3. Results

3.1. Isolation of CD4 T cell subsets

3.1.1. Identification and isolation of tonsillar CD4 T cell subsets based on co-expression of CXCR5 and ICOS

Upon activation in secondary lymphoid organs CD4 T cells upregulate chemokine receptor CXCR5 to facilitate migration into B cell follicles to foster B cell help (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000). This process is accompanied by regulation of several genes including molecules involved in costimulation such as ICOS. Freshly isolated CD4 T cells from tonsils when co-stained with CXCR5 and ICOS; a progressive up regulation was seen with subsets, which were clearly sub-dividable (Fig. 3A). Based on this staining pattern, tonsillar CD4 T cells were sorted into three subsets namely $CXCR5^{-}ICOS^{-/lo}$, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells (Fig. 3B). Approximately, 30% of the cells in the $CXCR5^{-}ICOS^{-/lo}$ subset expressed ICOS. Percentage distribution of each subset varied between samples (Fig. 3A). In general, the $CXCR5^{hi}ICOS^{hi}$ subset was the smallest with the other two having approximately the same size.

Individual subsets displayed distinct morphology when viewed under light microscope (Fig. 3C). $CXCR5^{-}ICOS^{-/lo}$ cells showed a largely rounded appearance as compared to $CXCR5^{hi}ICOS^{hi}$ cells. $CXCR5^{hi}ICOS^{hi}$ cells displayed an amoeboid appearance with a capacity to rapidly adhere to each other and form clumps within 4 hours of culturing. $CXCR5^{lo}ICOS^{int}$ cells had mixed morphology of round and amoeboid cells without adhering readily.

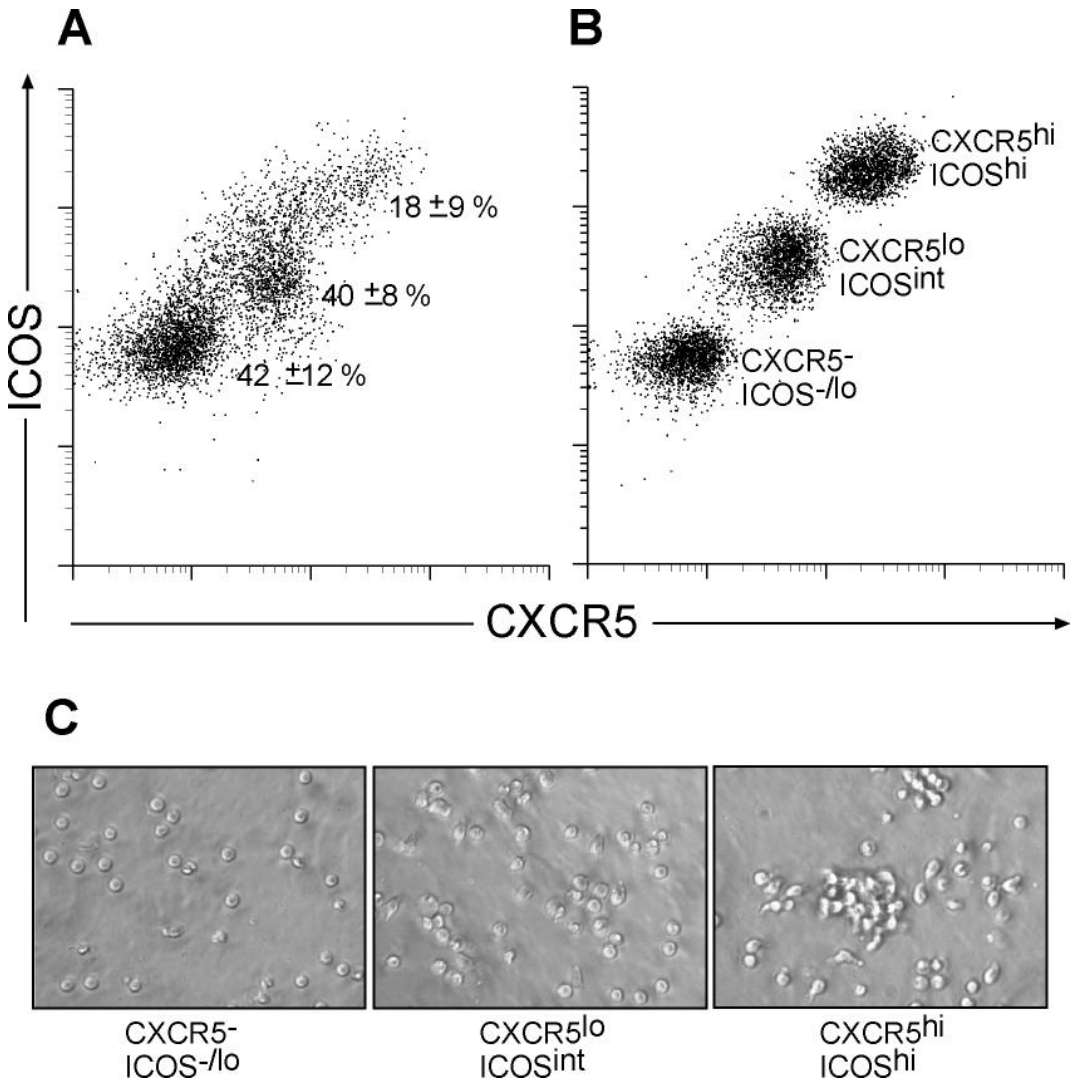

Fig. 3: Isolation of tonsillar B helper T cells. (A) Expression pattern of CXCR5 and ICOS on freshly isolated tonsillar T cells using flow cytometer. Population sizes are given relative to the total number of tonsillar CD4 T cells (mean ± SD; n=19). (B) Overlay of post-sort CD4 T cell subpopulations. MACS sorted tonsillar CD4 T cells were FACS sorted into three subsets namely $CXCR5^{-}ICOS^{-/lo}$, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$. Purity of the subsets in all the downstream experiments was always above 95%. ICOS was expressed on 30% of the $CXCR5^{-}$ cells rendering this subset to be named $CXCR5^{-}ICOS^{-/lo}$ cells. Other two $CXCR5^{+}$ subsets, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$, had intermediate and high levels of ICOS respectively. (C) Individual CD4 T cell subpopulations displayed distinct morphology when viewed under light microscope. $CXCR5^{-}ICOS^{-/lo}$ cells showed largely rounded appearance as compared to $CXCR5^{hi}ICOS^{hi}$. $CXCR5^{hi}ICOS^{hi}$ cells displayed amoeboid appearance with a capacity to rapidly adhere and form clumps within 4 hours of standing in culture. $CXCR5^{lo}ICOS^{int}$ had mixed morphology of rounded and amoeboid cells lacking adherence potential.

T cells switch the expression of CD45 isoforms based on their state of activation in peripheral blood. Expression of CD45RA and CD45RO was determined by flow

cytometry (Fig. 4). CD45RA was expressed on nearly 80% of $CXCR5^{-}ICOS^{-/lo}$ cells, which was about 15% on $CXCR5^{lo}ICOS^{int}$ cells and almost absent on $CXCR5^{hi}ICOS^{hi}$ cells. On the other hand 91% of $CXCR5^{lo}ICOS^{int}$ cells and 99% of $CXCR5^{hi}ICOS^{hi}$ cells had CD45RO on their surface. In peripheral blood CD45RA is a marker for naïve T cells and CD45RO is associated with classical memory T cells. Hence higher expression of CD45RA on $CXCR5^{-}ICOS^{-/lo}$ indicates a phenotype similar to naïve cells. Likewise higher expression of CD45RO on $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells indicates that their phenotype might be closely related to memory T cells.

These initial observations (basically staining pattern and cell morphology) led us to anticipate that tonsillar CD4 T cells are indeed a heterogeneous pool, which may differ in their functional properties with B cell help confined to either $CXCR5^{-}ICOS^{-/lo}$ or $CXCR5^{hi}ICOS^{hi}$ subsets. Hence tonsillar CD4 T cell subsets were further characterized based on their migratory potential towards CXCL13, chemokine receptor expression, capacity to stimulate B cells and secretion of CXCL13. Proliferative potential and susceptibility to undergo apoptosis was also determined. Large-scale gene expression profiles were generated and compared the transcript profiles with peripheral blood CD4 T cells subsets. In addition, suitability of CD57 as a follicular B helper T cell marker was evaluated.

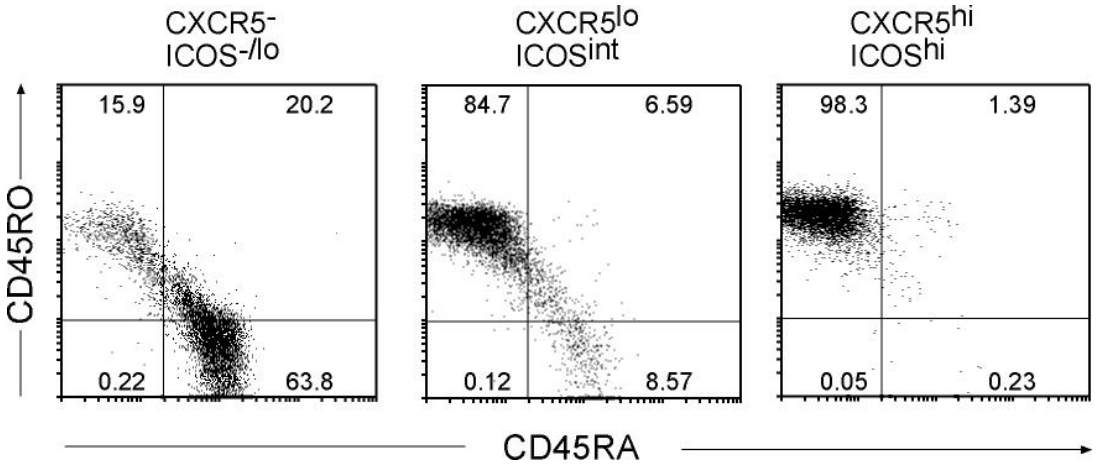

Fig. 4: Expression of CD45RA and CD45RO on tonsillar CD4 T cell subsets. Flow cytometry dotplots show CD45RA versus CD45RO staining. Tonsillar CD4 T cells were stained with CXCR5 and ICOS in combination with CD45RA and CD45RO monoclonal antibodies. CD45RA was prominently expressed on $CXCR5^{-}ICOS^{-/lo}$ cells where as CD45RO was expressed on $CXCR5^{+}CD4^{+}$ T cell subsets. Dotplots are representative of two independent tonsils.

3.1.2. Isolation of CD4 T cell subsets from peripheral blood

All peripheral blood CD4 T cell subsets – Naïve, T_{CM1}, T_{CM} and T_{EM} were sorted using the sorting strategy described in materials and methods. The phenotype of the sorted cells was as follows: naïve – $CD45RA^{+}CCR7^{+}$, T_{CM1} – $CD45RA^{-}CCR7^{+}CXCR5^{+}$, T_{CM} – $CD45RA^{-}CCR7^{+}$ and T_{EM} – $CD45RA^{-}CCR7^{-}$ Post-sort purity of peripheral blood subsets was routinely ≥ 95% (Fig. 5).

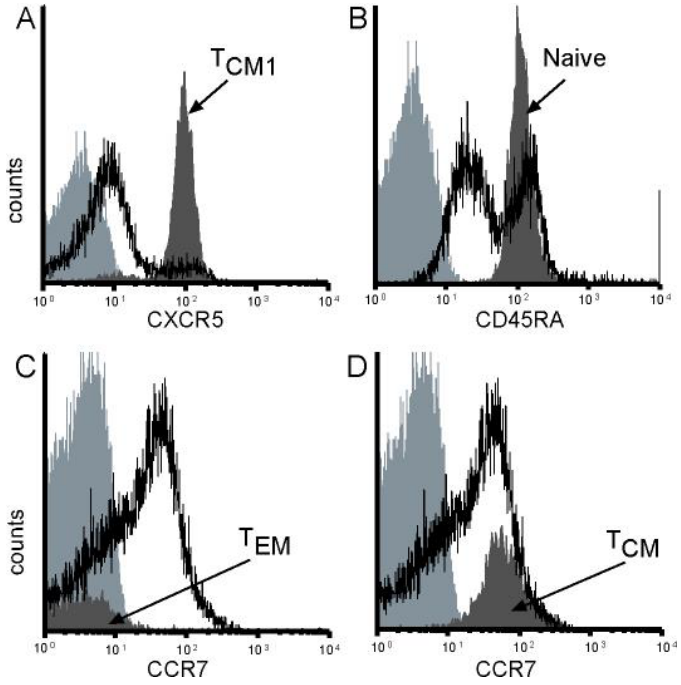

Fig. 5: Purity of peripheral blood subsets. Each figure shows an overlay of three histograms representing isotype control in light gray closed histogram, cells before sorting in black open histogram and sorted cells in dark gray closed histogram (pointed arrow) **(A)** About 15% of the human peripheral blood CD4 T cells express CXCR5 which were directly sorted from CD4 T cells after labeling with anti-human CXCR5. **(B)** Naïve cells isolated using CD45RA microbeads. **(C)** $CCR7^{+}$ T_{CM} cells and **(D)** $CCR7^{-}$ T_{EM} cells.

3.2. Varying levels of CXCR5 on $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells does not confer differential chemotactic potential

About 60% of tonsillar CD4 T cells expressed chemokine receptor CXCR5. A distinct feature of these cells is that the amount of expression of CXCR5 is highly variable as compared to peripheral blood T_{CM1} cells. The only known ligand for CXCR5 is CXCL13, expressed on follicular dendritic cells and stromal cells of B cell areas of secondary lymphoid organs. T cells expressing CXCR5 respond to CXCL13 and were shown to migrate into follicles for cognate interactions with B cells.

In order to determine the differential capacity of tonsillar CD4 T cell subsets, a migration assay was performed using CD4 T cells in a transwell assay. Before starting the actual experiment optimal concentration of CXCL13 was determined which was found to be about 300nM. Media without any chemokine was used as a negative control. As shown in figure 6, controls from all the three subsets as well as $CXCR5^{-}ICOS^{-/lo}$ cells showed a small number of spontaneously migrating cells. In contrast, $CXCR5^{hi}ICOS^{hi}$ cells display chemotaxis towards CXCL13 and surprisingly, does not differ much as compared to $CXCR5^{lo}ICOS^{int}$ cells. Hence, it was concluded that CXCR5 expression at varying levels on tonsillar CD4 T cells confer similar migratory potential.

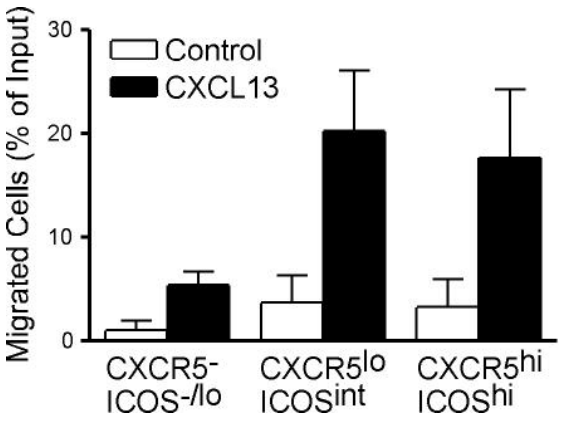

Fig. 6: CXCR5 expression at varying degrees confers similar chemotatic ability of CD4 T cell subsets. Migration potential of the CD4 T cell subsets towards CXCL13 (300nM) was tested in a Boyden chamber. $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ CD4 T cells did not differ in their chemotatic response to CXCL13. Chemotaxis of cells in $CXCR5^{-}ICOS^{-/lo}$ subset were similar to the levels of spontaneous migration in controls. Media alone without chemokine was used as negative control. Data shown is derived from 3 independent experiments (Mean ± SD).

3.3. Spectrum of chemokine receptor expression on tonsillar and peripheral blood CD4 T cell subsets

To determine expression of chemokine receptors on tonsillar CD4 T cells, which might be responsible for differential migratory capacities of $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells, freshly isolated cells were stained with subset specific markers, CXCR5 and ICOS, in combination with one of the chemokine receptor and analyzed on a flow cytometer. Thirteen chemokine receptors were tested, of which only CXCR3, CXCR4, CCR4 and CCR7 were expressed on tonsillar CD4 T cell subsets (Fig. 7A and B). Among CXC chemokine receptors only CXCR4 was expressed on all the subsets at higher levels. CXCR3 was expressed by a subpopulation, indicating that tonsillar CD4 T cell subsets identified based on the

co expression of CXCR5 and ICOS could be still heterogenous (Fig. 7E). $CXCR5^{-}ICOS^{-/lo}$ cells express high levels of CCR7, which allows them to retain in T cell areas. On the other hand $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells down-regulated CCR7 accompanied by up-regulation of CXCR5, which enables them to leave T cell areas and enter germinal centers. Very low levels of CCR7 on $CXCR5^{lo}ICOS^{int}$ cells might contribute to a different micro-anatomical positioning as compared to $CXCR5^{hi}ICOS^{hi}$ cells. CCR4 was induced in $CXCR5^{-}ICOS^{-/lo}$ cells and was upregulated on $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells (Fig. 7F).

The restricted expression of chemokine receptors by tonsillar CD4 T cell subsets prompted us to compare them with peripheral blood CD4 T cell subsets. Indeed, these subsets were found to express most of the receptors tested except CXCR1, CXCR2 and CXCR6 (Fig. 7C and D). So to summarize, naïve CD4 T cells express the homeostatic chemokine receptors CCR7 and CXCR4 that allow them to recirculate between secondary lymphoid tissues. In addition, naïve cells expressed lower levels of inflammatory chemokine receptors CXCR3 and CCR4. Within the central memory compartment, T_{CM} cells retained CCR7 and CXCR4 with the expression of high levels of CXCR3 and CCR4. T_{CM1} cells had a chemokine receptor expression pattern similar to T_{CM} cells, except that they had upregulated CXCR5. T_{EM} cells express CXCR3, CCR2, CCR3, CCR4 and CCR5, which allow these cells migrate to inflamed tissues. CCR1, CCR6 and CCR9 were common to all antigen-experienced cells.

Thus other than CXCR5, tonsillar CD4 T cell subsets differ, only in the expression of the chemokine receptors CCR4 and CCR7. Since CCR4 does not show a large difference between the subsets, low levels of CCR7 alone might play an important role in positioning $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells in the B cell follicles.

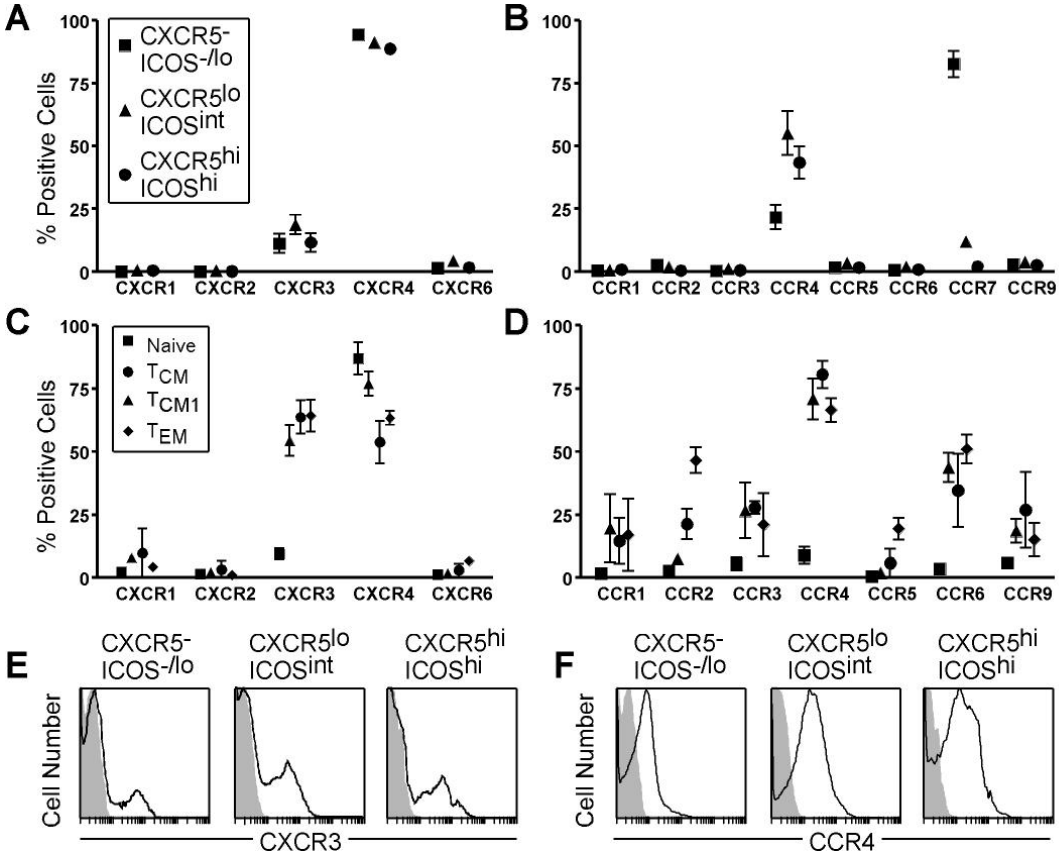

Fig. 7: Spectrum of chemokine receptor expression in tonsillar and peripheral blood CD4 T cell subsets. Chemokine receptors expressed by freshly isolated tonsillar (A, B) and peripheral blood (C, D) CD4 T cell subsets was determined by flow cytometry. Expression of CXC-chemokines receptors (A, C) and CC-chemokine receptors (B, D) is shown. CXCR5 and CCR7 in peripheral blood and CXCR5 in tonsillar subsets was not shown as these were used as sorting markers. Apart from CXCR4 and CCR7, only CXCR3 and CCR4 are expressed on tonsillar CD4 T cell subsets (E) CXCR3 was expressed on a small subset of all the three tonsillar CD4 T cell subpopulations whereas CCR4 (F) was expressed on all the tonsillar CD4 T cells at varying levels. Data shown is derived from 3 independent experiments (mean ± SD).

3.4. Follicular B cell help *in vitro* correlates with CXCR5/ICOS expression

Germinal center T helper cells are programmed to migrate into follicles for cognate interactions with B cells leading to enhanced immunoglobulin secretion. To assess the relative capacities of the tonsillar CD4 T cells subsets to stimulate B cells, T cell - B cell co-cultures were established. T cell subsets were cultured with autologous B cells for defined period of time and the amount of IgG secreted by B cells determined in the supernatant. Cell without stimulation and stimulated with anti-CD3/CD28 were tested and for negative control B cells were cultured in the absence of T cells.

Without additional T cell stimulation (Fig. 8A), from day 4 to day 7 there was no enhancement of IgG secretion by $CXCR5^{-}ICOS^{-/lo}$ cells, which was similar to the negative control. In contrast, $CXCR5^{lo}ICOS^{int}$ induced IgG production that increased from day 7 to day 10. Most efficient B cell helpers are $CXCR5^{hi}ICOS^{hi}$ cells, inducing IgG secretion form day 4 itself and reaching peak levels at day 10. In case of polyclonal stimulation (Fig. 8B), the induction of $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ was much more pronounced. Surprisingly, under these conditions the most efficient B cell helpers were $CXCR5^{lo}ICOS^{int}$ cells instead of $CXCR5^{hi}ICOS^{hi}$ cells. This was an unexpected observation that prompted to investigate the survival capacities of the subsets (shown below).

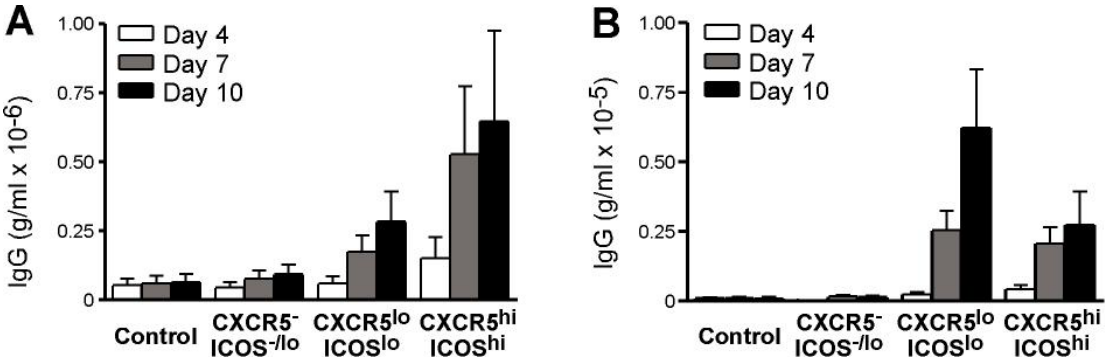

Fig. 8: Follicular B cell help *in vitro* correlates with CXCR5/ICOS expression. Equal number (1 x 10^5) of sorted $CXCR5^{-}ICOS^{-/lo}$, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells were cocultured with autologous B cells in 96 well culture plates for 4-10 days. Control represents monoculture of B cells. After incubating for indicated time points, culture supernatants were collected and immunoglobulin concentrations were determined by ELISA. **(A)** In the absence of stimulatory agent $CXCR5^{hi}ICOS^{hi}$ T cells were the most potent stimulatory of B cells, which is evident as early as day 4. **(B)** Upon stimulation with anti-CD3/CD28 antibodies, IgG secretion was enhanced in general for all the subsets. B cells in the presence of $CXCR5^{lo}ICOS^{int}$ cells secreted higher levels of IgG.

3.5. CXCL13 secretion *in vitro* correlates with CXCR5/ICOS

Expression of CXCL13 by $CXCR5^{+}$ T cells from tonsils was observed from global gene expression profiling of CD4 T cell subsets (also shown below). In order to check the secretion of CXCL13, co-cultures similar to IgG secretion were set and the cell free supernatant was used for ELISA. B cells cultured alone up to 10 days do not secrete CXCL13 (Fig. 9A). When $CXCR5^{-}ICOS^{-/lo}$ subset was cocultured with B cells without anti-CD3/CD28 stimulation, CXCL13 was not detected up to 10 days. In the presence of $CXCR5^{lo}ICOS^{int}$ cells CXCL13 secretion was detectable from day 4 on and gradually increased till day 10. In case of $CXCR5^{hi}ICOS^{hi}$ cells, highest levels were detected compared to other subsets, indicating that they are the most efficient producer of CXCL13. Upon T cell

stimulation with anti-CD3/CD28, CXCL13 secretion was still not detectable in $CXCR5^{-}ICOS^{-/lo}$ cells (Fig. 9B). But in case of $CXCR5^{lo}ICOS^{int}$ cells and $CXCR5^{hi}ICOS^{hi}$ cells, levels of CXCL13 secretion were enhanced as compared to cocultures without additional stimulation.

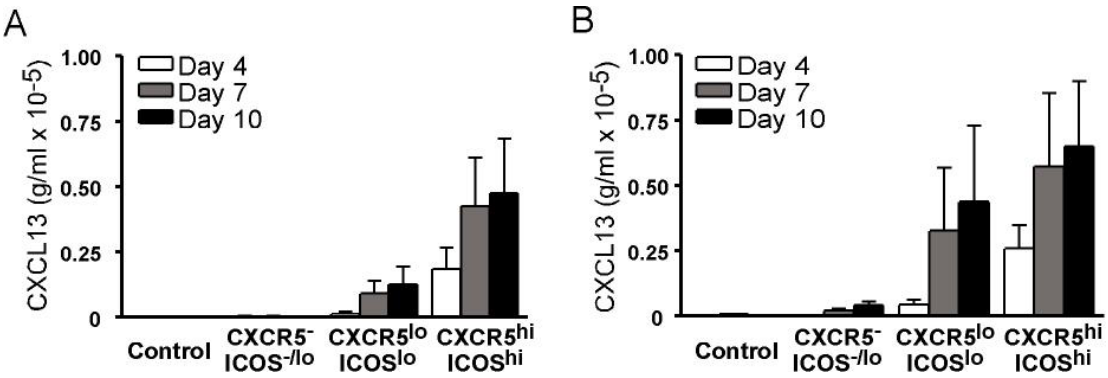

Fig. 9: CXCL13 secretion *in vitro* correlates with CXCR5/ICOS expression. Equal number (1 x 10^5) of sorted $CXCR5^{-}ICOS^{-/lo}$, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells were cocultured with autologous B cells in 96 well culture plates for 4-10 days. Control represents monoculture of B cells. After incubating for indicated time points, culture supernatants were collected and CXCL13 concentrations were determined by ELISA. **(A)** $CXCR5^{hi}ICOS^{hi}$ CD4 T cells secrete higher amount of CXCL13 in the absence of stimulatory agent. **(B)** Addition of anti-CD3/CD28 antibody enhanced CXCL13 secretion.

Endogenous expression of CXCL13 from freshly isolated tonsillar subsets was determined by western blotting (Fig. 10). In $CXCR5^{-}ICOS^{-/lo}$ cells there was no CXCL13 expression, whereas increasing amount was observed from $CXCR5^{lo}ICOS^{int}$ cells to $CXCR5^{hi}ICOS^{hi}$ cells. CXCL13 detection by western blotting and ELISA confirm that the expression is an inherent capacity of the subsets and is not influenced by activation or during involvement in cognate help to B cells.

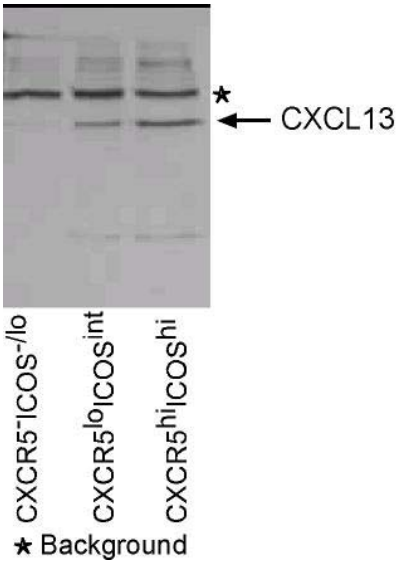

Fig. 10: CXCL13 protein expression on tonsillar CD4 T cell subsets. Sorted tonsillar CD4 T cells were lysed and concentration of total protein was determined on spectrophotometer using BSA as standard. Normalized volumes of each sample was used for western blotting. Arrow points to the a band of about 30 kDa corresponding to the dimerized form of CXCL13 which is about 12,6 kDa. Differential expression of CXCL13 in all the tonsillar CD4 T cell subsets was observed. Figure is representative of three independent experiments.

3.6. $CXCR5^{hi}ICOS^{hi}$ CD4 T cells have a reduced proliferative potential and enhanced susceptibility to apoptosis

CD4 T cells do not survive alone *in vitro* for long time without external stimulation or presence of cytokines. In the presence of B cells, CD4 T cells engage in cognate interactions leading to productive humoral immune responses. This process also enhances the survival capacity of T cells. Additionally, TCR triggering via CD3 and costimulation by CD28 leads to expansion and differentiation of CD4 T cells. To determine the differential proliferative capacity of the tonsillar CD4 T cell subsets, T cells were labeled with CFSE and then co-cultured with autologous B cell. Two different conditions were tested, without any external stimulation and stimulation with anti-CD3/CD28 antibodies. The cells were cultured up to 5 days and the degree of proliferation measured by flow cytometry.

In the absence of stimulatory agents (Fig. 11A), no proliferation was detected in any of the subsets even up to 5 days. Strikingly, when cells were anti-CD3/CD28 stimulated (Fig. 11B), the cells started to proliferate as early as day 3, with highest proliferation detected in $CXCR5^{lo}ICOS^{int}$ cells (59%) followed by $CXCR5^{-}ICOS^{-/lo}$ (45%) and then $CXCR5^{hi}ICOS^{hi}$ cells (24%). At day 4, still $CXCR5^{hi}ICOS^{hi}$ cells had less percentage of cells proliferating, about 24%, as compared to the other two subsets but interestingly; $CXCR5^{-}ICOS^{-/lo}$ cells attained the same percentage of dividing cells as $CXCR5^{lo}ICOS^{int}$ subset (85%). At day 5, only 50% of the $CXCR5^{hi}ICOS^{hi}$ cells had undergone more than one division as compared to about 90% in $CXCR5^{-}ICOS^{-/lo}$ subset and $CXCR5^{lo}ICOS^{int}$ subset. Furthermore, in $CXCR5^{hi}ICOS^{hi}$ cells only 9.8% of the cells had undergone 3-4 cell divisions. Whereas there were about 20% more cells that had undergone 3-4 cell divisions in the $CXCR5^{-}ICOS^{-/lo}$ subset compared to the $CXCR5^{lo}ICOS^{int}$ subset. This data

altogether suggests that CD4 T cells need an external stimulus apart from the presence of B cells to undergo proliferation. Among the tonsillar CD4 T cell subsets $CXCR5^{hi}ICOS^{hi}$ cells show diminished rate of proliferation. $CXCR5^{-}ICOS^{-/lo}$ followed by $CXCR5^{lo}ICOS^{int}$ has enhanced proliferation.

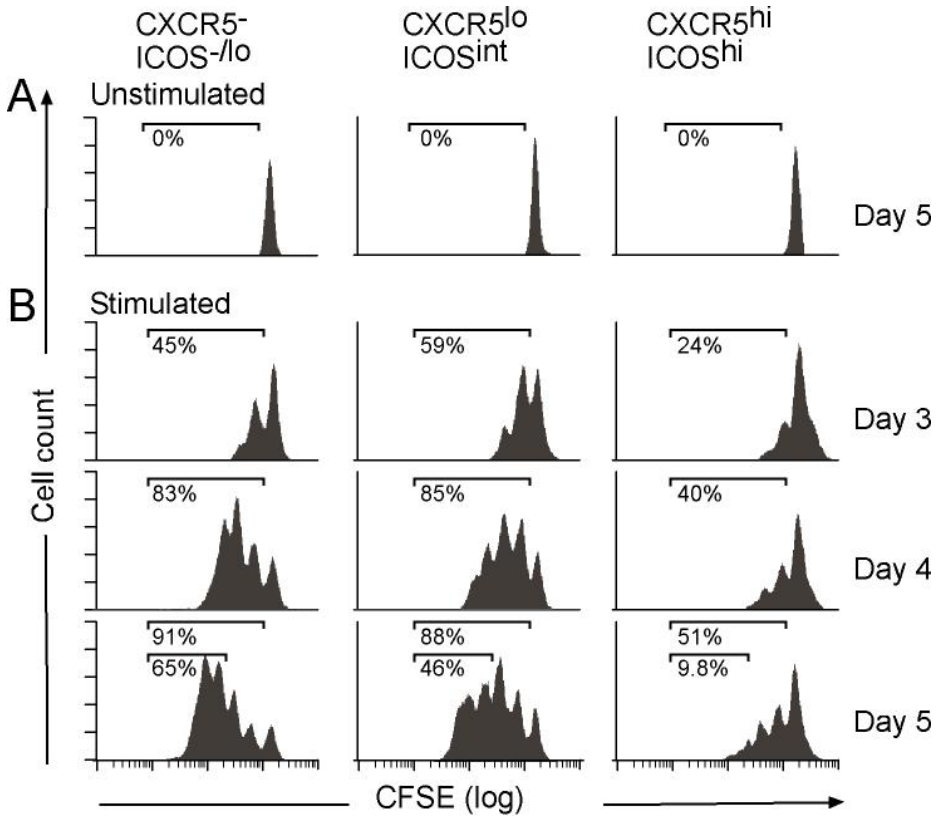

Fig. 11: $CXCR5^{hi}ICOS^{hi}$ CD4 T cells have a reduced proliferative potential and enhanced susceptibility to apoptosis. Tonsillar CD4 T cells were labeled with CFSE and equal numbers of T cells were cocultured with autologous B cells in 96 well culture plates. (A) In the absence of stimulatory agent, cells did not proliferate even up to 5 days. (B) In the presence of anti-CD3/CD28 antibodies, T cells showed proliferation as early as day 3. At day 5, 91% of the $CXCR5^{-}ICOS^{-/lo}$ cells had proliferated with 65% undergoing more than 3 divisions. Among $CXCR5^{lo}ICOS^{int}$ cells, 88% underwent proliferation with 45% of the cells having more than 3 divisions. In contrast, only half the $CXCR5^{hi}ICOS^{hi}$ cells proliferated with just 9.8% reaching more than 3 divisions.

As the cells undergo proliferation in cultures the expression of CXCR5 dramatically changed compared to ICOS. As shown in figure 12, tonsillar CD4 T cells show downregulation of CXCR5 starting from day 3 itself. On day 5, there was a considerable size of the CD4 T cell population that has downregulated CXCR5. On the other hand, expression of ICOS was found to be constant even when the cells divide more than 4 times. In contrast to the expression of CXCR5 and ICOS on

CD4 T cells, sorted subsets when cocultured with B cell did not show significant changes in the expression of the above markers (Fig. 13).

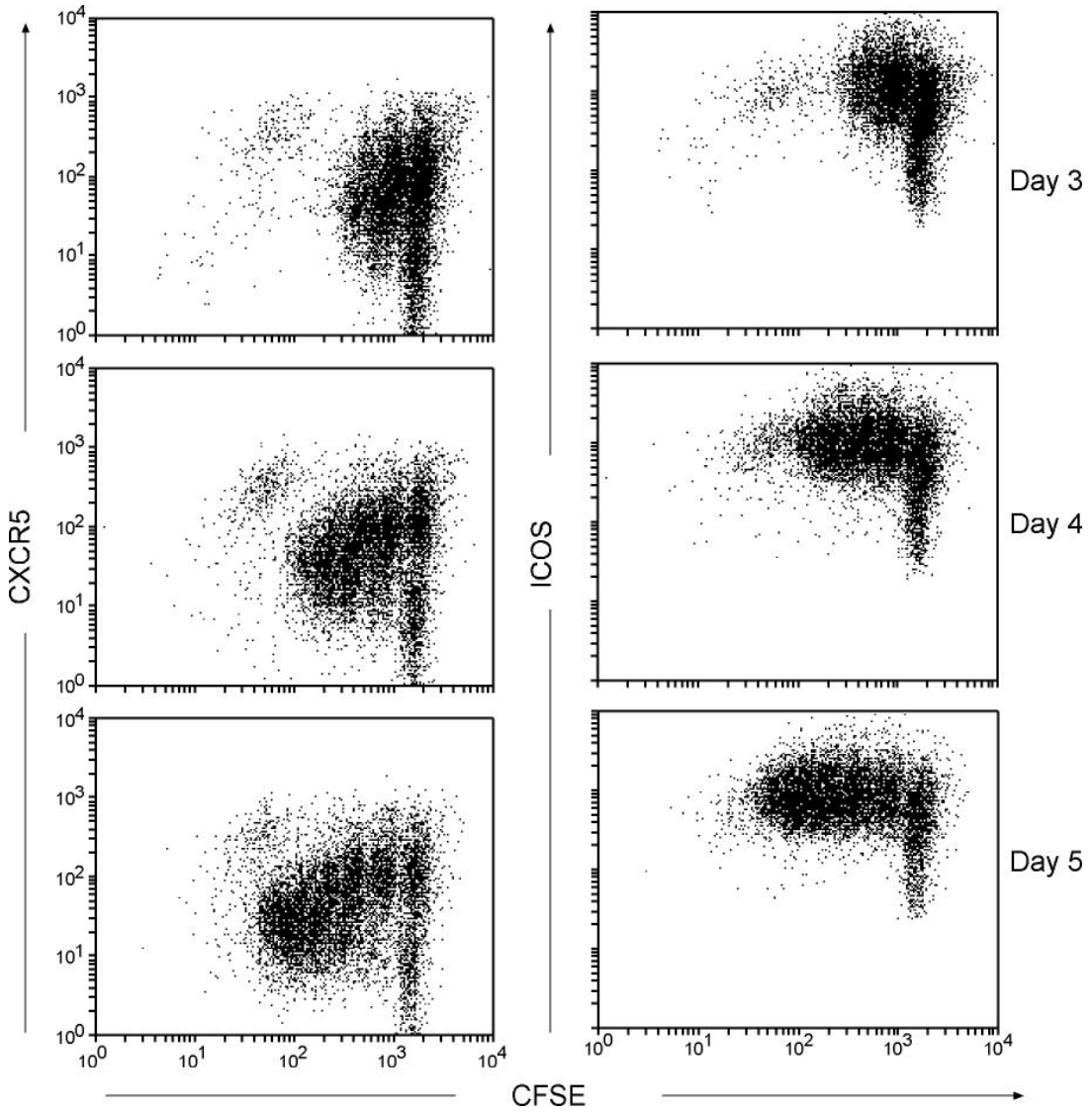

Fig. 12: Proliferating cell from CD4 T cell-B cell cocultures down regulate CXCR5 but maintain ICOS expression. Tonsillar CD4 T cells were labeled with CFSE and cocultured with autologous B cells. At day 3, 4 and 5, cells were co-stained with CXCR5-ICOS and read on flow cytometer. Each dotplot represent CFSE on x-axis and either CXCR5 (left panel) or ICOS (right panel) on y-axis. Proliferating cells loose CXCR5 starting from day 3. After day 5, a considerable size of T cells downregulated CXCR5. ICOS expression was however maintained on proliferating cells.

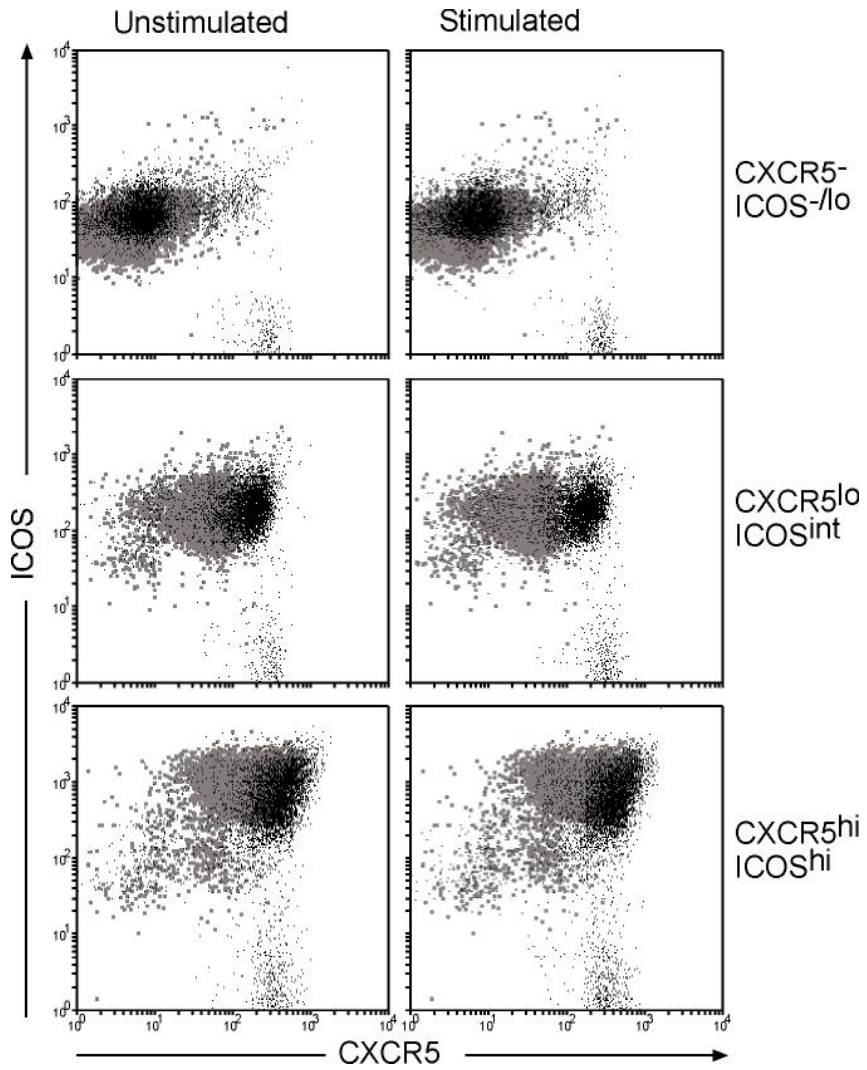

Fig. 13: Sorted subsets maintain CXCR5 and ICOS expression in cocultures. Dot plots represent CXCR5 versus ICOS staining of the CD4 T cell subsets. Each dot plot is an overlay of before (gray dots) and after co-culturing (black dots) of tonsillar CD4 T cell subsets with autologous B cells. Left panel shows unstimulated and right panel represents anti-CD3/CD28 stimulated. There was no significant difference between the expression of CXCR5 and ICOS either before culturing or after culturing in the presence or absence of stimulation. Data is representative of five independent observations.

After determining that $CXCR5^{hi}ICOS^{hi}$ cells have limited proliferative capacity, it was interesting to find out the susceptibility of the subsets to undergo apoptosis. To perform this, tonsillar CD4 T cells were sorted and co-cultured with B cells as done before in proliferation assays. Cells were stained with 7-AAD and Annexin V to determine live, early apoptotic and late apoptotic cells. At day 3 (Fig. 14), the percentage of apoptotic cells was remarkably enhanced in $CXCR5^{hi}ICOS^{hi}$ subset (42%) as compared to $CXCR5^{lo}ICOS^{int}$ subset (25%) and $CXCR5^{-}ICOS^{-/lo}$ subset (15%). In summary, the $CXCR5^{hi}ICOS^{hi}$ cells are prone to apoptosis as compared

to other tonsillar CD4 T cell subsets, again supporting our hypothesis that the terminally activated state of follicular B helper T cells.

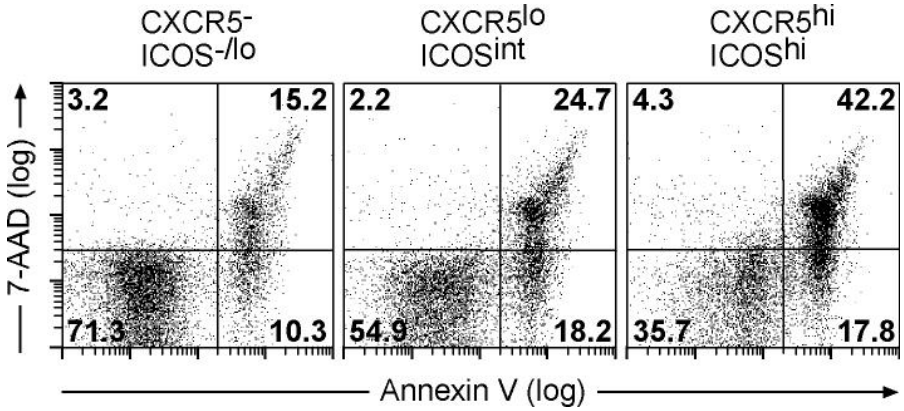

Fig. 14: CXCR5^{hi}ICOS^{hi} CD4 T cells are prone to apoptosis. Tonsillar CD4 T cell subsets were co-cultured with autologous B cells, each 1 x 10^5, in the absence of stimulatory agents. Cells were stained with Annexin V and 7-AAD to determine the proportion of apoptotic and necrotic cells. B cells were excluded from analysis by gating on CXCR5-ICOS dot plot. At day 3, the relative capacity of the cells to survive decrease from 71% in CXCR5^{-}ICOS$^{-/lo}$ subset to 54.9% in CXCR5^{lo}ICOS^{int} and 35.7% in CXCR5^{hi}ICOS^{hi} subset. Representative flow cytometry data for three independent experiments are shown.

3.7. Follicular B helper T cell activity is independent of CD57 expression

CD57, previously known as HNK-1 or Leu7 is a carbohydrate epitope expressed on Natural killer cells, B cells and a small subset of T cells. CD57 expressing CD4 T cells are exclusively found in germinal centers and hence it was proposed to be a surrogate marker for follicular B helper T cells. Approximately 12% of tonsillar CD4 T cells express CD57, which distributes to 2%, 18% and 35% in CXCR5^{-}ICOS$^{-/lo}$, CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} subsets respectively (Fig. 15A). Since there are considerable numbers of CD57 expressing cells in CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} cells (Fig. 15B) it was assumed that their presence might affect the observed B cell help in cocultures. To test this, both the subsets CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} cells were sorted based on the expression of CD57 (Fig.16). Sorted fractions were cocultured with autologous B cells for 7 days. As a control monoculture of B cell was used. Cell free supernatants were used for ELISA to check the concentration of IgG and CXCL13. In the absence of stimulation (Fig. 17A and C), CD57^{+} and CD57^{-} populations did not differ much with respect to IgG or CXCL13 secretion. Similarly when the cells received polyclonal stimulation (Fig. 17B and D) both IgG and CXCL13 secretion were enhanced in all the cell populations but the difference between CD57^{-} and

$CD57^+$ cell populations was similar to that observed in the absence of stimulation. The finding led us to conclude that CD57 expression is not required by follicular CD4 T cells for B cell help.

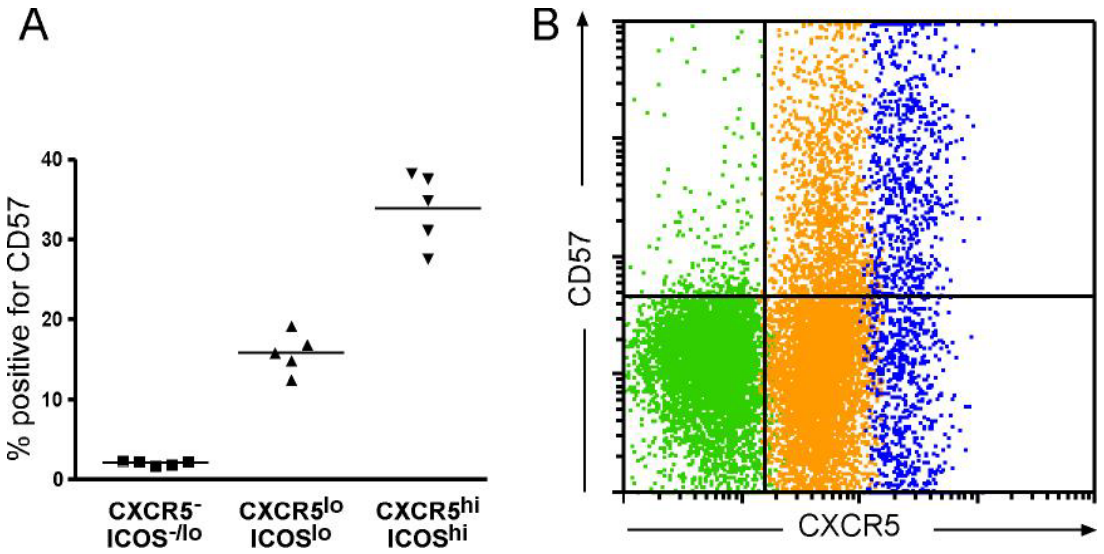

Fig. 15: Overlap of CD57 expressing cells in CXCR5-ICOS tonsillar CD4 T cell subsets. (A) Percentage of CD57 expressing cells increase from $CXCR5^-ICOS^{-/lo}$ to $CXCR5^{hi}ICOS^{hi}$ cells. (B) Dot plot showing CXCR5 and CD57 expression. CD57 expression was confined to a subset of CXCR5 positive cells in $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ subpopulations.

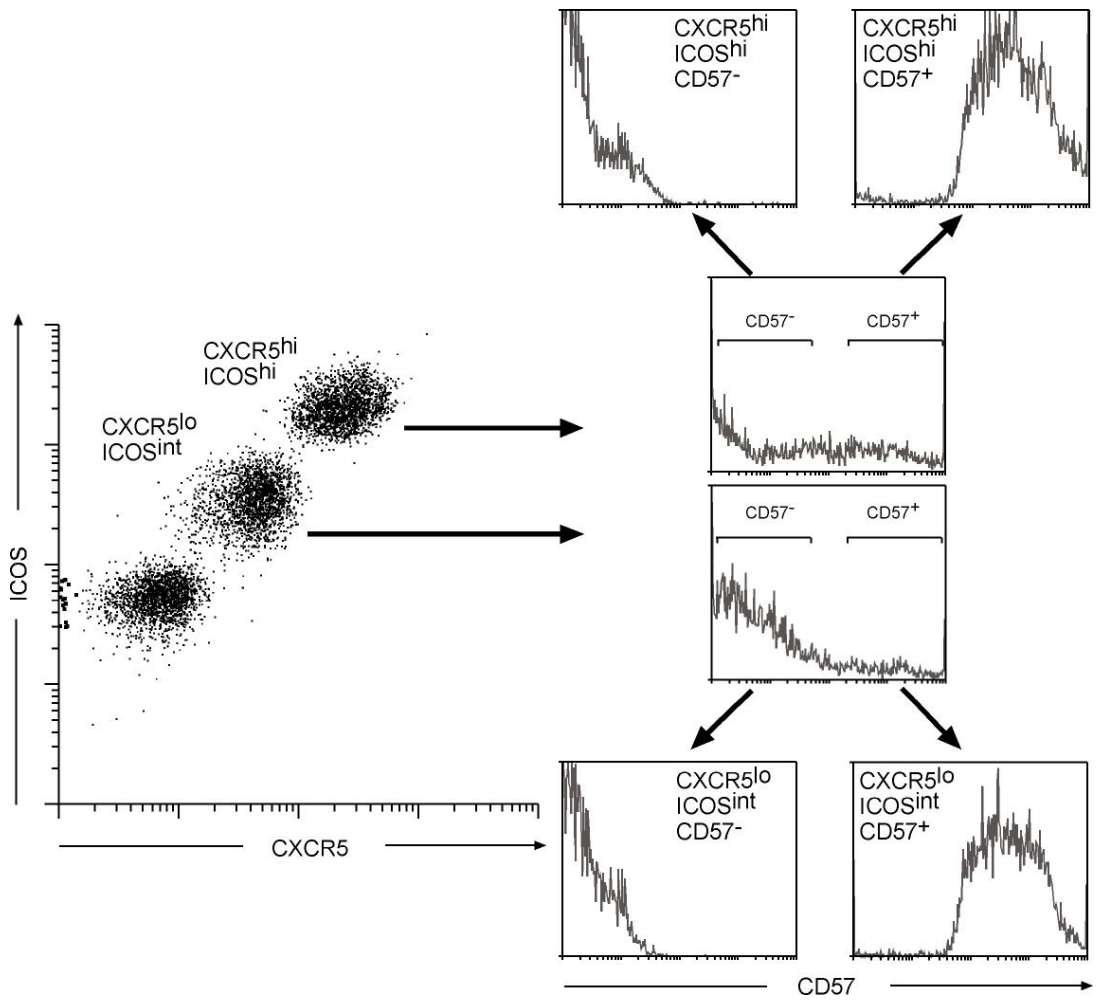

Fig. 16: CD57 subsets sorting. CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} cells were further subdivided based on the expression of CD57.

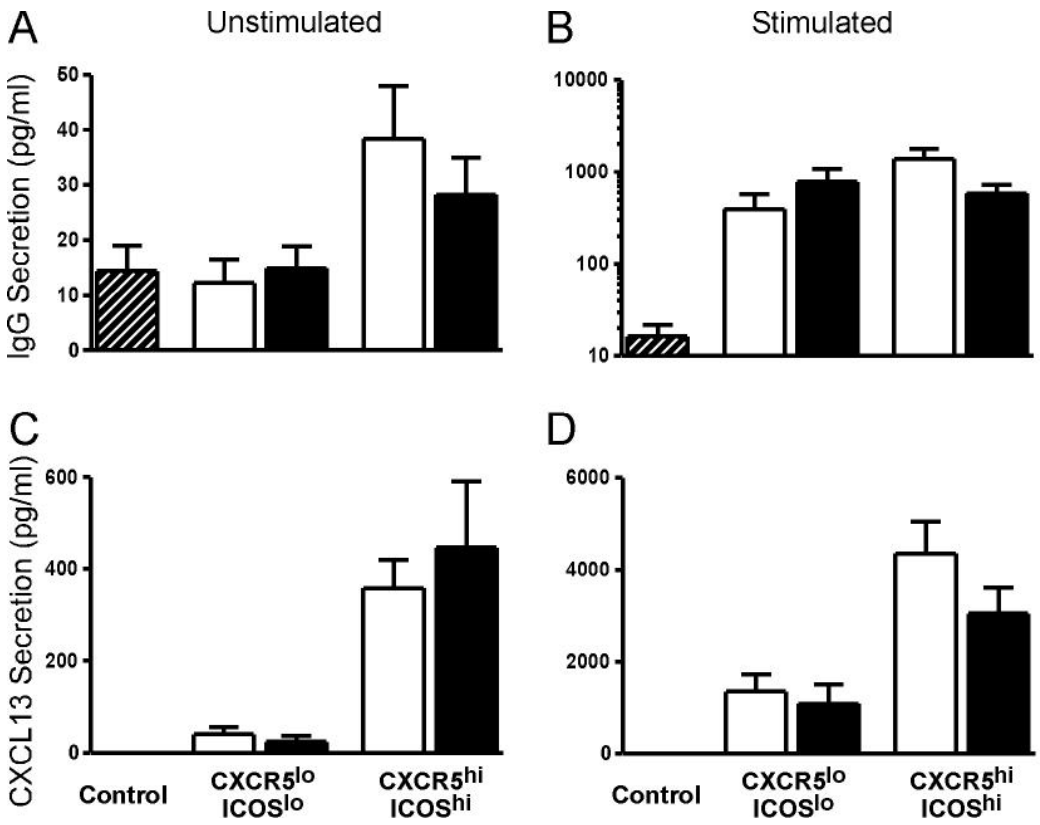

Fig. 17: Follicular B helper T cell activity is independent of CD57 expression. IgG (A, B) and CXCL13 (C, D) ELISA from $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ CD57 subsets in the presence of autologous B cells. B cells cultured in the absence of T cells served as control. Left panel shows unstimulated cocultures and right panel represents anti-CD3/CD28 stimulation. IgG as well as CXCL13 did not show significant difference between the CD57 subsets of $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells. Stimulation enhanced the cognate help as well as CXCL13 secretion but both the subsets remained equally potent.

3.8. Transcript signatures distinguished follicular B helper T cells from central memory and effector memory T cells

Affymetrix Human genome U133 genechip array was used to generate transcript profile of the sorted subsets.

3.8.1. Amount of RNA and quality of the labeled RNA

Total RNA was isolated from the freshly isolated CD4 T cell subsets. The RNA yield did differ between cell type and activation status and additionally, multistep sorting with high-level purity imposes a limit on ultimate yield. Using control experiments it was found that at least $3 - 4 \times 10^6$ CD4 T cells were required to synthesize about 15μg of labeled cRNA employing single round of amplification. In order to reach sufficient amount, RNA was pooled from three different donors, which eliminated the necessity for an additional round of amplification and also

minimized the variations arising from different donors. Appendix I shows the details of cRNA synthesized from tonsillar and peripheral blood subsets.

3.8.2. Array metrics showed standard data quality

Every sample was checked on test chip before hybridization on U133 set. The quality of hybridization was scrutinized using the 3'/5' ratio (Appendix II). Signal intensity ratio of the 3' probe set over the 5' probe set is often referred to as the 3'/5' ratio. This ratio gives an indication of quality of starting RNA and efficiency of cRNA synthesis. 3'/5' ratio for cRNA generated from tonsillar subsets was within 3 except for β-actin from $CXCR5^{-}ICOS^{-}$ subset. However GAPDH was within the recommended threshold of less than 3. Similarly, for cRNA synthesized from peripheral blood subsets, the ratio for GAPDH was always below 3. But the ratio of β-actin in some samples was exceeding the routine value of 3. Considering the fact that there is no standard cutoff to access sample quality and values for GAPDH being normal, the cRNA synthesized was considered to be suitable for hybridization.

3.8.3. Identification of differentially expressed genes

Affymetrix pivot data (excel files) generated using microarray suite were uploaded into GeneSpring and data analysis was performed as discussed in materials and methods. The total number of probes in U133A and U133B are 22,283 and 22,645 respectively with some common probes. To ease data mining, data from U133A and U133B chips was merged prior to analysis, which resulted in 44,760 for every subset. The number of probes “filtered out” at each step was as follows:

“PRESENT” CALLS			
$CXCR5^{hi}ICOS^{hi}$	14,827		
$CXCR5^{lo}ICOS^{int}$	14,403		
$CXCR5^{-}ICOS^{-/lo}$	14,773		
Naïve	13,429	All merged	18,206
T_{CM1}	12,837		
T_{CM}	12,488		
T_{EM}	13,855		

The probes with present calls from all the samples were merged to generate a single list of 18,206 probes. These probes were used in the next step to identify those with 2 fold up regulation by comparing: (1) Among tonsillar subsets and (2) Tonsillar subsets to peripheral blood subsets. Further analysis involved merging all

the 2 fold lists, resulting in 6787 pre-filtered probes, which were used for statistical analysis. Using 1 way ANOVA, 3272 probes were considered to be statistically significant differentially regulated among the tonsillar subsets and tonsillar subsets compared to peripheral blood subsets. The break-up of the 3272 probes for each comparison were presented in table 3

Table 3: Genes that are 2-fold upregulated between any two subsets

Among tonsillar subsets

	$CXCR5^{-}ICOS^{-/lo}$	$CXCR5^{lo}ICOS^{int}$	$CXCR5^{hi}ICOS^{hi}$
$CXCR5^{-}ICOS^{-/lo}$		194	506
$CXCR5^{lo}ICOS^{int}$	428		223
$CXCR5^{hi}ICOS^{hi}$	981	377	

Tonsillar subsets as compared to peripheral blood subsets

	$CXCR5^{-}$ $ICOS^{-/lo}$	$CXCR5^{lo}$ $ICOS^{int}$	$CXCR5^{hi}$ $ICOS^{hi}$	T_{CM1}	Naive	T_{CM}	T_{EM}
$CXCR5^{-}$ $ICOS^{-/lo}$				384	292	317	376
$CXCR5^{lo}$ $ICOS^{int}$				408	619	367	359
$CXCR5^{hi}$ $ICOS^{hi}$				930	1165	802	777
T_{CM1}	488	359	661				
Naïve	276	591	867				
T_{CM}	688	556	784				
T_{EM}	1049	791	984				

3.8.4. Sorting markers displayed expression values as expected

Markers used for sorting were the first set of genes, whose expression was confirmed on the gene chip data. Figure 18 shows, two probes for CXCR5, one probe each for CCR7 and ICOS. A progressive up-regulation of the transcript for CXCR5 and ICOS from $CXCR5^{-}ICOS^{-/lo}$ to $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ was observed in tonsillar subsets. In addition, mRNA for CXCR5 was abundantly present in T_{CM1} cells but absent from all other peripheral blood subsets. CCR7 transcripts are down regulated in both $CXCR5^{hi}ICOS^{hi}$ as well as T_{EM} subsets, and up regulated in naïve cells. Transcript abundance and protein expression of sorting markers match as expected, confirming the quality of the genechip data.

Subsequent analysis of the gene expression of 3272 probes included clustering, principal component analysis, dividing into functional sub-groups and generating individual gene lists with pair-wise combinations.

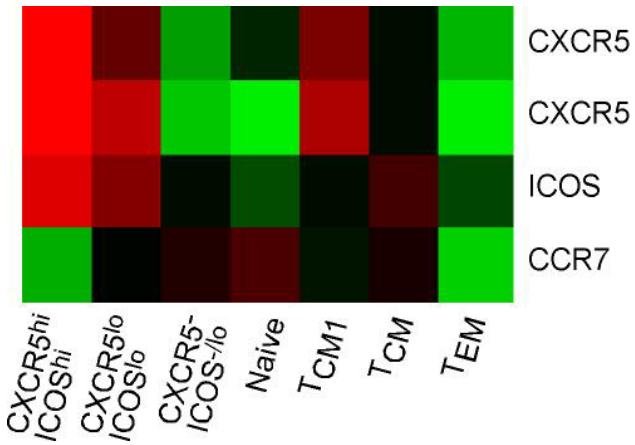

Fig. 18: Expression of sorting markers. mRNA levels of CXCR5, ICOS and CCR7 on all the tonsillar and peripheral blood CD4 T cell subsets. CXCR5 was represented with two probes on U133A chip. Expression markers were observed to be as expected. CXCR5 and ICOS transcripts were progressively up regulated on tonsillar subsets from $CXCR5^{-}ICOS^{-/lo}$ to $CXCR5^{hi}ICOS^{hi}$ cells. CXCR5 mRNA was also expressed in T_{CM1}. mRNA for CCR7 was expressed on memory and naïve subsets and downregulated on T_{EM} and $CXCR5^{hi}ICOS^{hi}$ cells.

3.8.5. Clustering showed distinct gene expression profiles of the CD4 T cell subsets

Normalized expression values of 3272 statistically significant differentially expressed probes were used for hierarchical clustering. Hierarchical clustering is most popular method for microarray data analysis, which is displayed as a heat map. Genes or samples with similar expression pattern were grouped together and are connected by a dendrogram. The heat map shows rows corresponding to probes and columns to samples (Fig. 19). The normalized expression value for each gene was color-coded. Green cells indicate low, red cells high and black cells normal expression levels. Within tonsillar CD4 T cell subsets, $CXCR5^{hi}ICOS^{hi}$ cells can be clearly distinguished from $CXCR5^{-}ICOS^{-/lo}$ and $CXCR5^{lo}ICOS^{int}$ T cells with a large number of genes being up regulated. $CXCR5^{-}ICOS^{-/lo}$ subset had relatively less number of genes up regulated with a pattern resembling Naïve T cells from peripheral blood. When all the subsets were compared, it was observed that in general, genes that were silent in tonsillar subsets were active in peripheral blood and *vice versa*. Numbers displayed under each branch of the dendrogram are r-values, which represent the distance between the sub-branches under the node. The values range between −1 and +1 with increase in correlation.

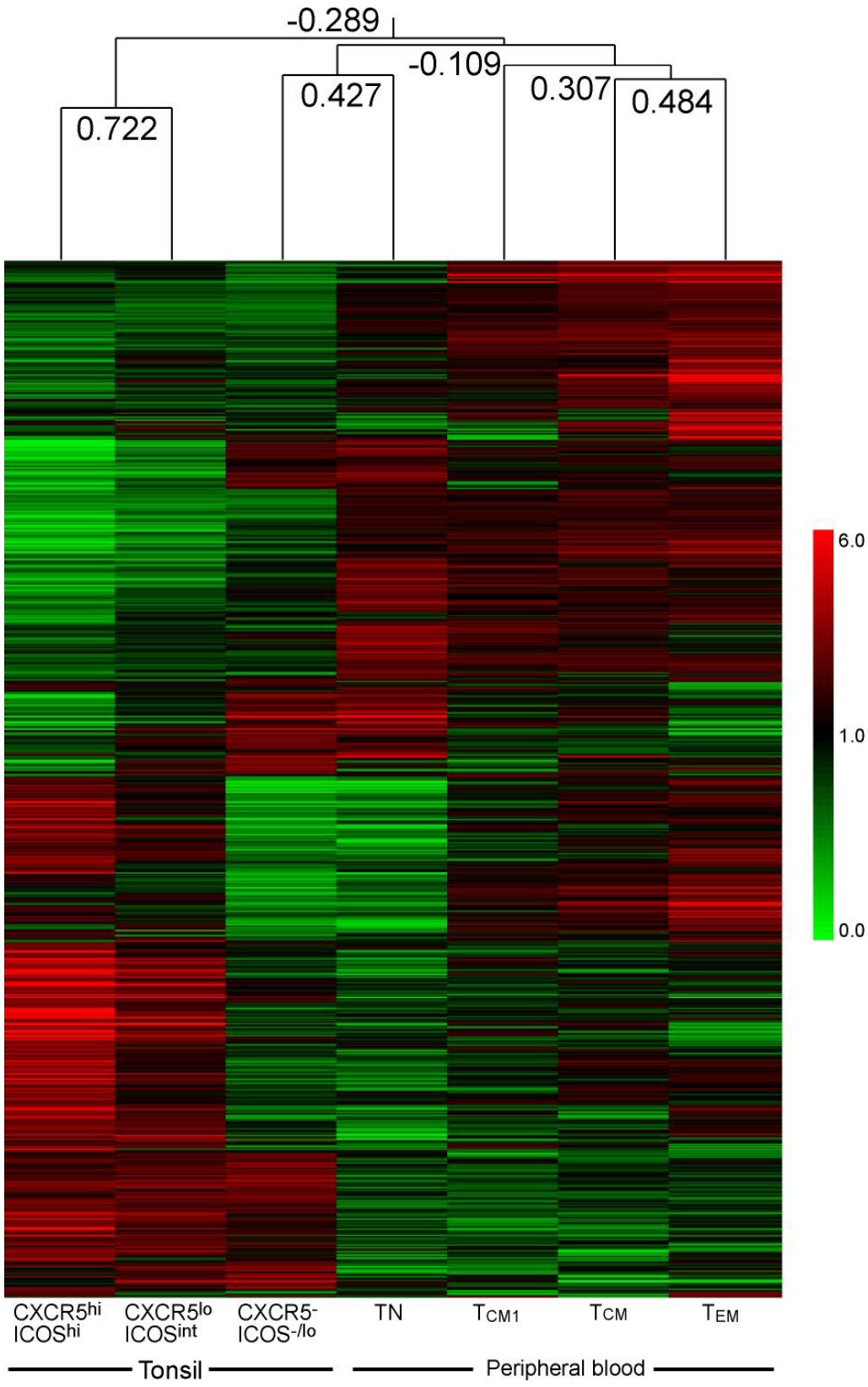

Fig. 19: Gene expression profiles distinguished Follicular B helper T cells from central memory and effector memory T cells. Hierarchical cluster analysis

of peripheral blood and tonsillar CD4 T cell populations. Depicted are the relative expression levels of 3272 probes that are ≥ 2-fold differentially expressed between at least two CD4 T cell subsets. Each row represent a probe for specific transcript and each column represent a subset with three biological replicates. The dendrogram indicates similarities in the expression pattern between experimental samples.

3.8.6. Principal component analysis (PCA) revealed distinctive features of the tonsillar and peripheral blood subsets

PCA is a data decomposition technique that reduces dimensionality by retaining those characteristics of the data set that contributes most to its variance. Thereby, simplifying the visual inspection of the relationship between the samples. PCA will simply find eigenvectors (principal components) and eigenvalues relevant to the data using a covariance matrix. The first principal component is laid along the direction with maximum variance and the second principal component lies in the subspace perpendicular to the first one. The number of components does not exceed the number of samples in the analysis. Eigenvalues indicate the variance as a percentage of total variance, which shows how much a particular component represents the data.

Using 3272 differentially regulated probes across 7 subsets, a PCA was performed by using Genespring software, which generated two components, PCA 1 with 48.6% and PCA 2 with 26% variance (Fig. 20). These components were plotted in a two dimensional space with component 1 on the X-axis and component 2 on the Y-axis to analyze relationship between the subsets. There were three prominent groups clearly segregated from each other. Naïve cells and $CXCR5^{-}ICOS^{-/lo}$ cells were placed close to each other in group A. T_{CM1} T_{CM} and T_{EM} formed group B, where as group C contained $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells. Since $CXCR5^{-}ICOS^{-/lo}$ represent the unprimed compartment of tonsillar CD4 T cells, they closely resemble naïve cells from peripheral blood. Hence, these two subsets lie in close proximity to each other and far away from other tonsillar and peripheral blood subsets (group B and C). Effector/memory subsets from peripheral blood were grouped together divided from tonsillar subsets on component 1 and naïve cells on component 2. Surprisingly T_{CM1}, the only CXCR5 expressing subset in peripheral blood was located distantly as compared to $CXCR5^{+}CD4^{+}$ T cells from tonsils. $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells were placed close to each other but still separated by PCA2. Thus, these results support the notion that follicular help is a distinct entity and the follicular B helper T cells are highly distinct from effector/memory T cells from peripheral blood.

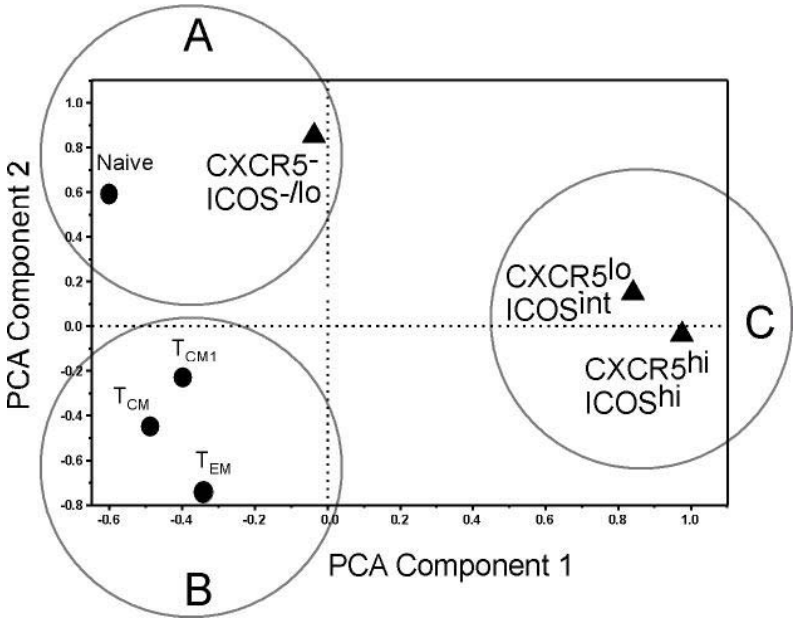

Fig. 20: Principal component analysis of microarray data from CD4 T cell subset. Follicular B helper T cell subsets, particularly the $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ were clearly distinct from effector cells of peripheral blood (T_{CM1}, T_{CM} and T_{EM}). $CXCR5^{-}ICOS^{-/lo}$ subset lie in close proximity to Naive. Surprisingly, T_{CM1} was placed in quadrant with T_{CM} and T_{EM} isolated from tonsillar T cell subsets.

3.8.7. Self-organizing maps on tonsillar subsets indicates $CXCR5^{hi}ICOS^{hi}$ cells might be derived from $CXCR5^{-}ICOS^{-/lo}$ cells

Data from three tonsillar subsets was separately analyzed using the filtering algorithm used above. This generated a list consisting of 2002 probes differentially regulated among the tonsillar subsets. Analysis using self-organizing maps (SOM) is shown in figure 21. Probes that were progressively up and down regulated from $CXCR5^{-}ICOS^{-/lo}$ to $CXCR5^{hi}ICOS^{hi}$ cells constituted the major groups. A total of 1,200 probes were up regulated and 763 probes were downregulated as the cells differentiate towards $CXCR5^{hi}ICOS^{hi}$ subset. $CXCR5^{lo}ICOS^{int}$ subset instead showed only 39 probes being differentially regulated. This indicates that $CXCR5^{hi}ICOS^{hi}$ cells might be derived from $CXCR5^{-}ICOS^{-/lo}$ cells via $CXCR5^{lo}ICOS^{int}$ T cells.

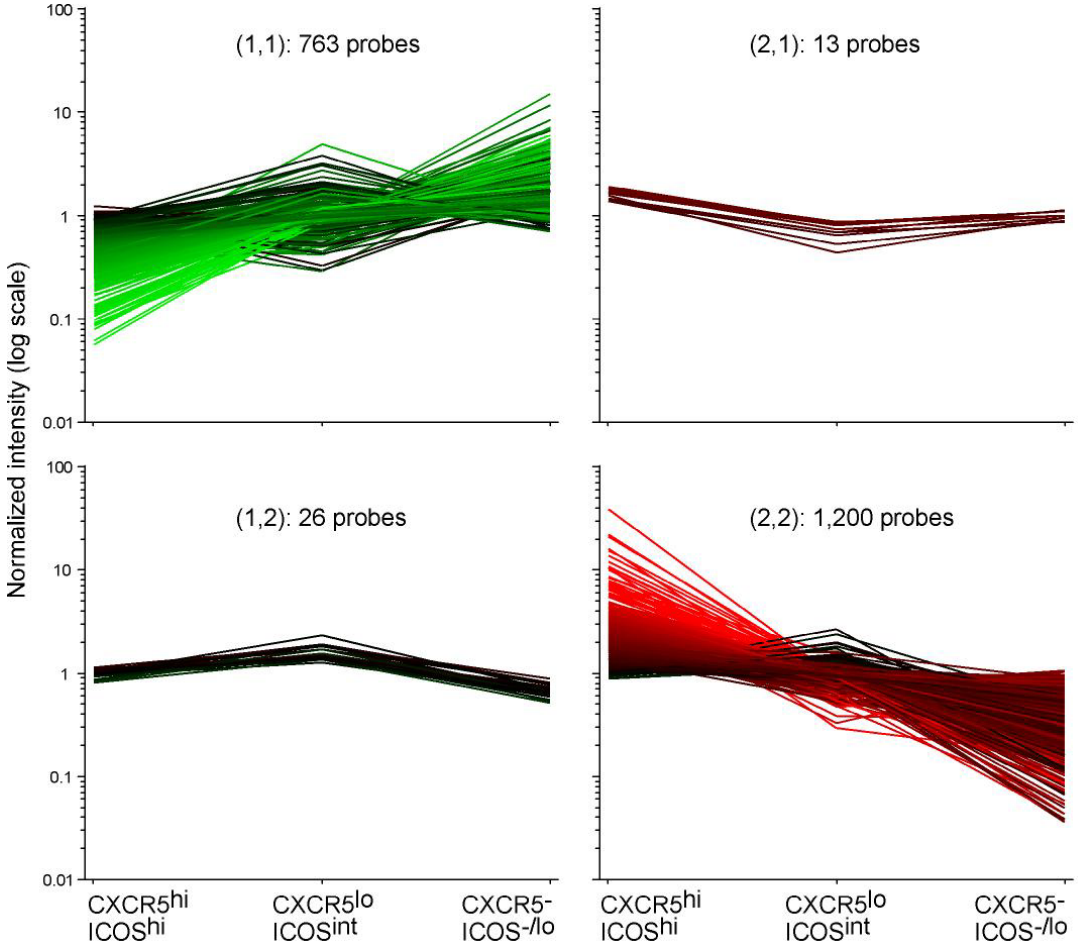

Fig. 21: SOM analysis of tonsillar CD4 T cell subsets. 2X2 SOM analysis of 2002 probes differentially regulated between tonsillar CD4 T cell subsets revealed that around 96% of the genes were either up (763 probes) or down (200 probes) regulated from $CXCR5^{-}ICOS^{-/lo}$ to $CXCR5^{hi}ICOS^{hi}$ subsets. Very few genes were differentially regulated in $CXCR5^{lo}ICOS^{int}$ cells, possibly indicating that $CXCR5^{hi}ICOS^{hi}$ cells are derived from $CXCR5^{-}ICOS^{-/lo}$ cells via $CXCR5^{lo}ICOS^{int}$ T cells.

3.9. Validation of microarray data by RT-PCR

Secondary validation of microarray data was pursued using real-time quantitative reverse transcription polymerase chain reaction (RT-PCR), which at present is the most reliable technique to verify microarray data at transcript level. A set of genes was selected based on differential regulation in $CXCR5^{hi}ICOS^{hi}$ and $CXCR5^{-}ICOS^{-/lo}$ subsets. Some of the genes selected were those that are previously not characterized in follicular B cell help. Expression data was obtained from three independent samples and compared with microarray data (Fig. 22). 15 differentially regulated genes were tested and the expression patterns as measured by RT-PCR are reconfirmed to those observed on microarray with the

exception of IL4R. Here real time data did not show a decreasing expression of the transcript as observed on microarray.

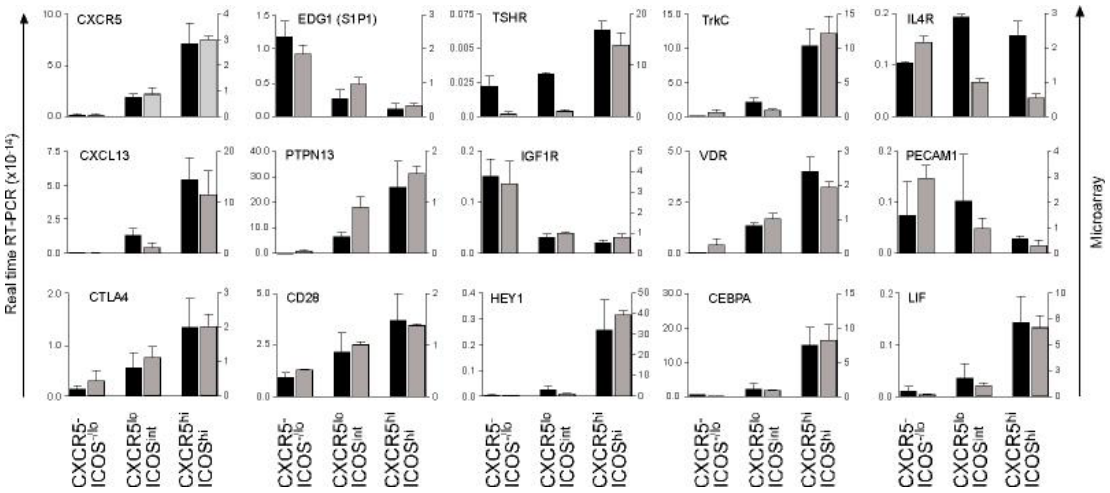

Fig. 22: Validation of microarray data by RT-PCR. Some of the selected genes were confirmed by real time RT-PCR shown in closed bars. Open bars indicate data derived by microarray analysis of the corresponding gene. Data is representative of three independent experiments.

3.10. Selected genes differentially expressed among tonsillar and peripheral blood subsets

Among the differentially regulated probes, a through search for genes which might contribute to T_{FH} biology were selected and presented according to their functional relevance in figure 23. Functional categories showing limited expression in tonsillar CD4 T cell subsets were chemokine and chemokine receptors, adhesion molecules and genes involved in apoptosis. Whereas costimulatory molecules, signaling molecules, transcription factors, Notch and frizzled genes were heavily expressed by $CXCR5^{hi}ICOS^{hi}$ cells and to some extent by $CXCR5^{lo}ICOS^{int}$ cells.

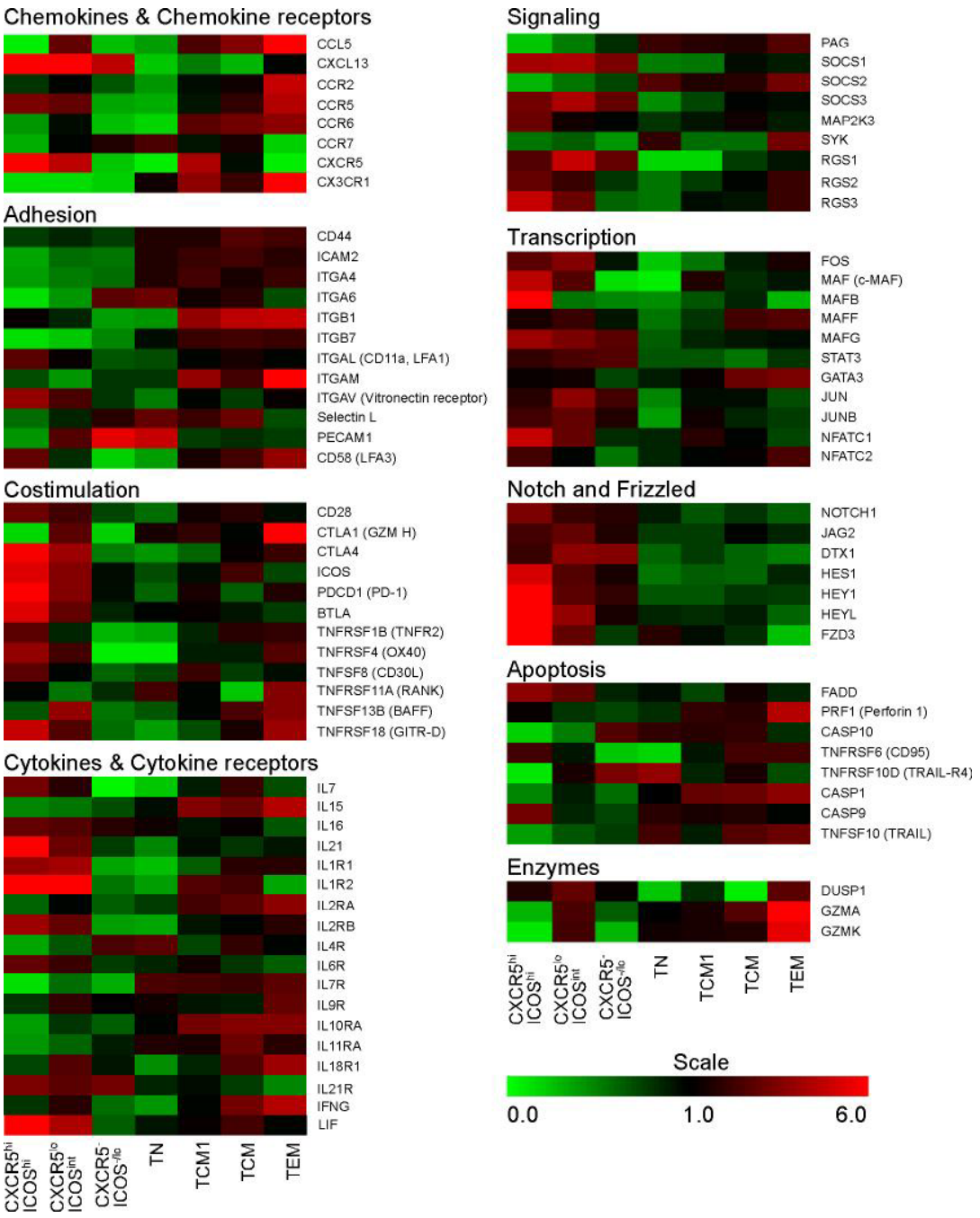

Fig. 23: Selected genes differentially expressed among tonsillar and peripheral blood subsets. Selected gene from various functional classes showed differential expression among the subsets. Chemokine receptors and adhesion molecules were restricted in tonsillar subsets. In contrast, costimulatory molecules, cytokines, cytokine receptors and signaling molecules were abundant in tonsillar subsets as compared to peripheral blood subsets.

3.10.1. Chemokines and chemokine receptors

Chemokine expression in CD4 T cell subsets was restricted to CCL5 and CXCL13. CCL5 was expressed to some extent by $CXCR5^{lo}ICOS^{int}$ and by peripheral blood

T_{CM} and T_{EM} cells. CXCL13, the ligand for CXCR5 was produced at high levels by CXCR5hiICOShi cells and CXCR5loICOSint cells followed by $CXCR5^{-}ICOS^{-/lo}$ cells and was totally absent in peripheral blood subsets. Real time RT-PCR and western blotting showed a clear difference of expression levels between $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells. With respect to chemokine receptors differential expression was more striking among all the peripheral blood subsets. Other notable observation was that tonsillar subsets did not express as many chemokine receptors as compared to peripheral blood subsets confirming to the flow cytometry data. Compared to all the effector cells, non-polarized peripheral blood naïve cells and tonsillar $CXCR5^{-}/ICOS^{-/lo}$ cells lack chemokine receptor expression except CCR7 and CXCR4, which probably allowed them to recirculate through secondary lymphoid organs. Within tonsillar CD4 T cells, CCR7 was down regulated from $CXCR5^{-}/ICOS^{-/lo}$ to $CXCR5^{hi}ICOS^{hi}$ cells. Among the peripheral blood CD4 T cells, CXCR5 was only present on a subset of central memory T cells (T_{CM1}). CCR2, CCR5 and CX3CR1 were abundant in effector memory T cells; T_{CM1} cells also expressed the latter. Central memory T cells showed an intermediate expression pattern between naïve on one side and effector memory on the other side.

3.10.2. Adhesion molecules

In addition to chemokine receptors, expression of adhesion molecules on the surface of lymphocytes plays a decisive role for the cells to pass through endothelial cell layer of blood vessels. Adhesion molecules interact with their ligands presented on the surface of vascular endothelial cells in the luminal side of the vessels. Keeping in line with chemokine receptors, the expression of adhesion molecules was also constrained on $CXCR5^{+}$ Tonsillar CD4 T cells except for LFA1, LFA3 and integrin $\alpha_{v.}$

Among other integrins subunits, β1 was present on T_{CM1}, T_{CM} and T_{EM} and αM on T_{CM1} and T_{EM}. L-selectin differentiated between naïve and T_{CM} from T_{EM} where as LFA3 and integrin α6 between naïve, central memory and T_{EM} cells. Interestingly, PECAM1 signals were detected on naïve cells from peripheral blood and were progressively diminished on tonsillar subsets from $CXCR5^{-}ICOS^{-/lo}$ to $CXCR5^{hi}ICOS^{hi}$.

3.10.3. Costimulatory/inhibitory molecules

Costimulatory molecules provide second signal to naïve T cells after TCR triggering to modulate activation and effector function. Unlike chemokine receptors and adhesion molecules, transcripts for costimulatory/inhibitory molecules were

enriched on CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} cells. These cells prominently upregulate most of the CD28 family members like CD28, CTLA4, ICOS, PD-1 and BTLA. Expression of CD28, CTLA4 and ICOS were confirmed by real time RT-PCR. Among the TNF/TNFR super-family members, TNFR2, OX40, CD30L and GITR-D were progressively up regulated in CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} cells. BAFF was uniquely present only on CXCR5^{lo}ICOS^{int} cells. Fewer number of costimulatory/inhibitory molecules were seen on peripheral blood subsets, CTLA1, RANK, BAFF and GITR-D were expressed on T_{EM}. In general most of the genes have average expression on central memory subsets and almost absent on naïve cells.

3.10.4. Cytokines and Cytokine receptors

Differential mRNA expression of cytokines and cytokine receptors was more pronounced among the CD4 T cell subsets. Interestingly expression of IL-7, IL-16, IL-21, IL-1R1, IL-1R2, IL-2RB, and IL-6R seems to be characteristic of CXCR5^{hi}ICOS^{hi} cells. IL-21R mRNA was present in all the tonsillar CD4 T cells. Furthermore, it is interesting to note that CXCR5^{hi}ICOS^{hi} cells express transcripts for both IL-21 and its receptor, IL-21R. In peripheral blood subsets, elevated levels of IL-15 and IL-10RA were confined to only antigen-experienced cells. Transcripts of IL-2RA, IL-7R, IL-9R, IL-18R1 were abundant in T_{EM} cells. IL-11RA mRNA was high in T_{CM} cells alone. Lower expression of IL-4R in CXCR5^{hi}ICOS^{hi} was confirmed by real time RT-PCR but the differences in CXCR5^{-}ICOS$^{-/lo}$ cells and CXCR5^{lo}ICOS^{int} cells as observed by microarray could not be detected. Hallmark Th1 cytokine, IFNγ mRNA was notably elevated in T_{EM} and to certain extent in T_{CM} cells but was found at average levels in CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} cells.

3.10.5. Signaling molecules

Analyses of signaling molecules revealed that genes belonging to the SOCS and RGS families are differentially expressed among CD4 T cell subsets. Suppressor of cytokine signaling (SOCS) regulates negative feedback of cytokine receptor signaling. SOCS1 and SOCS3 were present in all tonsillar CD4 T cells subsets; SOCS1 was more abundant in CXCR5^{hi}ICOS^{hi} and CXCR5^{lo}ICOS^{int} cells and SOCS2 in CXCR5^{lo}ICOS^{int} cells. SOCS2 seems to be characteristic of T_{EM} cells, which was down regulated in CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} cells. A Second set of intracellular signaling molecules differentially expressed is Regulator of G protein signaling (RGS), which also act as negative regulators specific to G protein-coupled receptors. RGS1 was highly up regulated in CXCR5^{lo}ICOS^{int} cells and RGS3 in CXCR5^{hi}ICOS^{hi} cells. MAP2K3 mRNA was abundant in CXCR5^{hi}ICOS^{hi} cells compared to all other subsets.

3.10.6. Transcription factors

In line with genes involved in intracellular signaling, expression profiles of tonsillar CD4 T cells included genes involved in transcription. For example c-MAF, MAFB and MAFG mRNA were highly elevated in $CXCR5^{hi}ICOS^{hi}$ cells. MAFB mRNA distinguishes $CXCR5^{hi}ICOS^{hi}$ cells from peripheral blood T_{EM} cells. Th2 specific gene GATA3 was highly expressed in central memory/effector memory CD4 T cells. On the other hand, JUN and JUNB mRNA was abundant in $CXCR5^{lo}ICOS^{int}$ cells. Among the NFATc proteins, NFATc1 was high in $CXCR5^{hi}ICOS^{hi}$ cells and $CXCR5^{-}ICOS^{-/lo}$ cells are completely devoid of NFATc2.

3.10.7. NOTCH and Frizzled

In humans, Notch signalling is mediated by four notch receptors and five ligands. The receptors are named Notch 1-4 and the ligands are named Jagged 1, 2 and delta 1-3. Several genes involved in NOTCH signaling pathway showed a prominent expression on $CXCR5^{hi}ICOS^{hi}$ cells. The ligands JAG2 and deltex 1 mRNA was elevated in $CXCR5^{lo}ICOS^{int}$ cells but the receptor Notch1 was abundant in $CXCR5^{hi}ICOS^{hi}$ cells. HES1, HEY1 and HEYL are bHLH-type transcription factors, which are target genes of Notch signaling, abundantly expressed in $CXCR5^{hi}ICOS^{hi}$ cells. HEY1 mRNA levels were also confirmed by real time RT-PCR. Frizzled 3 mRNA, a Wnt receptor was highly expressed by $CXCR5^{hi}ICOS^{hi}$ cells alone.

3.10.8. Apoptosis

In contrast to the apoptosis data presented earlier (Fig.14), $CXCR5^{hi}ICOS^{hi}$ cells did not express elevated levels of apoptosis related genes compared to other subsets, except caspase 9 and Fas-associated death domain (FADD). However, Fas (CD95) levels were raised in tonsillar $CXCR5^{+}$ $CD4^{+}$ T cells as compared to $CXCR5^{-}ICOS^{-/lo}$ cells. Caspase 10 was elevated in $CXCR5^{-}ICOS^{-/lo}$ cells and highly down modulated in $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells. TRAIL-R4 (TNFRSF10D) transcript was abundantly present in tonsillar $CXCR5^{-}ICOS^{-/lo}$ and naïve cells. It ligand was also present in all the subsets except $CXCR5^{hi}ICOS^{hi}$ cells and highly up regulated in T_{EM} cells. Finally, Perforin 1 mRNA was present at low levels in all the subsets and at relatively high levels in T_{EM} cells.

Overall, gene expression data suggests that $CXCR5^{hi}ICOS^{hi}$ cells belong to a distinct class of CD4 T cells, which can be easily distinguished from peripheral blood subsets.