4. Discussion

Humoral immune responses are essential for the generation of high affinity antibodies against invading pathogens. T cell dependent humoral immune responses evolve after a series of dynamic interactions occurring between immune cells within the anatomically distinct sites of lymphoid organs. Key cellular players are dendritic cells (DCs), which present antigen, T cells that mediate immune signals and B cells that differentiate into antibody producing plasma cells. T cells in particular CD4 compartment in secondary lymphoid organs is associated with helper activity for B cell stimulation and probably also generation of Th1 and Th2 cells. CD4 T cells orchestrate channeling cognate responses derived from dendritic cells to B cells in the form of B cell help during T cell dependent immune responses. Thus, T cell-B cell collaboration is essential in order to generate high affinity antibodies for T-dependent antigens.

4.1. Activation and differentiation of Naïve CD4 T cells in secondary lymphoid organs

Dendritic cells residing in peripheral tissues initiate immune responses by capturing antigen, upregulating CCR7 and finally migrating into the draining lymph nodes. Antigen-inexperienced CD4 T cells engage with dendritic cells and scan their surface in search of foreign antigens to initiate T cell response (Thery and Amigorena, 2001). Antigens are presented to naive T cells in the form of peptide fragments bound to major histocompatibility complex (MHC) molecules leading to the activation and differentiation of T cells. Costimulatory interactions by CD28 and ICOS are necessary for optimal T cell activation (Gonzalo *et al.*, 2001; Wang *et al.*, 2000). Activated T cells secrete cytokine IL-2 that is required for clonal expansion. The entire process results in the formation of a heterogenous pool of CD4 T cells within the T cell area of the secondary lymphoid organ. A subset of activated CD4 T cells, upregulate chemokine receptor CXCR5 and migrates towards B cell follicles to initiate cognate interactions with B cells. These cells were provisionally named as follicular B helper T cells (T_{FH}) (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000). B cells depend on the T cell help to get activated and form germinal centers. Germinal centers are the sites of B cell expansion, somatic hypermutation (SHM), isotype switching, affinity maturation, plasma cell and memory B cell generation (MacLennan, 1994). T_{FH} cells provide further help in sustaining the germinal center reaction (Kim *et al.*, 2001).

Apart from CXCR5, it is established that T_{FH} cells express the costimulatory molecule inducible costimulator (ICOS) (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000). Deficiency of ICOS in mice has been shown to drastically affect T dependent B cell immune responses.

4.2. CD4 T cell subsets in secondary lymphoid organs

Previous studies from our lab have shown that tonsillar CD4 T cells expressing the chemokine receptor CXCR5 provide B cell help (Breitfeld *et al.*, 2000). $CXCR5^{+}CD4^{+}$ T cells from tonsils but not from peripheral blood efficiently stimulate antibody secretion by B cells (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000). About 60% of tonsillar CD4 T cells express CXCR5 at varying levels indicating a considerable degree of heterogeneity among these cells. To identify specific subset carrying potential T_{FH} activity we considered the use of markers with functional relevance. Subsequently, tonsillar CD4 T cells were sorted based on the co-expression of CXCR5 and the costimulatory molecule ICOS. Three subsets of CD4 T cells, namely $CXCR5^{-}ICOS^{-/lo}$, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ were identified with distinct morphological features and their functional and molecular aspects were studied. $CXCR5^{hi}ICOS^{hi}$ cells were the most potent inducers of IgG by B cells, secreted highest amount of CXCL13, showed limited proliferation and enhanced apoptosis compared to other subsets. Large scale gene expression profiling revealed that tonsillar subsets constituted distinct subpopulations. In addition, expression of CD57 was found to be independent of T_{FH} function. Based on these findings, it is hypothesized that all the three subsets might represent different states of activation. $CXCR5^{-}ICOS^{-/lo}$ subset represents nonpolarized cells, $CXCR5^{lo}ICOS^{int}$ subset constitute recently activated cells and $CXCR5^{hi}ICOS^{hi}$ subset terminally differentiated cells. These findings also led us to propose linear differentiation pathway acting on $CXCR5^{-}ICOS^{-/lo}$ cells to differentiate into $CXCR5^{lo}ICOS^{int}$ cells representing a precursor population for $CXCR5^{hi}ICOS^{hi}$ cells. Hence $CXCR5^{hi}ICOS^{hi}$ cells are considered to represent actual T_{FH} population.

4.2.1. Balanced migration of activated CD4 T cell subsets into B cell follicles

Migration of activated CD4 T cells into B cell follicles is essentially required for B cell help, and activated CD4 T cells in turn, receive growth and survival signals when in contact with B cells. Tonsillar CD4 T cells expressing CXCR5 migrate readily in response to BLC/BCA-1, which is expressed in B cell follicles (Schaerli *et al.*, 2000). The process of migration is however tightly controlled by a balanced

expression of CXCR5 and CCR7, enabling activated CD4 T cells to acquire differential responsiveness towards CXCL13, CCL19 and CCL21 (Ansel *et al.*, 1999; Hardtke *et al.*, 2005). In agreement with these findings it is shown here that $CXCR5^{+}CD4^{+}$ T cell subsets readily migrated to CXCL13 as compared to $CXCR5^{-}$ T cells. But contrary to our expectation, both subsets, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells expressing CXCR5 at low and high levels respectively, did not differ in their response to CXCL13. This means varying levels of chemokine receptor CXCR5 on the cell surface do not confer differential migration abilities.

Thus, *in vivo* it is likely that the precise micro-anatomical positioning of $CXCR5^{lo}ICOS^{int}$ cells within the secondary lymphoid organs may be defined by remnant expression of CCR7. This assumption could be tested *in vitro* using a simple method. In the chemotaxis assay using CXCL13 in the bottom chamber, and then varying amounts of recombinant CCL19 or CCL21, the T cell zone chemokine, could be added along with CD4 T cells in the upper chamber. The expected pattern might show a significant difference in $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells. Nevertheless, it can be inferred that expression of a combination of chemokine receptors at different levels might dictate trafficking and anatomical positioning of lymphocytes within the tissues. In the case of tonsillar CD4 T cell subsets, variable expression of CXCR5 was not able to dictate differential migration potentials. Hence, we determined the pattern of chemokine receptor expression on tonsillar CD4 T cell subsets and compared it with the pattern of peripheral blood CD4 T cell subsets. This was intended to check if chemokine receptors other than CXCR5 are expressed which might influence the positioning of $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ CD4 T cell subsets.

4.2.2. Restricted expression of chemokine receptors on tonsillar CD4 T cell subsets

Based on their activation state, immune cells express an array of chemokine receptors, and use them to navigate through chemokine gradient present in the tissues. Chemokine receptor expression in tonsillar subsets was determined by flow cytometry and compared with peripheral blood CD4 T cell subsets. Naïve and central memory T cell subsets express CCR7, a receptor for lymphoid homing, which allows them to enter into secondary lymphoid organs (Sallusto *et al.*, 1999). Other chemokine receptors expressed on these subsets are CXCR4, CXCR3 and CCR4. CXCR3 and CCR4 expressing subsets in T_{CM} cells are reported to represent pre-Th1 and pre-Th2 cells respectively (Rivino *et al.*, 2004). In vitro, CXCR3 and CCR4 expressing T_{CM} cells differentiated into $CCR7^{-}$ Th1 and Th2

cells upon stimulation with homeostatic cytokines IL-7 and IL-15. On the other hand, $CXCR3^{-}CCR4^{-}$ T_{CM1} cells did not respond to the cytokine driven expansion but retained CCR7 expression and their nonpolarized state. Recently, it was also shown that CXCR3 and CCR4 on naïve cells from peripheral blood define cells with very early memory phenotype representing central memory and effector memory T cells (Song *et al.*, 2005). Expression of CXCR3 on Th1 cells was also shown to precede the expression of CCR5 (Rabin *et al.*, 2003). Although CCR5 was not detected on cell surface of tonsillar subsets, its mRNA was abundant in both $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells. T_{EM} cells down modulate the expression of CCR7 but upregulate chemokine receptors for inflammatory chemokines such as CCR2, CCR3, CCR4, CCR5 and CXCR3 that allow these cells to home to non-lymphoid tissues and carry out immediate effector responses (Kim *et al.*, 2003; Sallusto *et al.*, 1998b; Sallusto *et al.*, 1997).

In contrast to the wide range of chemokine receptors expressed on CD4 T cells in peripheral blood, tonsillar CD4 T cell subsets limit their usage irrespective of their state of activation. Only CXCR3 and CXCR4 were expressed among CXC chemokine receptors whereas CCR4 and CCR7 were expressed among CC chemokine receptors. $CXCR5^{-}ICOS^{-/lo}$ cells express high levels of CCR7 allowing them to recirculate between secondary lymphoid organs. When these cells interact with DCs and undergo activation and differentiation, CCR7 is downregulated with concomitant upregulation of CXCR5, which is a marker for homing to B cell follicles. A portion of the cells from all the three subsets express CXCR3 and CCR4 and the pattern of CXCR3 expression indicate an additional subset within these cells. Rabin et al., reported that $CXCR3^{+}CD4^{+}$ T cells are localized around and within germinal center light zone (Rabin *et al.*, 2003). Ligands for CXCR3 and CCR4, CXCL9 and CCL22 respectively, are localized to distinguishable but adjacent foci in T cell areas of the tonsil (Song *et al.*, 2005). Specific expression of these two chemokines together with our results on chemokine receptors CXCR3 and CCR4 suggest that there could be a considerable amount of functional heterogeneity prevailing in the existing subsets. CXCR3 is expressed on both peripheral blood and tonsillar activated CD4 T cells (Rabin *et al.*, 2003). Extending the findings from Song et al., and Rivino et al., it is of considerable interest to compare the cells expressing CXCR3 and CCR4 from secondary lymphoid organs with that of peripheral blood to check the if they have common lineage. Hence, flow cytometry data revealed that tonsillar CD4 T cells had a restricted chemokine receptor usage as compared to peripheral blood subsets. Particularly, $CXCR5^{hi}ICOS^{hi}$ cells from tonsils had diminished expression of chemokine receptors as compared to central memory and effector memory CD4 T cells. This

indicates that tonsillar CD4 T cell subsets as compared to central memory and effector memory CD4 T cells, might have limited trafficking ability mostly confined to secondary lymphoid tissues.

Furthermore, limited expression of chemokine receptors in tonsillar CD4 T cells also suggests that the degree of heterogeneity is far less compared to peripheral blood CD4 T cells. Apparently, activated CD4 T cells in secondary lymphoid organs are solely dedicated to B cell help unlike effector cells in peripheral blood carrying out divergent functions.

4.2.3. $CXCR5^{hi}ICOS^{hi}$ cells cluster readily in spite of poor expression of adhesion molecules

$CXCR5^{hi}ICOS^{hi}$ cells appear amoeboid, possibly to increase the surface area for cognate interactions with B cells. *In vitro* these cells were also observed to adhere remarkably and form clusters within a short span of time as compared to $CXCR5^{-}ICOS^{-/lo}$ and $CXCR5^{lo}ICOS^{int}$ cells. Hence it was assumed that $CXCR5^{hi}ICOS^{hi}$ T cells might express a variety of cell-cell adhesion molecules. Instead, only vitronectin receptor (ITGAV) mRNA was highly expressed in these cells and moderate levels of lymphocyte function associated-1 (LFA-1, CD18 or integrin beta2) and lymphocyte function associated-3 (LFA-3, CD58) transcripts were found. Follicular dendritic cells and B cells in human tonsils coexpress Vitronectin receptor and its ligand vitronectin and it is known for mediating homophilic cell adhesion interactions (Lange *et al.*, 2001). LFA-1 binds to Intracellular adhesion molecule 1 (ICAM-1, CD54) which is important for naïve T cell to form immunological synapse with dendritic cells (Grakoui *et al.*, 1999). LFA-1 mRNA is progressively up regulated on $CXCR5^{hi}ICOS^{hi}$ cell, which might indicate additional functions in the context of B cell help. LFA-3 is known to be expressed on dendritic cells, which interacts with CD2 on T cells. Its expression on $CXCR5^{hi}ICOS^{hi}$ cell might also be important during cognate interactions with B cells. L-selectin (CD62L) is downregulated from $CXCR5^{-}ICOS^{-/lo}$ and $CXCR5^{lo}ICOS^{int}$ to $CXCR5^{hi}ICOS^{hi}$ T cells but it is abundantly expressed in peripheral blood subsets except T_{EM} cells. P selectin ligand (CD162) is highly expressed in peripheral blood subsets as compared to tonsillar subsets. Among other integrins mRNA for ITGA4 (integrin α4, CD49d) and ITGB7 (integrin β7) are highly expressed on peripheral blood subsets compared to tonsillar subsets. Integrins from noncovalent heterodimers made of alpha and beta subunits. Expression of integrin $\alpha4\beta7$ on T cells allow them to interact with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and facilitate extravasation from blood to secondary lymphoid organs

and inflamed tissues. Integrin ITGA6 (integrin alpha 6, CD49f) mRNA is abundant in $CXCR5^{-}ICOS^{-/lo}$ and Naïve cells where as ITGAM (integrin alpha M, CD11b) is abundant in T_{EM} cells. ITGAM and ITGB2 heterodimerise to form Mac-1 that binds to ICAM-1. ITGA6 and ITGAM are also important molecules for stabilizing the process of rolling on endothelial cells during lymphocytes extravasation.

4.3. Dynamic control of proliferation and apoptosis in Tonsillar CD4 T cell subsets

Since sorted tonsillar CD4 T cell subsets did not proliferate in cocultures with autologous B cells, unsorted cells were used which also did not show proliferation in the absence of stimulatory agents. Upon anti-CD3/CD28 stimulation proliferation was observed on day 3 in all the CD4 T cell subsets. This was followed up until day 5 where 90% of $CXCR5^{-}ICOS^{-/lo}$ and $CXCR5^{lo}ICOS^{int}$ cells had divided. Sorted cells did not proliferate in cocultures even after anti-CD3/CD28 activation and prolonged incubation. Nevertheless these were potent enough to stimulate autologous B cells for immunoglobulin production. This indicates that the individual capacity of the tonsillar CD4 T cell subsets to stimulate B cells is independent of proliferation.

Several reasons could be attributed for the complete lack of proliferation in cocultures using sorted subsets. Primarily, certain cytokines secreted by one subset may act as growth factors for others. Early experiments by others have shown that CD57 expressing germinal center T cells require exogenous IL-2 to stimulate B cell immunoglobulin production (Andersson *et al.*, 1996). In another study ICOS expressing peripheral blood CD4 T cells were also shown to produce low levels of IL-2 that are required for initial proliferation but not sufficient for sustained growth (Riley *et al.*, 2001). IL-2 was absent in freshly isolated $CD57^{-}$ and $CD57^{+}$ $CD4^{+}$ T cells, but was upregulated in both the subsets upon stimulation with pokeweed mitogen or a mixture of PMA and ionomycin (Butch *et al.*, 1993; Johansson-Lindbom *et al.*, 2003). However, both at mRNA and protein levels expression of IL-2 was low in $CD57^{+}$ subset as compared to $CD57^{-}$ subset. In line with this our gene expression data generated from resting tonsillar CD4 T cell subsets did not show any detectable levels of IL-2 mRNA. Thus, when sorted cells were used in cocultures, scanty levels of IL-2 produced by one of the subsets might have been further diluted resulting in the absence of proliferation. In case of unsorted CD4 T cells, there could be certain level of IL-2 sufficient for inducing proliferation upon TCR ligation. *In vivo*, the process of activation and proliferation

could be occurring in functional microenvironments where T cell subsets might efficiently share additional cytokines apart from IL-2.

In case of IL-2, a simple approach of observing the changes would be to add exogenously recombinant IL-2 to either sorted T cell subsets or to the whole CD4 T cell population cocultured with autologous B cells. It would be noteworthy to observe if there is any change in the B cell stimulatory capacity or the differentiation of $CXCR5^{-}ICOS^{-/lo}$ and $CXCR5^{lo}ICOS^{int}$ CD4 T cells. In case of complete CD4 T cells another possible approach could be to sort three subsets and label with a long-term cell tracking dye individually. Recombine all the three subsets proportionately as in original CD4 T cells and coculture with B cells. Since the cells are labeled, changes occurring in $CXCR5^{-}ICOS^{-/lo}$ and $CXCR5^{hi}ICOS^{hi}$ cells could be easily tracked. This approach would allow us to provide further evidence for our proposed pathway of differentiation from $CXCR5^{-}ICOS^{-/lo}$ to $CXCR5^{hi}ICOS^{hi}$ cells.

During an immune response, a subset of CD4 T cells gets activated, proliferates and differentiates in order to home into B cell follicles. This process leads to an expansion of the CD4 T cell pool, which is followed by a contraction phase to remove the cells that have outlasted their usefulness. Analyses of the programmed cell death in CD4 T cell subsets revealed that there is a progressive increase in the degree of apoptosis as the cells upregulate CXCR5 and ICOS. In the extrinsic pathway of apoptosis, Fas-FasL interaction recruits caspase-8 and caspase-10 with the binding of adaptor molecule Fas-associated death domain (FADD) to mediate the formation of death-inducing signaling complex (DISC) (Tibbetts *et al.*, 2003). In the DISC, proteolytic activation of caspase-8 and caspase-10 occurs which in turn activates downstream executioner proteases like caspase-3, caspase-6 and caspase-7. Once activated, these caspases modulate proteolysis of nuclear and cytoskeletal components to develop the phenotypic characteristics of apoptosis. Although Fas is expressed on the majority of follicular B helper T cells (Breitfeld *et al.*, 2000), mRNA for caspase 8 and caspase 10 are unexpectedly low. Unlike caspase-8 and caspase-10 mRNA that was not detectable in $CXCR5^{lo}ICOS^{int}$ cells and $CXCR5^{hi}ICOS^{hi}$ cells, the downstream caspase-3 and caspase-6 mRNA was “present” in all the subsets. It means that there is basal level of mRNA expression for caspase-3 and caspase-6.

Interestingly, mRNA for caspase-9, an initiator caspase of intrinsic pathway of apoptosis, is elevated in $CXCR5^{hi}ICOS^{hi}$ cells. Caspase-9 is activated by apoptotic protease activating factor 1 (APAF-1), which in turn has been oligomerized by

cytochrome c released from mitochondria (Green, 2005). Activated caspase-9 cleaves and activates executioner protease caspase-3 and caspase-6, which are finally responsible to mediate breakdown of cytoskeletal proteins and degradation of DNAse inhibitor ICAD. The absence of initiator caspases of the extrinsic pathway and upregulation of caspase-9 along with executioner caspases suggests that the mitochondrial gated pathway or intrinsic pathway might be active in tonsillar CD4 T cells undergoing apoptosis. Furthermore, $CXCR5^{hi}ICOS^{hi}$ cells represent the smallest proportion among the three subsets. Thus, during T cell dependent humoral immune responses, expansion of follicular B helper T cells might be efficiently controlled by a mechanism possibly involving the intrinsic pathway of apoptosis.

The generation of memory T cells is an important aspect of the adaptive immune system to mount an immediate response upon re-challenge with a specific antigen. Antigen experienced CD4 T cells must receive survival signals and exhibit resistance to apoptosis in order to develop into long-lived memory cells. To date, it is not known if a functional memory compartment develops during the process of T_{FH} cell generation. Expression pattern of certain markers associated with memory might support this view. CD45RO, the classical memory marker is expressed on approximately 90% of $CXCR5^{lo}ICOS^{int}$ cells, and on almost all $CXCR5^{hi}ICOS^{hi}$ cells. On the other hand, CD45RA, a marker for naïve T cells, is expressed in about 80% of $CXCR5^{-}ICOS^{-/lo}$ cells. The receptor for IL-7, as shown in mice both *in vitro* and *in vivo*, regulates the survival and generation of memory CD4 T cells (Kondrack *et al.*, 2003; Li *et al.*, 2003). IL-7R mRNA levels in $CXCR5^{lo}ICOS^{int}$ cells are far less as compared to peripheral blood subsets but 4.5 fold higher when compared to $CXCR5^{hi}ICOS^{hi}$ cells. Another interesting gene is CTLA-4, which is implicated in attenuation of T cell responses to induce tolerance. Expression of CTLA-4 also results in inhibition of proliferation and initiates survival of already activated T cells (Brunner-Weinzierl *et al.*, 2004). CTLA-4 expressing cells are proposed to represent a subset, which differentiate into memory T cells (Brunner-Weinzierl *et al.*, 2004). In $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ CD4 T cells, CTLA-4 is highly expressed, which may contribute to the initiation of memory formation. Again the most likely T cell subset responding to CTLA-4 could be $CXCR5^{lo}ICOS^{int}$ cells as these cells have intermediate proliferative capacity and susceptibility to apoptosis.

Furthermore, $CXCR5^{lo}ICOS^{int}$ cells retain expression of CCR7 to some extent, a chemokine receptor required by T cells to recirculate between secondary lymphoid organs. Contrary to this, CXCR5 expressing peripheral blood CD4 T cells (T_{CM1})

differ in their pattern of gene expression as compared to tonsillar CD4 T cell subsets; rather their expression signatures are more closely related to T_{CM} and T_{EM} cells. The intensity of the expression of CXCR5 as detected by microarray analysis is highest in $CXCR5^{hi}ICOS^{hi}$ cells followed by $CXCR5^{lo}ICOS^{int}$ cells and then T_{CM1} cells (Fig 23). Hitherto, using the results obtained from this study, the origin of T_{CM1} cells could not be linked to cells expressing CXCR5 from tonsils. But as discussed earlier there appears to be a substantial amount of heterogeneity in tonsillar CD4 T cell subsets. Hence it cannot be completely ruled out that T_{CM1} cells are derived from CXCR5 expressing cells from secondary lymphoid organs.

4.3.1. Expression of positive and negative costimulatory molecules might contribute to the rate of proliferation and apoptosis

All the members of CD28 and some TNF/TNFR family are expressed at varying mRNA levels in tonsillar subsets. The differences observed in the rate of proliferation of the three subsets could also be due to the engagement of negative regulators like CTLA-4, PD-1 and BTLA. These molecules are abundantly expressed by $CXCR5^{hi}ICOS^{hi}$ cells, which could act as a counterbalance to activating signals resulting in inhibition of proliferation (Riley *et al.*, 2001). Expression of all the known CD28 receptor family members is one of the most striking features of $CXCR5^{hi}ICOS^{hi}$ and to some extent $CXCR5^{lo}ICOS^{int}$ cells. CD28 alone is constitutively expressed on all CD4 T cells (Lenschow *et al.*, 1996) and others are sequentially expressed after activation. CD28 and ICOS provide positive costimulatory signals, which are counterbalanced by the coinhibitory signals generated from CTLA-4 and PD-1 (Carreno and Collins, 2002). CTLA-4 has greater affinity towards CD80 and CD86 compared to CD28 (Collins *et al.*, 2002). In tonsillar subsets CD28 is gradually upregulated from $CXCR5^{-}ICOS^{-/lo}$ to $CXCR5^{hi}ICOS^{hi}$ subset. Whereas, CTLA-4 is expressed on $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells, which might contribute in dampening immune responses.

Resting CD4 T cells express CD28 but once cells are activated ICOS is upregulated and its signaling takes over to regulate cytokine production (Coyle *et al.*, 2000; McAdam *et al.*, 2000; Ogawa *et al.*, 2001). In mice following immunization with keyhole limpet hemocyanin (KLH), treatment with B7.2-Fc fusion protein, which activates CD28, did not alter antibody production. Whereas injection of B7h-Fc, which activates ICOS signaling, enhanced anti-KLH antibody production (Guo *et al.*, 2001). CD28 has been shown to bind the lipid kinase phosphatidylinositol 3-kinase (PI3-K) (Prasad *et al.*, 1994; Truitt *et al.*, 1994) and Grb2 (Schneider *et al.*, 1995) by means of a phosphotyrosine-based motif,

pYMNM. ICOS instead possess YMFM motif in the corresponding region of CD28 that binds PI3-K but not Grb2. This inability to bind Grb2 leads to failure in IL-2 promoter activity after ICOS ligation (Harada *et al.*, 2003). Hence ICOS upregulation does not result in IL-2 secretion as seen in case of CD28.

CD28 and ICOS expressed at different stages of activation share some common signaling pathways (Rudd and Schneider, 2003). Evidence of similarity comes from transcriptional profiles generated from human CD4 T cells comparing CD28 and ICOS costimulation in the presence of TCR engagement (Riley *et al.*, 2002). Both conditions induced nearly identical transcription profiles except for a few transcripts like IL-2, IL-9, ICOS, Myelin and lymphocyte protein (MAL) and myosin F1 (MYO1F) were differently regulated by CD28. After the initial activation CD28 is not required for regulating CD4 T cell responses (Coyle *et al.*, 2000; McAdam *et al.*, 2000; Ogawa *et al.*, 2001). Hence, in order to sustain the ongoing immune response, high expression of ICOS by $CXCR5^{hi}ICOS^{hi}$ cells might be required to counteract negative signals generated by CTLA-4, and PD-1.

PD-1, an immunoglobulin superfamily member related to CD28 and CTLA-4, is also highly upregulated in $CXCR5^{hi}ICOS^{hi}$ cells. Using human tonsils it was reported that PD-1 is expressed on most T cells and a small subset of B cells in the light zone of germinal centers (Iwai *et al.*, 2002). PD-L1 and PD-L2 are the known ligands, of which the latter has a higher affinity for PD-1. In mice, germinal center B cells or FDCs lacked expression of both the ligands (Liang *et al.*, 2003). However in humans, tonsillar FDCs were shown to express PD-L1 and PD-L2 (Brown *et al.*, 2003). Hence it is of considerable interest to determine if PD-1 expression on T_{FH} is required for T-cell–FDC-interaction to dampen immune responses.

BTLA is functionally similar to CTLA-4 and PD-1. In contrast to CTLA-4 and PD-1, which are expressed on activated cells, BTLA is constitutively expressed on naïve T cells and is upregulated upon activation. Initially it was considered to be Th1 specific but its expression is also found on B cells (Hurchla *et al.*, 2005). BTLA expression on B cells and $CXCR5^{hi}ICOS^{hi}$ cells might back signal in both cells in order to stabilize immune responses.

A second set of costimulatory molecules tightly involved in cross-regulation with CD28 family are the members of TNF/TNFR family. For example signaling via ICOS induces CD40L expression on activated T cells to provide cognate B cell

help via CD40 (Hutloff *et al.*, 1999; McAdam *et al.*, 2001). Furthermore in $ICOS^{-/-}$ mice class switching was restored by CD40 stimulation.

4.4. Potent Follicular B helper activity resides in $CXCR5^{hi}ICOS^{hi}$ cells

It is well established that CXCR5 expressing CD4 T cells provide efficient B cell help for immunoglobulin production (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000). Subset of $CXCR5^{+}CD4^{+}$ T cells leave T cell areas and migrate into B cell follicles to engage in germinal center reaction (Hardtke *et al.*, 2005). When freshly isolated tonsillar CD4 T cell subsets were co-cultured with autologous B cells a considerable differences were observed in the stimulation capacities of all the three subsets. The stimulatory activity was directly correlating with the expression levels of CXCR5 and ICOS, with $CXCR5^{hi}ICOS^{hi}$ cells exerting highest stimulation, on all the days tested. However, upon activation with anti-CD3/CD28 antibodies, the stimulatory activity was observed to be prominent by $CXCR5^{lo}ICOS^{int}$ cells. This reversed effect could simply be attributed to the enhanced survival of $CXCR5^{lo}ICOS^{int}$ cells as compared to $CXCR5^{hi}ICOS^{hi}$ cells. Shifting the ratio of B and T cells in co-cultures results in B cells acquiring a greater degree of costimulation and enhanced IgG production. It was also observed that there were no obvious changes in the expression of CXCR5 and ICOS on T cells during the coculture in the presence or absence of stimulation. Considering the efficient stimulatory capacity of $CXCR5^{hi}ICOS^{hi}$ cells we speculate that this might represent the actual T_{FH} subset. Figure 24 shows a model depicting the activation of naïve CD4 T cells to differentiate into T_{FH} cells.

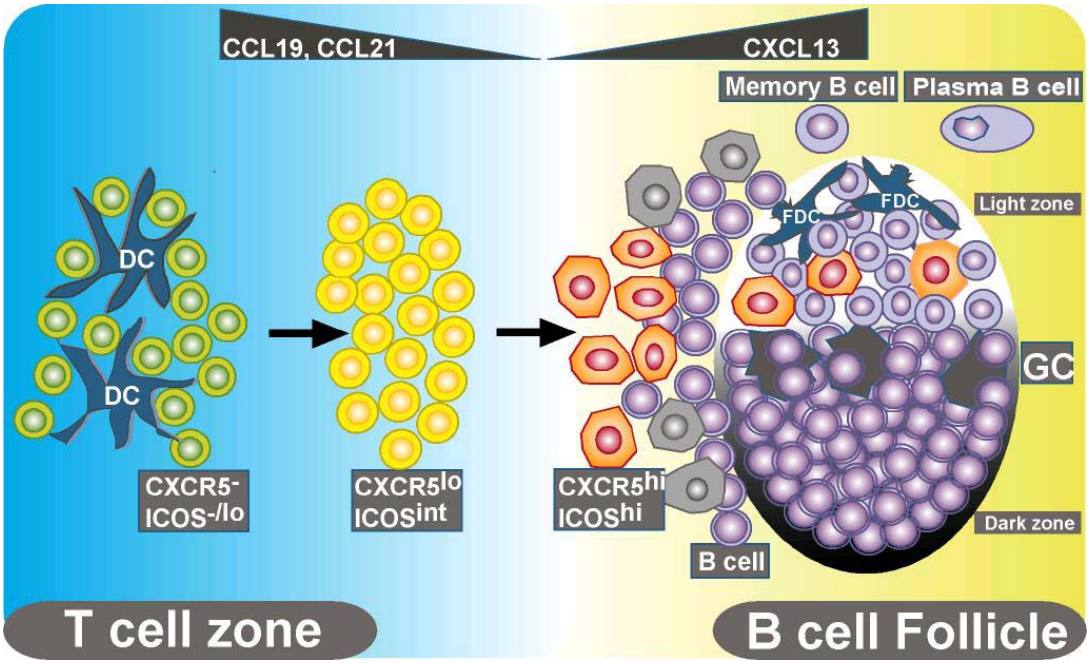

Fig. 24: Model depicting cognate cellular interactions and differentiation of follicular B helper T cells. Secondary lymphoid organs are compartmentalized into T cell zones and B cell follicles. T cell zones are rich in chemokines CCL19 and CCL21 that retains T cells expressing CCR7. B cell follicles contain chemokine CXCL13, which attracts B cells and a subset of T cells expressing CXCR5. CCL19 and CCL21 gradients are shown in blue and the CXCL13 gradient in yellow. Within the T cell zone, CD4 T cells not expressing CXCR5 but low levels of ICOS on their surface are designated as $CXCR5^{-}ICOS^{-/lo}$ CD4 T cells (green). These cells are also characterized by their lack of capacity to support immunoglobulin production and CXCL13 secretion but proliferate upon TCR-CD28 stimulation with considerable resistance to undergo apoptosis. $CXCR5^{-}ICOS^{-/lo}$ cells recognize antigen on DC (blue) in the form of peptide:MHC complexes in combination with costimulatory signals and upregulate CXCR5 and ICOS to differentiate into $CXCR5^{lo}ICOS^{int}$ CD4 T cells (yellow). These cells have intermediate levels of proliferation and apoptosis compared to $CXCR5^{-}ICOS^{-/lo}$ and $CXCR5^{hi}ICOS^{hi}$ cells. They also support immunoglobulin production by B cells but only after prolonged incubation. With these features $CXCR5^{lo}ICOS^{int}$ cells are hypothesized to represent precursors of T_{FH} cells. Alternatively, they may represent the memory compartment for T_{FH} cells. $CXCR5^{lo}ICOS^{int}$ cells further up regulate CXCR5 and ICOS to differentiate into $CXCR5^{hi}ICOS^{hi}$ cells (red), which constitute the actual follicular B helper T cells (T_{FH}). T_{FH} cells are efficient stimulators of B cell immunoglobulin production and secrete large amount of CXCL13 that act as chemoattractant for B cells and other T_{FH} cells. Cells undergoing apoptosis in $CXCR5^{hi}ICOS^{hi}$ subset are shown in grey color. Some of the T_{FH} cells migrate into the light zone of the germinal center to help B cells (violet) in survival and affinity maturation.

Ligation of CD28 followed by ICOS expression provides further signals for optimal T cell activation and proliferation. All members of the CD28 and some of the TNF/TNFR family of co-stimulatory molecules are expressed at moderate to high

levels in CXCR5^{lo}ICOS^{int} cells. For example, compared to other tonsillar subsets, BAFF is highly expressed in CXCR5^{lo}ICOS^{int} cells. It is a member of the TNF ligand superfamily expressed in T cells and dendritic cells, either membrane bound or processed and secreted (Schneider *et al.*, 1999). Three known receptors for BAFF are BAFF-R, TACI and BCMA, all expressed at various stages of B cell development (Ng *et al.*, 2005; Zhang *et al.*, 2005). TACI is expressed predominantly on memory B cells and BCMA is expressed on germinal center B cells, plasma blasts and plasma cells. BAFF binds with high affinity to BAFF-R and TACI and with lower affinity to BCMA to promote isotype switching, germinal center maintenance and plasma cell survival (Schneider *et al.*, 1999). Less than 5% of the CD4 T cells isolated from murine spleen and lymph nodes express BAFF-R (Ng *et al.*, 2005; Ye *et al.*, 2004). This expression was increased upon anti-CD3/CD28 stimulation or treatment with plate bound BAFF-Ig or anti-BAFF-R mAb. *In vivo*, BAFF/BAFF-R signaling is shown to be involved in CD4 T cell proliferation and activation. BAFF-R expression on tonsillar and peripheral blood CD4 T cells could not be checked, as it is not represented on the Affymetrix genechip U133 set used in this study. It will be of particular interest to check the function of BAFF-R if it is expressed on tonsillar CD4 T cells. It is particularly interesting that BAFF transcripts are abundant in CXCR5^{lo}ICOS^{int} cells suggesting an important contribution of this molecule, which needs to be investigated. A second TNF family member, that signals via BCMA and TACI is APRIL. Neither expression of neither APRIL nor its receptor BCMA or TACI could be detected on tonsillar CD4 T subsets by microarray analysis.

CXCR5^{hi}ICOS^{hi} cells are not only efficient stimulators of IgG secretion by B cells but also secrete 10-fold higher amounts of CXCL13 as compared to CXCR5^{lo}ICOS^{int} cells. Endogenous CXCL13 was determined by western blotting on resting tonsillar CD4 T cell subsets. CXCR5^{hi}ICOS^{hi} T cells contained highest levels of CXCL13 as compared to CXCR5^{lo}ICOS^{int} T cells. CXCR5^{-}ICOS$^{-/lo}$ T cells were negative for CXCL13. At mRNA level CXCR5^{hi}ICOS^{hi} T cells had 56-fold higher CXCL13 transcript as compared to CXCR5^{-}ICOS$^{-/lo}$ T cells. CXCR5^{lo}ICOS^{int} T cells were 13-fold higher as compared to CXCR5^{-}ICOS$^{-/lo}$ T cells. Activation of T cells by anti-CD3/CD28 antibodies in coculture led to an enhanced CXCL13 secretion in both CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} T cells. This indicates that activation does increase CXCL13 secretion on both subsets, but CXCR5^{hi}ICOS^{hi} cells are still the most efficient producers of chemokine. This indicates that anti-CD3/CD28 stimulation does not reverse the pattern of CXCL13 expression in CXCR5^{lo}ICOS^{int} and CXCR5^{hi}ICOS^{hi} cells as observed for IgG secretion by B cells. Kim et al., have shown that apart from anti-CD3/CD28, other stimulating

agents like PMA and ionomycin also enhanced CXCL13 secretion (Kim *et al.*, 2004). This was shown using $CD57^{+}CXCR5^{+}CD4^{+}$ T cells, which represent a subset of $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells. In addition, they demonstrated that CXCL13 secreting $CD57^{+}CD4^{+}$ T cells are located in germinal centers surrounded by follicular dendritic cells and activated B cells. As CXCR5 is expressed on both B cells and a subset of T cells, the expression of its ligand, CXCL13 by $CXCR5^{hi}ICOS^{hi}$ cells might be important in drawing these cells closer in order to deliver efficient T cell help to B cells.

$CXCR5^{hi}ICOS^{hi}$ cells also show unique expression pattern of mRNA for genes involved in notch signaling. Notch signaling is essentially mediated by four notch receptors (notch 1-4) and five notch ligands (Jagged 1 and 2; Delta 1, 3 and 4) (Artavanis-Tsakonas *et al.*, 1999). Notch receptor interaction with its ligand leads to cleavage of the intracellular domain of notch (NIC) by γ-secretase activity of presenelin. NIC is translocated to the nucleus where it binds to RBP-J□ (also known as CBF-1 and CSL) and converts it from transcriptional repressor to transcriptional activator by displacing a corepressor complex and recruiting coactivators. The end result of notch signaling events is transcription of several target genes like Hey1, Hes1, Hes5, pTα, deltex-1 and notch1.

Upregulation of these genes is required for lineage commitment during hematopoiesis, T cell and B cell development (Radtke *et al.*, 2004). Dendritic cells, T cells and B cells express different notch receptors and their ligands. Expression of notch receptor and its ligand has differential effects depending on the cell type. For example delta-like 1 and Notch-RBP-J signaling is required for the generation of marginal zone B cells (Hozumi *et al.*, 2004; Tanigaki *et al.*, 2002). Interactions between immune cells during antigen presentation and B cell help may involve various notch-ligand signaling events, which are important for cytokine secretion and expression of specific cell surface receptors (Hoyne *et al.*, 2001; Maillard *et al.*, 2003). Preferential expression of notch1, Hes1, Hey1, HeyL and FZD3 in $CXCR5^{hi}ICOS^{hi}$ cells as compared to other subsets indicates active notch signaling in this cell subset. Furthermore, expression of Hey1 has also been confirmed by real time RT-PCR. It is quiet tempting to determine the functional role played by active notch signaling in T_{FH} cells. Knock down of the transcript in T cells using interfering RNA and studying the consequences could be one possibility. Other approach would be to switch the model system to knockout mice. It would be more flexible to study T-B interactions extensively by eliciting various immune responses.

4.5. Follicular B helper T cells might represent a non-Th1/Th2 cell subset

T_{FH} cells are poor cytokine producers whose profile does not resemble either Th1 or Th2 cells. But upon stimulation they secrete IL-2, IL-4 and IL-10 (Butch *et al.*, 1993; Toellner *et al.*, 1995). Inefficiency to secrete cytokines by T_{FH} cells could be attributed to the expression of the members of suppressors of cytokine signalling (SOCS) family. These molecules are cytoplasmic proteins that act in a negative feedback loop via direct binding or preventing access to components of cytokine signaling cascade. T_{FH} cells express mRNA for SOCS1 and SOCS3. Both SOCS 1 and SOCS3 were shown to share 30% sequence homology (Alexander, 2002). SOCS1 is preferentially expressed on Th1 and SOCS3 on Th2 cells (Ilangumaran and Rottapel, 2003). Expression of both these molecules on T_{FH} cells might be responsible for tightly regulated expression of cytokines.

Transcription factors are another class of molecules whose expression is specific for Th1 and Th2 cells. Polarization towards Th1 or Th2 lineage is driven by the induction of T-bet and GATA3, respectively. But in case of T_{FH} cells mRNA for a number of molecules are either expressed at average levels or upregulated. GATA3 is highly expressed by peripheral blood T_{CM} and T_{EM} cells. Unlike Th1/Th2 cells, a master switch transcription factor for T_{FH} cells is not yet defined. Upregulation of MAF, MAFB, MAFG and NFATC1 in $CXCR5^{hi}ICOS^{hi}$ cells indicate that unlike Th1/Th2, there could be several different transcription factors driving T_{FH} differentiation. Hence, at this juncture considering the differential expression of signaling molecules and transcription factors it is likely that the T_{FH} cells might as well represent a non-Th1/Th2 cell type with a different lineage.

The expression pattern for sphingosine-1-phosphate 1 (S1P1) receptor distinguishes T_{FH} cells from rest of the tonsillar and peripheral blood subsets. This receptor is completely downregulated from $CXCR5^{-}ICOS^{-/lo}$ to $CXCR5^{hi}ICOS^{hi}$ T cells as also confirmed by realtime RT-PCR. S1P1 is essential for lymphocyte recirculation regulating exit from thymus and peripheral lymphoid organs. Hence, it is can be speculated that S1P1 downregulation could be one of the mechanisms used by $CXCR5^{hi}ICOS^{hi}$ T cells to retain in secondary lymphoid organs.

4.6. CD57 is coexpressed on a subset of CXCR5 and ICOS expressing cells

Follicular B helper T cells are poorly defined in terms of specific markers expressed on their surface. As compared to Th1 and Th2 cells, which can be easily distinguished using their cytokine profiles, T_{FH} cells are not associated with specific cytokines. Nevertheless, CD4 T cells with T_{FH} function are associated with expression of CD57 (Bowen *et al.*, 1991; Kim *et al.*, 2001; Kim *et al.*, 2005). Studies performed by Kim et al., on CD4 T cell from human tonsils have demonstrated that CD57 expressing cells stimulate immunoglobulin production and class switch recombination in B cells more efficiently than $CD57^-$ cells. In contrast, gene expression profiles of $CD57^+$ and $CD57^-$ cells were highly similar indicating that they do not represent a distinct subset (Chtanova *et al.*, 2004). CD57 association with T_{FH} is also controversial, as others have shown that $CD57^+$ GC T cells represent anergized cells, which failed to express IL-2 when activated by TCR stimulation (Johansson-Lindbom *et al.*, 2003). These cells were shown to induce immunoglobulin production of B cells only when exogenous IL-2 was added in the presence of pokeweed mitogen or Staphylococcal enterotoxin A (Andersson *et al.*, 1996).

Furthermore, the exact role of CD57 expression for CD4 T cell biology still remains unclear. However, CD57 is predominantly present in the nervous system, and its expression is spatially and temporally regulated during the development of nervous system (Schwarting *et al.*, 1987; Yoshihara *et al.*, 1991). Unlike in the immune system, its role is well defined in cell-cell interactions in the nervous system (Kunemund *et al.*, 1988; McGarry *et al.*, 1983). Studies on HNK-1 deficient mice have shown that it is important for higher functions of the brain such as synaptic plasticity and spatial learning (Yamamoto *et al.*, 2002). Initially it was thought that $CXCR5^+ICOS^+CD57^+$ cells might be the actual subset harboring follicular T cell help. In contrast, it was observed that $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ CD4 T cells when separated based on CD57 expression, differ neither in their B cell stimulatory capacity nor in CXCL13 secretion. Instead, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ cells displayed obvious differences in B cell help as well as CXCL13 expression.

Although $CD57^+CD4^+$ T cells were shown to be a distinct subset as compared to either Th1 and Th2 or T_{CM} and T_{EM} cells, the plausibility of this marker specific for T_{FH} cells stands invalid in light of present study. $CD57^+CD4^+$ T cells constitute about 16% in $CXCR5^{lo}ICOS^{int}$ and 34% in $CXCR5^{hi}ICOS^{hi}$ subsets. $CD57^+CD4^+$ T

cells are all CXCR5 expressing cells and $CD57^-$ cells constitute a mixture of $CXCR5^+$ and $CXCR5^-$ cells. Hence, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ subsets, which supported IgG secretion by B cells are unequally distributed among $CD57^-$ $CD4^+$ and $CD57^+CD4^+$ T cells, apparently leading to the differences in the follicular helper activity. Taken together, these results confirm that the stimulatory activity of T_{FH} cells is not associated with CD57 expression, but instead correlates with the expression levels of CXCR5 and ICOS.

4.7. Gene expression profiles reveal that T_{FH} cells are a distinct subset compared to central memory and effector memory T cells

Large-scale gene expression analysis was performed on all the three tonsillar CD4 T cell subsets and peripheral blood subsets. Gene expression data from all the 7 subsets was analyzed in order to gain insight into the specific pathways involved in cell differentiation and T_{FH} commitment. Most of the functional categories of differentially expressed genes were already discussed along with related functional aspects. An overview of differentially expressed genes and their possible role with respect to the relationship between the subsets is discussed here.

Using this data, principal component analysis (PCA) revealed three spatially separated distinct groups. Naïve cells from peripheral blood and $CXCR5^-ICOS^{-/lo}$ CD4 T cells define group A. group B and group C constitute rest of the peripheral blood and tonsillar subsets, respectively. Terminally differentiated T_{EM} and $CXCR5^{hi}ICOS^{hi}$ T cell subsets are located far away from their naïve counterparts. $CXCR5^{lo}ICOS^{int}$ T cells, which might represent an immediate precursor population for $CXCR5^{hi}ICOS^{hi}$ T cells or a memory compartment (as discussed before) for follicular help, are located in close proximity to $CXCR5^{hi}ICOS^{hi}$ T cells. One more important conclusion drawn from this analysis is that CXCR5 expressing T_{CM1} cells are closely related to peripheral blood subsets rather than tonsillar $CXCR5^+CD4^+$ T cells. Again it means the origin and function of T_{CM1} cells still remains unsettled.

Another interesting aspect that needs to be addressed is the function of $CXCR5^{lo}ICOS^{int}$ cells. Weather these cells are only precursor population of T_{FH} cells or if they have additional roles depending on the type of immune response. For a given aspect, $CXCR5^{lo}ICOS^{int}$ cells display similar behavior either to $CXCR5^-$ $ICOS^{-/lo}$ or $CXCR5^{hi}ICOS^{hi}$ cells. To start with, $CXCR5^-ICOS^{-/lo}$ and $CXCR5^{lo}ICOS^{int}$ cells constituted similar percentage distribution. Additionally, proliferative potential is also comparable between these cells if all the cells that

have been at least divided once are considered. In contrast, $CXCR5^{lo}ICOS^{int}$ cells share similarities with $CXCR5^{hi}ICOS^{hi}$ cells: not differing much in chemotaxis to chemokine CXCL13, ability to provide cognate help to B cells after prolonged incubation and CXCL13 secretion. In spite of these similarities with $CXCR5^{-}ICOS^{-/lo}$ on one side and $CXCR5^{hi}ICOS^{hi}$ on the other, $CXCR5^{lo}ICOS^{int}$ cells had intermediate expression for the majority of the genes that are differentially regulated. This makes them probably a mixture of T cell types, which might be flexible in a way that there could be multiple fates apart from being precursors for $CXCR5^{hi}ICOS^{hi}$ cells.

A pair-wise comparison of peripheral blood and tonsillar subsets revealed 3272 probes statistically significant and differentially regulated. The differences in tonsillar subsets and peripheral blood subsets (Probes 568-2032) were far more pronounced as compared to the subsets within tonsils (probes 622-1487), followed by subsets within peripheral blood (probes 67-464). The differences between subsets from peripheral blood are rather small as compared to tonsillar subsets. Peripheral blood memory/effector cells are polyclonal arising from various stimulations during the lifetime of an individual. As a result they may still contain a considerable amount of heterogeneity resulting in less obvious differences. This can be addressed effectively if gene expression data is generated from memory/effector cells specific for a selected antigen. The second reason could be that the tonsillar CD4 T cells were isolated from inflamed tonsils that are highly activated as compared to the resting peripheral blood subsets, which were isolated from healthy donors. In any case, the present study and as shown by others (Chtanova *et al.*, 2004; Kim *et al.*, 2004) it can be concluded that T_{FH} cells have distinct differentiation and functional programs as compared to peripheral blood memory/effector T cell subsets.

This study was taken up in order to better characterize and understand the biology behind follicular B helper T cells. Tonsillar CD4 T cells were fractionated based on the expression of CXCR5 and ICOS into 3 subsets namely $CXCR5^{-}ICOS^{-/lo}$, $CXCR5^{lo}ICOS^{int}$ and $CXCR5^{hi}ICOS^{hi}$ T cells. A set of *in vitro* functional assays and large-scale gene expression analysis was performed on tonsillar CD4 T cell subsets. Compared to other tonsillar subsets, $CXCR5^{hi}ICOS^{hi}$ T cells were the most efficient in inducing immunoglobulin production by B cells in spite of reduced potential to proliferate and higher susceptibility to apoptosis. In addition, $CXCR5^{hi}ICOS^{hi}$ cells displayed restricted chemokine receptor expression and secreted highest amount of CXCL13. $CXCR5^{hi}ICOS^{hi}$ cells emerged as a subset, which might represent the actual *in vivo* T_{FH} population. These cells are highly

distinct from peripheral blood central memory and effector memory in terms of gene expression. Generation of gene expression signatures has opened several future directions to study unresolved molecular mechanisms responsible for T_{FH} cell differentiation. Modulation of cognate help by T_{FH} cells has therapeutic role in autoimmune diseases and immunodeficiency. Identification of key molecules and signaling pathways might help to intervene cognate help to B cells and hence the T-cell-dependent humoral immune responses.