TH17 RESPONSE IN MURINE VL
**Introduction**

Interleukin-17 (IL-17) is primarily produced by T helper 17 (Th17) cells, which are known as a subset of T helper cells that are different from the classic T helper 1 (Th1) and 2 (Th2) cells. Apart from IL-17 secretion, Th17 is characterised by the expression of the chemokine receptor CCR6 and the transcription factor RORγt (Retinoic-related Orphan Receptor gamma t) (Kugyelka et al., 2016). There are two distinct Th17 cell subsets reported under in vitro stimulation conditions: the conventional Th17 cells generated following TGF-β/IL-6 stimulation, and inflammatory Th17 cells generated following IL-6/IL-23/IL-17 stimulation (Ghoreschi et al., 2010). The subsets of Th17 cells play a pivotal role in autoimmunity and chronic inflammatory diseases by boosting inflammatory response and participating in defence mechanisms against certain pathogens (Soong, Henard, and Melby, 2012). These seminal studies opened up a new area for investigating the role of Th17 as an inductor of inflammatory response during various auto-immune, cancer and infectious diseases. Association of IL-17 with inflammatory diseases was further authenticated by the presence of elevated IL-17 levels in the human serum during various auto-immune diseases such as rheumatoid arthritis, atherosclerosis and systemic lupus erythematosus (Erbel et al., 2014; Sherlock, Taylor, and Buckley, 2015). It has been shown that IL-17 modulates immune cell trafficking, mainly through induction of chemokines (CXCL1, CXCL5 and CXCL10), thereby initiating inflammation and cytokine production in these diseases (Freches et al., 2013). IL-17 modulates the expression of several proinflammatory molecules (TNF-α, IL-1β, and MCP-1) and matrix metalloproteinases, generating a strong inflammatory milieu during auto-immunity (Sherlock et al., 2015; Freches et al., 2013; Erbel et al., 2014). Moreover, it was demonstrated that IL-17 plays an oncogenic role by inhibiting tumor cell apoptosis, impairing antitumor responses, promoting tumor angiogenesis, tumor metastasis and invasion (Qian et al., 2015). Reports suggest that IL-17 producing cells not only regulate the progression of tumors but also protect against *Candida albicans* and *Trypanosoma cruzi* infection (Miyazaki et al., 2010; Korn, Bettelli, Oukka, & Kuchroo, 2009; Pandiyan et al., 2011). The proinflammatory effects of IL-17 occur as a result of the activation of inducible nitric oxide synthase (iNOS) and the induction of granulocyte macrophage colony-stimulating factor, IL-6, IL-8, TNF-α, and several...
chemokines, potentiating the inflammatory reaction (Kolls and Lind 2004). It was further reported that IL-17 along with IFN-γ is indispensable for neutrophil mediated long-term control of Mycobacterium tuberculosis infection (Freches et al., 2013). However, the role of Th17 in leishmaniasis is less well understood. In human Leishmania braziliensis infections, Th17 cells were reported to play a protective role along with a Th1 response by recruiting neutrophils to combat the disease (Novoa et al., 2011). Contrary to this, in a model of cutaneous leishmaniasis, caused by L. major, IL-17 production was shown to be associated with disease progression because the lesion sizes in infected IL-17 deficient BALB/c mice were smaller than controls (Terrazas et al., 2015). Besides, a correlation of IL-17 production from PBMCs of patients with cutaneous leishmaniasis and mucocutaneous leishmaniasis progression has been reported (Bacellar et al., 2009; Boaventura et al., 2010). A similar conflicting picture exists in case of visceral leishmaniasis (VL) too. Here larger amounts of IL-17 was reported in the serum of asymptomatic individuals with a delayed-type hypersensitivity reaction to L. donovani than in the serum of symptomatic patients (Pitta et al., 2009), emphasizing the role of IL-17 as complementary to Th1 cells in protection against VL. Additionally, Nascimento et al. demonstrated in resistant C57/Bl6 mice that during VL, IL-17 works synergistically with IFN-γ to trigger the NO production by infected macrophages and promote parasite killing (Nascimento et al., 2015). Contrary to this, in the same model of L. donovani infection, it was reported that IL-17 helps in disease progression by limiting the effectiveness of monocytes in controlling parasite growth and/or killing (Sheel et al., 2015). Therefore, the possibility of Th17 association with disease persists. Moreover, priming of Th17 cells through TGF-β, a disease promoting cytokine, further makes portrayal of Th17 in VL debatable. Therefore there is a need to explore the underlying mechanisms and possible roles of Th17 in the disease progression and combating immunosuppressive milieu created by Treg cells. Here we have made an attempted to investigate the profile of Th17 cells and the cytokines secreted from them during the progression of VL in susceptible BALB/c mice, and their role if any in combating infection against disease promoting responses generated by Treg cells and immunosuppressive cytokines.
Results

IL-17 secretion decreased with advancement of the disease

To explore the involvement of Th17 cells during progressive murine VL, we performed a kinetic study of IL-17 production with progressive *L. donovani* infection in BALB/c mice, a susceptible model for VL. BALB/c mice infected with virulent *L. donovani* parasites develop a progressive disease, mimicking human VL at least up to 4 months, as we have observed earlier (Fig. 3.1). We sacrificed the infected animals at various times of infection and spleens were taken out aseptically. Leishmanial antigen (LAG) stimulated splenocytes were cultured and supernatants were investigated for the production of IL-17 by ELISA. We found that production of IL-17 is low in the normal mice (~5 pg/ml). However, it increased dramatically at the initial phase of infection (~20 pg/ml) (Fig. 4.1 a). Nevertheless, as the disease progressed, the IL-17 production gradually decreased, reaching its basal level at the peak of infection (4 months of time). A similar observation was made with serum ELISA for IL-17 (Fig. 4.1 b). Here also higher levels of IL-17 were detected at the beginning of infection. It reached its peak at 2 months post infection (~27 pg/ml) and then decreased to basal levels with the disease advancement. The suppression of IL-17 in culture supernatants and serum was in concordance with the repression of protective Th1 response with exacerbation of infection (Fig. 3.8 & 3.16).

![Figure 4.1: Estimation of IL-17 by ELISA.](image)

Normal as well as infected mice of indicated post infection times were sacrificed and splenocytes were stimulated with LAg (10 µg/ml) and cultured. After 72 hrs culture supernatants were collected for ELISA (a). Various groups of the mouse were bleed through tail vein and serum was collected for cytokine ELISA (b). Data represented as means ± SE for three mice per group and are representative of two independent experiments with similar results.
Th17 cell population decreases with progression of the disease

A similar time kinetic study (as mentioned above for cytokine ELISA) was performed for evaluating Th17 cell profile through flow cytometry and production of IL-17 from them. Typically Th17 cells are characterised by the expression of CCR6 at the surface of CD4+ T cells and a transcription factor, RORγt in mice (Kugyelka et al., 2016) (Pandiyan et al., 2011). Therefore, CD4+ T cells were gated from CD3+ T cell population of single cell lymphocytes and Th17 cells were characterised by the expression of CCR6 along with RORγt. **Fig. 4.2** represents the identification of CD4⁺CCR6⁺RORγt⁺ Th17 cells from mice splenocytes.

![Th17 cell identification through flow cytometry](image)

**Fig 4.2**: Representation of Th17 cell identification through flow cytometry.

By profiling CD4⁺CCR6⁺RORγt⁺ Th17 population at various infection times, we found that with the inception of *L. donovani* infection, Th17 cells tend to increase (~3.2 %) compared to normal (~2.8 %) and remain enhanced till two months of infection (**Fig. 3.1** and **4.3**). This was observed in percent (~3.2 %) as well as an increase in the cell number (~29×10⁵ cells/spleen compared to ~4×10⁵ cells/spleen) of infected versus normal animals. However, during the later phase of intensified infection, Th17 cell population progressively reduced. At four months of infection,
CD4⁺CCR6⁺RORγt⁺ Th17 cells population shrunk to less than 2 % of total CD4⁺ T cells and the total number of CD4⁺CCR6⁺RORγt⁺ Th17 cells reduced to <1.7×10⁵ cells/spleen.

**Figure 4.3: Profile of Th 17 cells subsets at various times of infection.** Normal as well as infected mice were sacrificed at various time points as indicated and splenocytes were processed for FACS analysis of various Th 17 cell subsets. Data expressed as means ± SE for six mice per group and are representative of at least three independent experiments with similar results.

*Production of IL-17 and IFN-γ from Th17 cells decreased with progression of VL*

After monitoring the profiles of IL-17 and Th17 cells with disease kinetics, we wanted to study the production of IL-17 from these cells at these post infection times. We found that IL-17 producing CD4⁺ T cells and CD4⁺CCR6⁺RORγt⁺ Th 17 cells increased dramatically at one month of infection (~3%) compared to controls (~0.5%). However, with infection, a decline in IL-17 producing Th17 cells were observed reaching basal levels both in percentage as well as in the total number of cells (Fig. 4.4 & 4.5). At four months of infection, when the levels of immunosuppressive cytokines were high (Fig. 3.16), IL-17 producing CD4⁺ T cell and Th17 cells were lowest in number compared to the other time points (~2.7×10⁴ cells/spleen and ~1.6×10⁴ cells/spleen respectively). It is intriguing to note that some CD4⁺CCR6⁺RORγt⁺ Th 17 cells were producing IFN-γ as well at the commencement
of infection, but this IFN-\(\gamma\) producing Th17 population also gradually decreased with the advancement of the disease and were almost negligible at 4 months of infection. Similarly, IL-17 and IFN-\(\gamma\) producing double positive CD4 and Th17 cells increased dramatically at the beginning of infection (~2.8% and ~1.8% respectively) compared to normal (~0.5% and ~0.4% respectively). These double positive cells remain increased till two months of infection, then decreased probably due to the onset of immunosuppressive cytokine at later phases of fierce infection. This increase and then decrease was very discernibly visible both in percent as well as in total number of cells. Interestingly, at 4 months of infection, when immunosuppressive cytokines were highly elevated, CD4\(^{+}\)CCR6\(^{+}\)ROR\(\gamma\)t\(^{+}\)IL-17\(^{+}\)IFN-\(\gamma\)\(^{+}\) Th17 cells were scarce both in percent and cell numbers. The decrease of IL-17 and/or IFN-\(\gamma\) producing cells at 4 months of infection was very prominent in both percentages as well as in total numbers of these cells implying a suppressed state of Th17 response along with a repressed Th1 response in immuno-suppressed milieu with the disease exacerbation (previous chapter).
Figure 4.4: IL-17 and IFN-γ Profile of CD4+ T cells. Normal as well as infected mice were sacrificed at various time points as indicated and splenocytes were stimulated with LAg (10 µg/ml) and cultured. After 12 hrs cultured cells were collected for cytokine profiling (IL-17 and IFN-γ) from CD4+ cells through flow cytometry. Data represented as means ± SE for six mice per group and are representative of three independent experiments with similar results.
Figure 4.5: Cytokine Profile of various Th 17 cell subsets. Normal as well as infected mice were sacrificed at various time points as indicated and splenocytes were stimulated with LAg (10 µg/ml) and cultured. After 12 hrs cultured cells were collected for cytokine profiling (IL-17 and IFN-γ) from various Th 17 cell sub-sets through flow cytometry. Data represented as means ± SE for six mice per group and are representative of three independent experiments with similar results.
Suppression of Th17 cells proliferation by Treg cells during ex-vivo culture

Our flow cytometry and ELISA based studies strongly emphasised the co-relation between the suppression of Th17 cells and its cytokines with the aggravation of the disease. At two months post infection time when the immunoprotective Th1 response was downregulated due to the emergence of Treg-mediated suppressive immune responses, Th17 and its cytokine, IL-17 remain enhanced. But at a later time of infection, when Treg cells and their cytokines dominated, the Th17 response also failed to sustain. Therefore we wanted to investigate the effect of Treg cells on the proliferation of Th17 cells by ex-vivo co-cultivating Th17 and Treg cells isolated from infected mice spleen. To do this, we purified CD4^+ T cells as well as CD4^+CD25^+ Treg cells from infected and un-infected mice splenocytes (~95% purity) and polarised the CD4^+ T cells into Th17 cells by treating them with IL-6, TGF-β, αIFN-γ and αIL-4 for 24 hrs. After 24 hrs of incubation, polarised cells were confirmed for phenotypic expression of CCR6 and RORγt and secretion of IL-17 through flow cytometry (~40% cells isolated from infected mice spleen were polarised and almost 20% of them were IL-17 producer compare to 0.6% IL-17 production from unpolarised cells). Fig. 4.6 shows the polarisation of CD4^+ T cells, isolated from infected mice splenocytes, into IL-17 secreting Th17 cells. Then these bona fide Th17 cells were co-cultured with Tregs in the presence of anti-CD3 and anti-CD28 at various ratios, and Th17 cells proliferation was studied through CFSE labelling.
Figure 4.6: Polarisation of CD4+ T cells to Th17 cells. CD4+ and CD4+CD25+ T cells were isolated from 4 months infected mice splenocytes as described earlier. CD4+ T cells were polarised into Th17 cells by treating them with IL-6, TGF-β, αIFN-γ and αIL-4 for 24 hrs. After 24 hrs of incubation polarised cells (right side in the figure) were confirmed for phenotypic expression of Th17 cells and secretion of IL-17.

Our co-culture study from one month infected mice splenocytes revealed that infected Tregs did not show any prominent suppressive effect on the proliferation of Th17 cells from the same mouse, compared to normal (Fig. 4.7 a). However, similar study at four months post infection time led to the substantial suppression of Th17 cell proliferation with increasing proportion of Treg cells. We performed two types of Th17 and Treg cells co-cultivation at 4 months post infection. In the first set-up, total cell number was kept constant with increasing proportion of Treg cells (Fig. 4.7 b) while in another condition increasing the number of Treg cells were cultured with a fixed number of Th17 cells (Fig. 4.7 c). The suppression of Th17 by Treg cells was clearly observed in either way of culture. It was interesting to note that the parallel co-cultivation studies with the normal cell types showed the consistent activity of Treg cells in suppressing the proliferation of Th17 cells (Fig. 4.7 b & c). Additionally, the
co-culture of Treg cells from 4 months infected mice spleen with the normal mice splenic Th17 cells and vice-versa at various indicated ratios, did not bring any considerable difference in the profile of Th17 cells proliferation (Fig. 4.7 d). The proliferative response of Th17 cells followed a similar trend in both the experimental set-ups, whether it was the culture of normal Th17 with Tregs obtained from infected mice or cultivation of Th17 cells from infected mice with normal mice splenic Tregs.

Figure 4.7: Suppression of Th17 cells by the presence of CD4+CD25+ Treg cells. Normal as well as one month and four months infected mice were sacrificed and spleen was removed aseptically. Splenic CD4⁺CD25⁻ and CD4⁺CD25⁺ Treg cells were purified through magnetic sorting according to manufacturer’s protocol. CD4⁺CD25⁻ were labelled with CFSE and polarised to Th17 cells in the presence of IL-6, TGF-β, αIFN-γ and αIL-4. After 24 hrs, polarised cells were stimulated with anti-CD3 & anti-CD28 in 96 u bottom plate and cultured with CD4⁺CD25⁺ Treg cells at various ratios. After 96 hrs cells were collected for CFSE based CD4⁺CD25⁺ proliferation assay through flow cytometry. (a) Co-culture of Th17 and Treg cells from one month infected mice. Co-cultivation of splenic Th17 with Treg cells of 4 months infected mice, (b) with constant total cell number or (c) with increasing proportion of Treg cells. (d) Co-culture of normal mice Th17 with 4 months infected mice splenic Treg cells and vice-versa. Data represented as means ± SE for three mice per group and are representative of two independent experiments with similar results.
Dominance of immunosuppressive cytokines in co-culture of Treg and Th17 cells pave the way for repression of Th17 response

The cytokine profile of culture supernatant of Th17 and Treg cell co-culture demonstrated that with the progressive inhibition of Th17 cells proliferation, the production of IFN-γ and IL-17 decreased (Fig. 4.8 a & b) and the secretion of immunosuppressive cytokines, IL-10, TGF-β and both the chains that participate in IL-35 formation (IL-12 p35 and EBI-3) increased (Fig. 4.8 c, d, e & f). Interestingly, elevated levels of both these chains of IL-35, with the enhanced suppression of Th17 cells in-vitro, implying that these chains probably were coming from IL-35 and not from other sources, as the production of IL-12p40 (complements with IL12p35 to make IL-12) and IL-27p28 (conjugates with EBI-3 for formation of IL-27) was very low and there was no prominent difference in their production with changing culture conditions (Fig. 4.9).
Figure 4.8: Profiles of various cytokines secreted during Th17 and Treg cells co-cultivation. Normal as well as four months infected mice were sacrificed and spleen was removed aseptically. Splenic CD4⁺CD25⁻ and CD4⁺CD25⁺ Treg cells were purified through magnetic sorting according to manufacturer’s protocol. CD4⁺CD25⁻ were polarised to Th17 cells in the presence of IL-6, TGF-β, αIFN-γ and αIL-4. After 24 hrs, polarised cells were stimulated with anti-CD3 & anti-CD28 in 96 u bottom plates and cultured with CD4⁺CD25⁺ Treg cells at various ratios. After 96 hrs culture supernatants were collected for cytokine ELISA. Data represented as means ± SE for three mice per group and are representative of two independent experiments with similar results.
In-vivo neutralisation of immunosuppressive cytokines leads to upregulation of IL-17

In the previous chapter, we have shown that TGF-β and IL-35 were indispensable for immuno-suppressive function of CD4⁺CD25⁺FOXP3⁺ Treg cells and with the advancement of the disease, these cytokines were elevated. Therefore, we blocked these cytokines by injecting anti-CD25, anti-TGF-β and anti-IL-35 neutralising antibodies intra-peritonially into various groups of BALB/c mice. These mice were then infected with *L. donovani* and the antibody concentration was maintained in the mice by weekly administration of these till the animals were sacrificed. After two months of infection, mice were sacrificed and splenocytes were cultured for cytokines analysis as described earlier. We found that administration of αTGF-β+αIL-35 led to the maximum production of IL-17 both in the culture supernatant and serum from these mice, followed by αIL-35, αTGF-β and αCD25 treated groups respectively (Fig. 4.10). The group treated with control antibody registered lowest production of IL-17. The group with highest IL-17 production (αTGF-β+αIL-35 treated) also recorded elevated IFN-γ and TNF-α levels, with lowest parasite burden (Fig. 3.30 & 3.31).
Figure 4.10: Estimation of IL-17 by post in-vivo treatment of neutralising antibodies. Mice were administered with various doses of neutralising antibodies and infected with *L. donovani* strain AG83 as described earlier. Normal as well as variously treated and infected mice were sacrificed after 2 months of infection. Splenocytes were isolated from these mice and cultured post LAg stimulation (10 µg/ml). After 72 hrs culture supernatants were collected for ELISA (a). Various groups of the mouse were bleed through tail vein and serum was collected for cytokine ELISA (b). Data represented as means ± SE for three mice per group and are representative of two independent experiments with similar results. A group of mice administrated with αTGF-β+αIL-35 was showing statistical significance with all other groups and depicted as *** p < 0.0001.
Discussion

Th17 is known to participate in the induction of inflammation during infection but the pathogenic or protective role of IL-17 in *L. donovani* infection is not well understood. In this study, we found that the production of IL-17 was induced against *L. donovani* infection. Our work suggests that though IL-17 was produced by a small population of CD4^+^ T (Th17) cells, it was potentially combating *L. donovani* infection even when the Th1 response was suppressed. Although some reports indicated that *L. donovani* infection induced the expression of IL-17 in resistant C57BL/6 mice (Terrazas et al., 2015; Sheel et al., 2015), Nascimento et al. have recently associated IL-17 with pro-inflammatory response in VL (Nascimento et al., 2015). But its role during the progression of *L. donovani* infection is not well understood. Besides, information is scanty regarding its encounter with the disease-promoting Treg cells during *L. donovani* infection in BALB/c mice. In this regard, we established, by co-culturing Th17 cells with Treg that IL-17 counteract immunosuppressive responses generated by Tregs and remain dominant till full-fledged immunosuppressive responses develop, post established infection. Furthermore, among various immuno-suppressive cytokines secreted from Treg cells, IL-35 was the most effective cytokine for downregulation of Th17 response. Interestingly, neutralization of IL-35 along with TGF-β resulted in upregulation of IL-17 response leading to disease resolution. Therefore, it appears that successful resolution of infection was achieved only after downregulation of suppressive immune responses that resulted in upregulation of Th17 along with the Th1 response.

The proinflammatory and host protective functions of IL-17 in other infectious disease models are well established. This includes models of *Mycobacterium tuberculosis* (Freches et al., 2013; Lockhart, Green, & Flynn, 2006), *Salmonella* (Schulz et al., 2008), *Trypanosoma cruzi* (Miyazaki et al., 2010) and *Listeria monocytogenes* (Hamada et al., 2008) infections where IL-17 deficiency was associated with an enhanced disease severity. Moreover, IL-17 is said to be a promoter of Th1 reaction and has been used in combination with vaccine and drugs for improving their effectiveness in tuberculosis and other infectious diseases (Gopal et al. 2014; Asad & Ali, 2014). In VL, injection of recombinant IL-17 with curdlan...
enhanced its therapeutic effect, thereby causing a marked increase in generation of IFN-γ along with NO and a significant suppression of the organ parasite burden during murine VL (Ghosh et al., 2013). In contrast, few recent reports emphasizing IL-17 secreted from γδ T cells suggest that IL-17 promotes VL progression in C57BL/6 mice (Terrazas et al., 2015; Sheel et al., 2015). However, these studies were mainly focused on IL-17 production from γδ T cells rather than Th17. Contrary to this, our work highlights the involvement and functioning of IL-17 produced by Th17 cells during _L. donovani_ infection of BALB/c mice, a susceptible model, which mimics human VL at least up to 4 months. We found that IL-17 secretion from splenocytes decreased with the dissemination of the infection. This decrease in IL-17 production was evident in both supernatants of splenocytes as well as in the serum. These data are consistent with the earlier report in human VL that IL-17 transcripts were expressed in low abundance (Ansari et al., 2011). The low or downregulated expression of Th17-associated cytokines in patients with active disease may be particularly significant in light of clinical studies in the Sudan showing leishmanial-antigen stimulated the production of IL-17 by PBMCs was strongly associated with protection against VL (Pitta et al., 2009). Our observations of IL-17 profiles in infected mice splenocyte culture supernatant and serum may lead to speculate that IL-17 may be involved in controlling the disease hence downregulation of IL-17 was orchestrated the disease progression.

We next investigated the contribution of Th17 in IL-17 production and found that a small portion of CD4⁺ T cell are CCR6⁺RORγt⁺ Th17 too, but they are highly significant for IL-17 production particularly at the beginning of infection. IL-17 produced by Th17 cells were diminished with the dissemination of infection, emphasising the involvement of Th17 and IL-17 in inducing protective host response during the disease. This was consistent with the earlier reports that IL-17 generates inflammatory responses during VL (Nascimento et al., 2015; Pitta et al., 2009). In the same line, it was reviewed that IL-17−/− mice infected with _L. infantum_ failed to control parasitemia and increased the proliferation of Treg cells and production of IL-10 (de Freitas, et al. 2016). Our kinetic data revealed that Treg cells and its immunosuppressive cytokines increased with disease progression, which governed the
inhibition of protective inflammatory responses including Th1 as well as Th17. Interestingly, at two months of established infection, when IL-12, IFN-γ and other immune-protective cytokines were repressed, the IL-17 level was still elevated. Hence, it appears that IL-17 may be providing some hindrance to disease promoting cells and cytokines. Thus, at this time, IL-4 and IL-10 levels were under check. Later at 3 months of infection, when Th17 response turned off, all the disease promoting cytokines got upregulated leading to enhancement of *L. donovani* infection.

From our study, it seems to be obvious that Th17 response is proinflammatory thus protective in the case of VL. But being an inflammatory cytokine, IL-17 may contribute to CL and ML pathogenesis through several mechanisms, including neutrophil activation for generating an inflammatory response which enhances tissue injury at the wound/lesion. It appears that a regulated production of IL-17 contributes to infection control while excessive IL-17 can promote neutrophil influx and tissue damage, thereby increasing the risk of ML (Boaventura et al., 2010, Soong et al., 2012). Therefore, additional studies to reveal the production of IL-17 at early versus late stages of infection are important to understanding the involvement of IL-17 in the disease. Our work not only showed the kinetics of IL-17 production from Th17 cells at various times of infection but our ex-vivo co-cultivation studies demonstrated that at the early time of infection (when IL-17 production from Th17 was highest), Treg cells were unable to suppress Th17 proliferation. In such a situation, elevated levels of IFN-γ and TNF-α were also observed. It may be due to amplification in IL-17 production and downregulation of disease promoting cytokines, IL-10, TGF-β and the most importantly IL-35. IL-35 is a heterodimeric cytokine composed of IL12p35 and Ebi3 chains. Its secretion appears to be restricted to Tregs and is required for their maximal suppressive capacity in autoimmunity and some infectious diseases such as hepatitis and Chagas disease (Xiang and Xie, 2015; Gee & Kumar, 2009; Gravano and Vignali, 2012). But its role in VL has not been studied. Our work of the previous chapter shows IL-35 as the most effective immunosuppressive cytokine secreted from CD4⁺CD25⁺FOXP3⁺ Treg cells. In the present study, Th17 and Treg cells co-culture studies revealed that at 4 months of intensive infection, Treg cells could effectively suppress IL-17 proliferation. This suppression of Th17 proliferation was accompanied
by elevated levels of immunosuppressive cytokines, IL-10, TGF-β and IL-35. This was in accordance with the earlier report in *L. major* infection that exacerbation of disease leads to alleviation in IL-17 production due to enhanced levels of IL-10 and IL-27 (Anderson, et al. 2009). IL-27 and IL-35 both belong to the IL-12 family of cytokines, where EBI-3 is a common chain to both IL-27 and IL-35. The probably presence of EBI-3 is the reason for immuno-suppressive capacity of IL-27 as well as IL-35, as another chain of IL-27 (IL27p28) was reported to impart inflammatory response (Basset et al., 2015). Additionally, Ansari et al. (Ansari et al., 2011) showed elevated circulating levels of IL-27 and elevated expression of IL-27p28 and EBI-3 transcripts in VL patients. But in our co-culture the levels of both IL-27p28 and IL-12p40 (a chain which makes cytokine, IL-12) were negligible, emphasizing IL-35 as the chief source of EBI-3. It further signifies that IL-35 along with TGF-β is instrumental in downregulating Th1 and Th17 responses and creating suppressive milieu during disease.

Further, for achieving a convincing conclusion, we studied the protective role of Th17 in BALB/c model where Tregs and immunosuppressive cytokines, TGF-β and IL-35 were blocked by administration of neutralising antibodies against them in various combinations. Interestingly, the outcome highlights the most potent control of infection obtained only after injection of TGF-β+IL-35 neutralising antibodies. The protection achieved not only due to increased Th1 response but TH17 response as well synchronised with depressed suppressive cytokines levels.

In conclusion, we can say that *L. donovani* infection generates a mixed immune response with anomalous Th1/Th2 cytokine levels. It may or may not cause suppression of IL-17 response. Here in our work, we obtained a coherent picture of cytokine profile of Th17 cells during progression of VL in mice model. We found at the beginning of infection, CD4⁺CCR6⁺ROTY⁺ Th17 cells enhanced with increased number of IL-17 and IFNγ positive cells. But as the infection progressed, the Th17 cell sub-sets and their cytokines decreased, reaching a basal level at four months infection time. At this time point, immunosuppressive cytokines, IL-4, IL-10, IL-35 and TGF-β were high. Therefore, it appears that the rise of these cytokines leads to suppression of IFN-γ producing cells. The initial rise in the number of Th17 cells and
their cytokines, IL-17 and IFN-γ, is an outcome of host immune response against the infection, but due to overwhelming Th2 response, later on, Th17, as well as Th1 response, fade away. It appears that may be a Th17 function is synergistic to Th1 cytokines thus; its suppression also causes the infection to prevail. Therefore, ex-vivo and in-vivo blocking of disease promoting cytokines increase Th1 as well as Th17 responses, leading to cure.