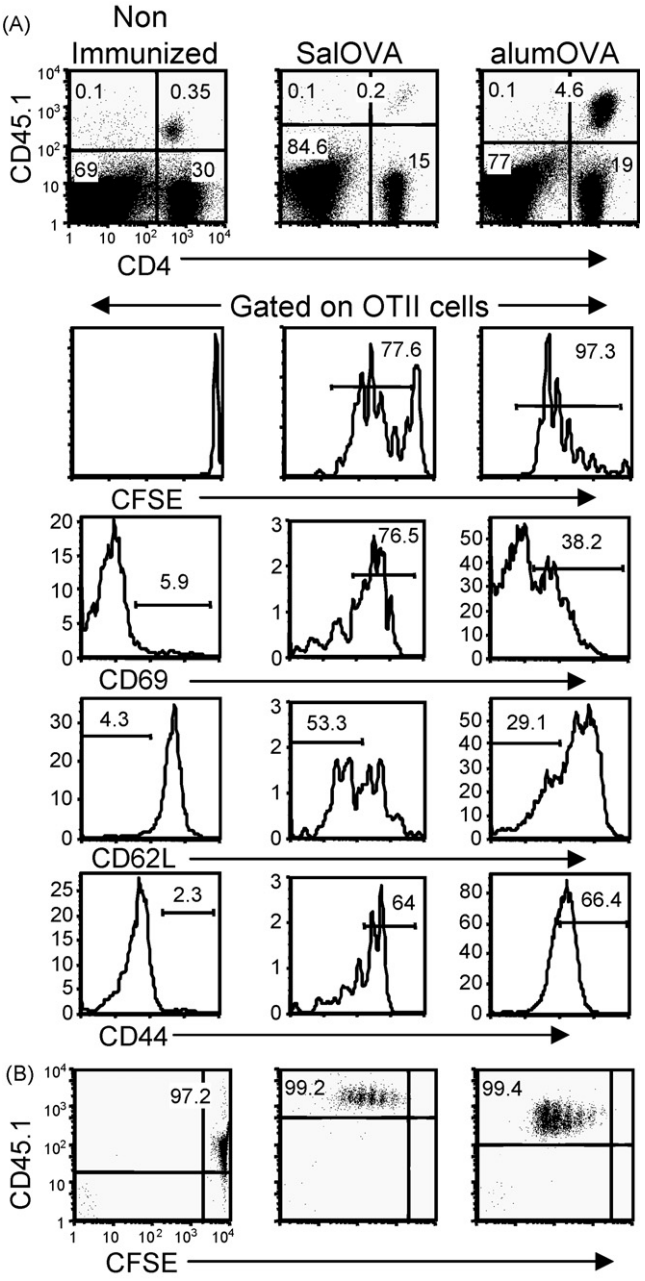

Fig. 2. Phenotype and isolation of OTII cells differentiated *in vivo*. One day after receiving CFSE-labeled CD45.1+ OTII cells congenic CD45.2+ B6 mice were immunized either with SalOVA or alumOVA in the footpads. (A) Three days after immunization OTII cells in the draining popliteal LN, identified by CD4 and CD45.1 co-expression were assessed for the expression of CFSE, CD69, CD62L and CD44. CFSE dilution reflects proliferation induced in OTII cells by immunization. The numbers above the horizontal bars in the histograms indicate the percent of cells with fluorescence intensity in the range indicated by the bars. (B) Shows representative sorts of OTII cells that had completed at least 1 cell cycle sorted from responding LN 3 days after immunization. These were used for the gene expression profiling studies shown in Figs. 3 and 4.

remain stable between the naïve and the activated cells. Three genes constitutively expressed by CD4 T cells (β-actin, 18S RNA, β2-microglobulin) were selected to assess their stability of expression in the context of the present study of CD4 T cell differentiation *in vivo*. While β-actin and 18S RNA are highly expressed genes, β2-microglobulin is expressed at more modest levels (Fig. 3A). When data were normalized according to β-actin mRNA levels (or 18S RNA not shown), activated OTII cells appeared to down regulate the majority of the genes compared to naïve OTII cells (Fig. 3B, red dots). By contrast, when normalized to β2-microglobulin mRNA levels the activated CD4 T cells show a slight general increase in the level of gene expressions (Fig. 3B, black dots). As the genes on these microfluidic cards were selected for their likely differential expression by activated T cells, the latter appears to reflect more faithfully the acquisition of immunological functions by activated CD4 T cells.

These observations at the population level led us to compare the behavior of β2-microglobulin and β-actin expression at the single OTII cell level at various stages during the differentiation process by real time RT-PCR. OTII cells were isolated as single cells as a function of the number of division accomplished, as visualized by CFSE dilution, at the following stages (Histograms in Fig. 3C): non-dividing naïve OTII cells from non-immunized mice (Naïve OTII); and from mice 3 days after immunization; (i) activated cells, as assessed by high CD69 expression (gates as shown in Fig. 2A), that had not yet divided (0 division); (ii) cells that had divided 2–3 times (2–3 divisions); (iii) cells that had divided 6 times (6 divisions). RT-PCR was performed on the single cells derived from each of these 4 sequential OTII subsets. When monitored at the single cell level, although both β2-microglobulin and β-actin were increased during the first cell cycles, β-actin showed the greatest level of change for activated OTII cells that had not divided or had undergone 2–3 rounds of division (Fig. 3C). This is shown by the increase in the trendline slopes for OTII cells that have just started dividing. In addition, the standard deviation obtained varies to a greater extent for β-actin than for β2-microglobulin, indicating a cell cycle state-dependent heterogeneity of the β-actin expression. This might reflect the role of β-actin in both: (i) in the formation of the immunological synapses necessary for DC and CD4 T cell interaction and (ii) during the cell division process as part of the cytoskeleton complex. These considerations, along with published evidences of the limitations of β-actin as internal control for RT-PCR (Selvey et al., 2001), have led us to use β2-microglobulin in preference to β-actin to normalize the data in the current study. Statistical significance of differentially expressed genes was then calculated as described in Section 2.

3.5. Differential gene expression analyzed by real-time RT-PCR

There were selectively higher levels of mRNA for IFN-γ, T-bet, Hlx, CIITA, IL-12Rβ2, in OTII cells responding to SalOVA and IL-4, IL-13, GM-CSF, GATA-3 from alumOVA responders. These expected results provide further positive controls for the gene expression data. Among the 248 genes showing significant profiles, the 194 of most interest are displayed in Fig. 4. When both groups of activated cells are compared to naïve cells for the expression of these 194 genes, the expression of 82 (42%) was up-regulated and 10 (5%) down regulated. Fifty three of the 194 (27.3%) were specific to SalOVA-responding OTII cells, whereas 52 (26.8%) were specific to alumOVA-responding cells.

The array of cytokines produced by activated CD4 T cells strongly influences the effectiveness of an immune response. SalOVA-responding CD4 T cells expressed the broader repertoire of cytokines. These included IL-1α, IL-1β, IL-6, IL-12α, IL-15, IL-27 (IL-30), IL-18, and IFN-γ. In contrast, mRNA for only a few cytokines including IL-4, IL-13, GM-CSF, TGFβ3 were detected in alumOVA-responding cells. OTII cells were induced by both Ag to up-regulate IL-2, IL-10, IL-17, IL-17C, IL-21, IL-27 (EBI3) and TGFβ1. In addition, SalOVA-responding OTII cells expressed many cytokine receptors: IL-1R1, IL-1R2, IL-1Rrp (T1/ST2), IL-12Rβ1, IL-12Rβ2, IL-15Rα, IL-17Re, IL-18R1, IL-18Rap, IL23R and GM-CSFRβ2. In contrast, alumOVA-responding OTII cells selectively up-regulated only IL-6Rα and IL-17Rb. Isolates from SalOVA-responding OTII cells also contained high levels of mRNA for a wide array of

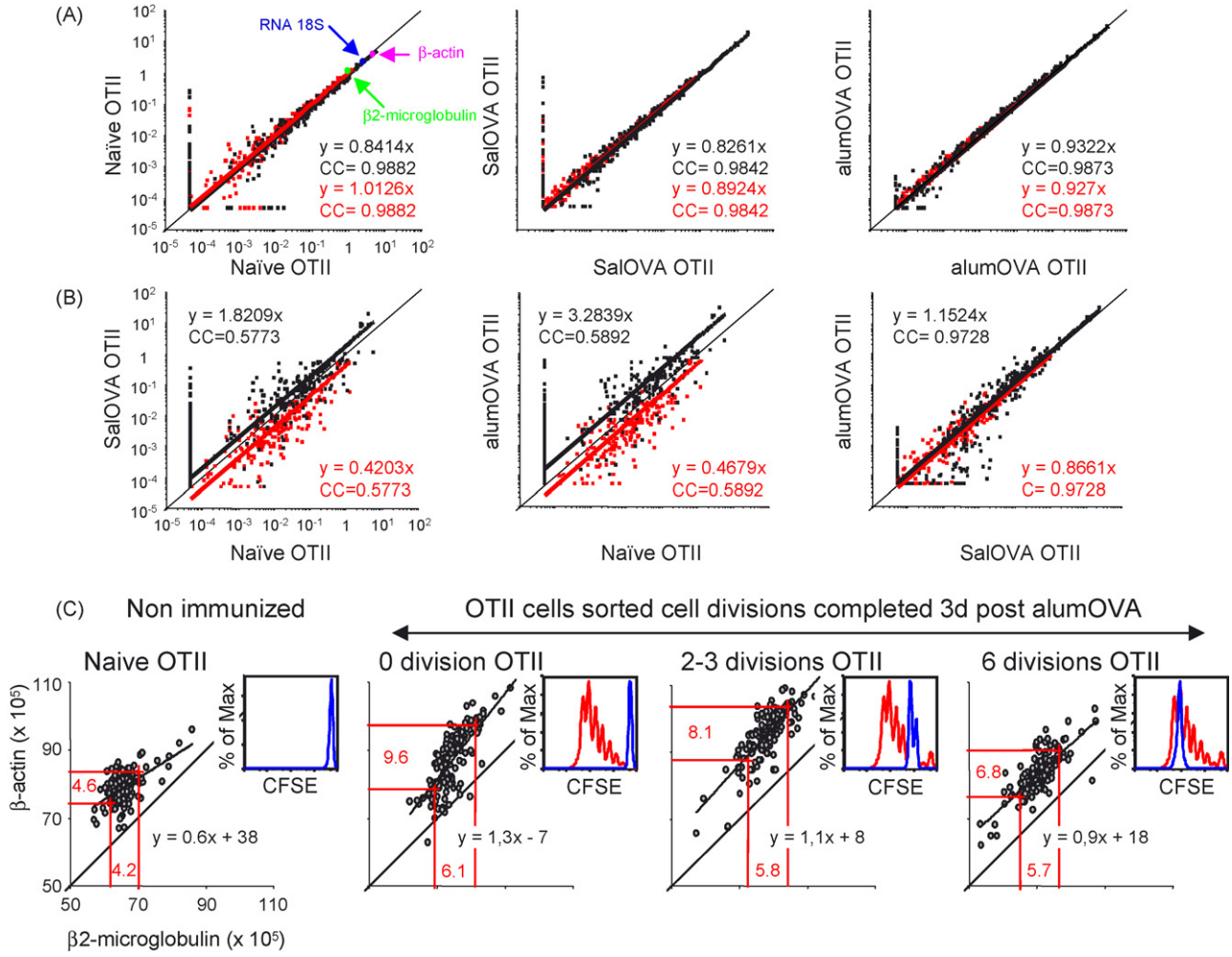

Fig. 3. Reproducibility and normalization of the gene expression data obtained by semi-quantitative real-time RT-PCR. (A and B) OTII cells (characterized in Fig. 2B) were isolated from popliteal LN either of non-immunized mice or mice that had been immunized 3 days before with either SalOVA or alumOVA. Specific mRNA levels were assessed by real time RT-PCR using microfluidic cards. The number of cycles required to reach a threshold signal – C_t (see Section 2) for each of the target genes minus either the Ct for β2-microglobulin (black dots), or the C_t for β-actin (red dots), was expressed as $2^{-\Delta C_t}$. The values were plotted: (A) for two samples of a similar population or, (B) for two samples of different populations. Dots specific for housekeeping genes (β-actin, RNA 18S, β2-microglobulin) are highlighted by color. The equation of the linear trendline, and the correlation coefficient (CC) calculated using β2-microglobulin (black), or the β-actin (red) as reference are specified. (C) Mice received CFSE-labeled OTII cells and were either non-immunized or immunized with alumOVA. Three days later, single OTII cells from popliteal LN were isolated on the basis of the number of cell cycles they had completed (see histograms top right corner) as follows: naïve OTII cells, OTII cells that had not divided, but were CD69+ (0 division), OTII cells that had completed 2–3 divisions, or those that had completed 6 divisions. Relative quantification of cDNA species specific of β-actin and β2-microglobulin genes was carried out by a duplex RT-PCR on single cells. Dot plots represent mRNA transcript level for the two genes, calculated as 2^{-C_t}. About 130 cells from each subset were analyzed. Equation of the trendline and standard deviation values (red) are shown.

chemokines (CCL2, CCL3, CCL4, CCL7, CCL9, CXCL1, CXCL2, CXCL4, CXCL9, CXCL11). The chemokine receptors – CCR1, CXCR3 and CX3CR1 – were also selectively associated with SalOVA-responding cells. On the other hand CCR4, CCR8, CCRL2 and CXCR5 expression was a feature of OTII-populations induced by both Ag.

B7 and TNF members expressed by CD4 T cells regulate the outcome of an immune response. A large number of B7 and TNF family members, including PD-1, ICOS, 4-1BBL, DR6, Fas, GITR, Light, Light-R, mDcTrailR2, OX40L, TNF-α, Troy were up-regulated in mRNA preparations from OTII cells activated by either Ag. Interestingly, DR3, LtβR and TWEAK appeared to be highly specific to SalOVA-responding OTII cells. Furthermore, cell surface molecules such as CD38, SLAMF7, SLAMF9, TLR5 and TLR-9 were associated with SalOVA-responding OTII cells whereas CD59a, CD62L, CD83, CD200, CD223 (Lag-3), CD224, Edg1, EphA3, Galectin-3, Itga2, and Neuropilin-1 were found in alumOVA-responding OTII cells. No obvious differential expression was observed for molecules involved in intracellular transduction signal (such as kinases, phosphatases), or the caspases and bcl-family molecules involved in cell survival/death. Nevertheless, many of these were increased in responding compared to naïve OTII cells.

Finally, there was clear differential expression of several genes encoding transcription factors. CIITA, Hlx and T-bet were associated with SalOVA-responding OTII cells, whereas Fosl1, GATA-3, Gfi1, Helios and Nfatc1 were associated with alumOVA-responding OTII cells.

4. Discussion

Despite the importance of CD4 Th cells in the regulation of immune responses, there is much more to be learnt about the diversity of effector functions induced *in vivo* by different types of immunization. Transfer of naïve OTII cells into normal mice, and subsequent immunization with SalOVA or alumOVA allowed the emergence of distinct effectors to be monitored during Th1

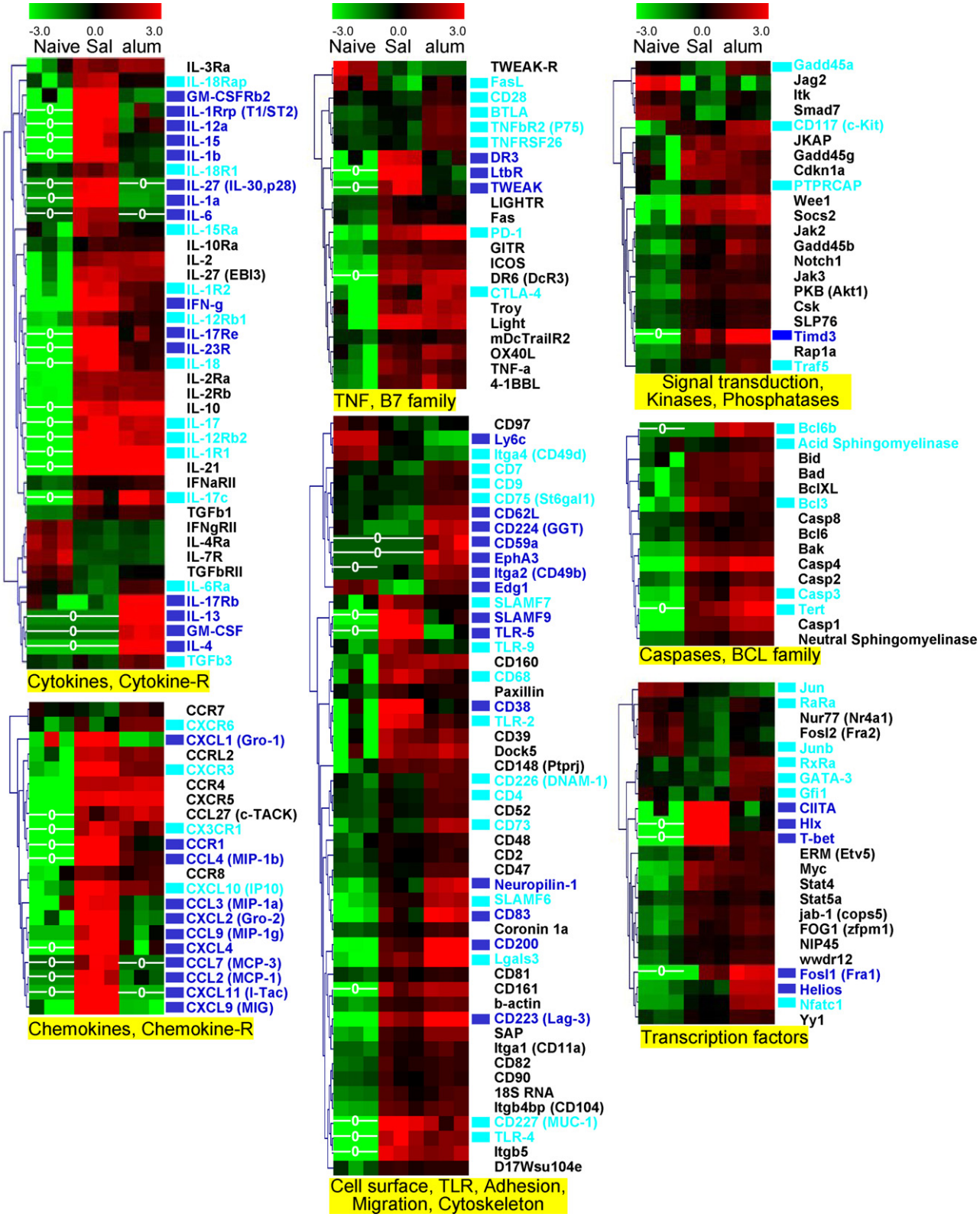

Fig. 4. Genes differentially expressed by OTII cells responding either to SalOVA or alumOVA. The mRNA was extracted from OTII cells that were purified from three independent groups of ten naïve, or SalOVA-immunized or alumOVA-immunized chimeric mice. Mean-centered cluster analysis is performed on differentially expressed genes within functional families assessed by real time RT-PCR using microfluidic cards. Turquoise and blue boxes/text highlight mRNA levels, respectively, with 2–5 or more than 5-fold differences between SalOVA- and alumOVA-responding OTII cells. Zeros notify the absence of gene expression in one condition.

and Th2 types of immune responses. Both SalOVA- and alumOVA-responding OTII populations contain restricted subsets of highly specialized cells producing either IFN-γ or IL-4, respectively. Importantly, the perspective of Th1- or Th2-polarization, defined as either IFN-γ- or IL-4-producing cells does not reflect the diversity of effector Th subsets generated during polarized immune responses *in vivo*. Indeed, analysis of the transcriptomes of naïve or activated OTII at the small scale of 384 genes of immunological relevance reveals global differences in both the diversity of T effectors cells and the factors leading to their generation in responses to SalOVA or alumOVA. Consistent with the acquisition of a range of effector functions, the majority of differentially expressed genes was up-regulated in activated compared to naïve OTII cells either selectively to one or another Ag (54%), or induced in both responses (42%). The results might reflect the generation by these two Ag of common pool of CD4 Th cells in addition to selective highly specialized primed cells; both of these are discussed below.

This work shows that OTII cells already acquired selective and divergent effector functions by 3 days depending on the Ag – SalOVA or alumOVA. The production of large number of cytokines and chemokines was associated with SalOVA-responding OTII cells. These included IL-1α, IL-1β, IL-6, IL-12α, IL-15, IL-27 (IL-30), IL-18, and IFN-γ, and CCL2, CCL3, CCL4, CCL7, CCL9, CXCL1, CXCL2, CXCL4, CXCL9, and CXCL11. In addition, they specifically expressed three TNF family members – DR3, LtbR, and TWEAK. Although these molecules have been shown to potentiate Th1 (Bamias et al., 2003; Migone et al., 2002; Papadakis et al., 2004), or pro-inflammatory cytokines (Chicheportiche et al., 2002; Stopfer et al., 2004), their potential role in the initiation or maintenance of the Th1 phenotype and IFN-γ production during *Salmonella* infection remains to be addressed. In contrast, alumOVA-responding OTII cells selectively produce only IL-4, IL-13, GM-CSF.

The possibility that some common CD4 Th cells develop is suggested by the finding that both alumOVA and SalOVA induce a proportion of the OTII cells to produce some or all of the following cytokines: IL-2, IL-10, IL-17, IL-17C, IL-21, and IL-27 (EBI3). In addition, genes that are associated with follicular helper functions, such as Bcl6, CD200, CXCR5, ICOS, IL-21, SAP, PD-1 (Chtanova et al., 2004; Vinuesa et al., 2005) were up-regulated in both SalOVA- and alumOVA-responding OTII cells. Although selective up-regulation of CD200 was more marked in alumOVA-responding OTII cells, the results indicate that follicular Th cells might develop in responses to Th1 or Th2 types of Ag, as previously suggested (Smith et al., 2004).

Our results show that great diversification from a naïve CD4 T cell population has already occurred within 3 days after immunization. The precocity of this divergent commitment highlights the importance of identifying the first signals delivered by dendritic cells that drive CD4 T cells to differentiate and acquire effector functions. The model system used has identified differential gene expression that deserve further study: IL-17Re, CD38, SLAMF7, SLAMF9, TLR-5, among the SalOVA-responding OTII cells and CD59a, CD62L, CD83, CD200, CD223, CD224, IL-6Rα, IL-17Rb, Edg1, EphA3, Galectin-3, Itga2, Neuropilin-1, SLAMF6, Helios and Fosl1 among the alumOVA-responding OTII cells. The expression of IL-6Rα and IL-17Rb restricted to alumOVA-responding OTII cells, for example, is of interest regarding the issue of what, if not IL-4 itself, is the trigger for IL-4 production by CD4 T cells in response to alum-precipitated antigen *in vivo* (Brewer et al., 1999; Cunningham et al., 2002, 2004b). IL-6 has been shown to induce CD4 T cells to become Th2 (Dodge et al., 2003; Rincon et al., 1997). IL-17Rb has been found in human (Angkasekwinai et al., 2007; Bosco et al., 2006; Chtanova et al., 2004; Wang et al., 2007) and mouse (Angkasekwinai et al., 2007) CD4 Th2 cells. IL-17Rb binds to IL-17B and IL-25 (IL-17E). IL-25 injection or systemic over-expression in transgenic mice provokes the up-regulation of Th2-features or proallergic pathologies (Angkasekwinai et al., 2007; Fallon et al., 2006; Kim et al., 2002; Pan et al., 2001). IL-25 has been shown to take part in the initiation of Th2 differentiation (Angkasekwinai et al., 2007) or in the maintenance and restimulation of Th2 memory cells (Wang et al., 2007). Although the mechanism of action of IL-25 has been shown to be IL-4-independent, IL-4 synergize with IL-25 to induce Th2 features. It is plausible that alum has the ability to induce IL-17Rb expression in responding CD4 T cells rendering them sensitive to IL-25. Potential sources of IL-25 identified are CD4 Th2 cells (Fort et al., 2001) (although this study failed to show selective IL-25 expression in OTII cells), mast cells (Ikeda et al., 2003), dendritic cells (Wang et al., 2007), and lung epithelial cells (Angkasekwinai et al., 2007), suggesting that innate immune cells may first produce this cytokine. Therefore, it is possible that alum promotes Th2 immune response through the induction of IL-25 and/or IL-6.

Conflict of interest

The authors have no conflicting financial interests.

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