CHAPTER 5

Sphingosine-1-phosphate inhibits flagellin – induced chemokine secretion from human T cells
5.1. Introduction

The cellular responses produced through activation of TLRs play a vital role in immunity against microbial pathogens including *Salmonella* (Akira *et al.*, 2006). TLR4 deficient and TLR2 deficient mice are highly susceptible to infection with *Salmonella* and *Staphylococcus* respectively (Takeuchi *et al.*, 2000). TLR5 has been shown to regulate humoral and cellular gut immunity by lamina propria dendritic cells during infection with *Salmonella* (Uematsu *et al.*, 2008), and deficiency of this TLR is associated with susceptibility to legionnaires' disease (Hawn *et al.*, 2003). TLR3 recognizes double-stranded RNA and transmits signals to activate NF-κB and production of Type I interferons (IFNs) (Alexopoulou *et al.*, 2001). Double stranded RNA (dsRNA) is a molecular pattern associated with viral infections. Most viruses produce dsRNA at some point during their replication. TLR3 and TLR9 have been shown to be essential components of innate immunity against mouse cytomegalovirus infection (Tabeta *et al.*, 2004). TLR3 – dependent induction of IFNs is critical for immunity to HSV-1 in the central nervous system in children (Zhang *et al.*, 2007). These and many other studies highlight the importance of TLRs in innate immunity against different pathogenic microorganisms. However, excessive responses through these pathogen sensors can lead to tissue damage which would be detrimental to the host (Liew *et al.*, 2005). Therefore, the inflammatory and innate immune responses produced through TLRs need to be regulated.

Several mechanisms that could downregulate responses induced through TLRs have been previously reported in macrophages and DCs. These include downregulation of the TLR receptor following stimulation with a specific ligand (Akashi *et al.*, 2000; Nomura *et al.*, 2000), downregulation of intracellular serine threonine kinase IRAK-1 that is required for intracellular signaling through TLRs (Siedlar *et al.*, 2004; Sato *et al.*, 2002; Li *et al.*, 2005).
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2000) and induction of IRAK-M, an inhibitor that prevents complex formation between IRAK-1 and TRAF-6 (Kobayashi, 2002). PI-3 kinase and SOCS family of molecules activated in the course of activation through TLRs have also been shown to suppress responses through these receptors (Fukao, 2002; Kinjo, 2002; Nakagawa, 2002). In addition, a number of membrane associated molecules with ability to downregulate responses from TLRs have also been reported. Some of these membrane receptors like SIGIRR and ST2 act as orphan receptors. These receptors contain TIR domain which is involved in negative regulation of TLR signaling (Brint, 2004; Wald, 2003); these receptors do not induce any stimulatory signals on their own. Chris Karp and colleagues have described a role of TLR homologue RP105 in negative regulation of TLR4 signaling in DCs (Divanovic et al., 2005). TRAIL-R acts as a negative regulator by stabilizing IκB-α (Diehl, 2004). In addition to these regulators, TRIAD3A, an E3 ubiquitin-protein ligase, which is constitutively expressed by most cells and tissues, ubiquitinylates some TLRs and leads to their degradation (Chuang and Ulevitch, 2004). Whitmore et al showed that ATF3 (activating transcription factor-3) acts as a negative regulator of Toll-like receptor responses (Whitmore et al., 2007). They showed that ATF3 is rapidly induced by various TLRs in mouse macrophages and plasmacytoid dendritic cells (DCs) as well as in plasmacytoid and myeloid subsets of human DCs. Macrophages from ATF-3 knockout mice secreted higher levels of IL-6 and IL-12 than wild type mice when activated with TLR ligands. In vivo administration of CpG in ATF-3 mice induced enhanced production of cytokines from splenocytes. Calcitonin gene-related peptide (CGRP), a neuropeptide released from sensory nerves during inflammation has been shown to negatively regulate TLR - stimulated production of inflammatory mediators such as TNF-α and CCL4 from murine dendritic cells (Harzenetter et al., 2007). Inhibition of TLR responses was
independent of IL-10 and did not involve perturbation of canonical TLR signaling, including activation of MAP-kinase and NF-κB pathways. Instead, the inhibitory activity of CGRP was mediated by the cAMP / protein kinase A pathway leading to rapid upregulation of the transcriptional repressor, inducible cAMP early repressor (ICER).

The results described in the previous section illustrated the mechanism by which TLR5 might co-stimulate IL-2 secretion from activated T cells. This section presents a regulatory pathway by which TLR5 - mediated chemokine secretion in human T cells might be modulated by serum - derived host factor(s).

5.2. Results

5.2.1. Flagellin – induced IL-8 secretion from human T cells is inhibited by serum

To study innate immune activation of human T cells with flagellin, model human T cell lymphoma line, Jurkat, was stimulated with this TLR ligand. Jurkat expressed TLR5 and showed a concentration - dependent binding to flagellin (Fig. 1 a & b). Flagellin induced IL-8 secretion from these cells in a dose - dependent manner (Fig. 1 c). Interestingly, it was observed that IL-8 secretion in response to flagellin was significantly inhibited in the presence of serum (Fig. 2 a). This inhibition was not due to reduced binding of flagellin to Jurkat cells or due to down regulation of surface TLR5 expression in the presence of serum (Fig. 2 b & c).

5.2.2. Sphingosine-1-phosphate suppresses TLR - induced inflammatory responses from human T cells

To identify the nature of the serum - derived component(s) that might be responsible for inhibiting flagellin – mediated responses from T cells, we tested if albumin that is the most
Figure 1: (a) Expression of TLR5 on Jurkat. Cells were incubated with goat anti-TLR5 antibody followed by FITC-labeled rabbit anti-goat IgG antibody and analyzed by flow cytometry. Shaded curve shows staining of cells with an unrelated goat antibody. (b) Binding of flagellin to Jurkat cells. Cells were incubated with 5 µg/ml (---), 25 µg/ml(-----), 50 µg/ml (---) and 100 µg/ml (—) flagellin-FITC for 1 h at 4°C and analyzed in a flow cytometer. Shaded curve shows unrelated FITC antibody as a control. (c) Flagellin induces IL-8 secretion from Jurkat. Cells were washed extensively with RPMI and plated at a density of 10^5 per well of a 96 well cell culture plate. Cells were stimulated for 16-18 h with different concentrations of flagellin at 37°C (in a humidified atmosphere with 5% CO2). IL-8 was determined in cell culture supernatants by ELISA.
**Figure 2:** (a) Flagellin-induced IL-8 secretion from Jurkat is inhibited by serum. Cells were washed extensively with serum-free RPMI, plated at a density of $10^5$ cells/well in a 96 well cell culture plate and stimulated for 16-18 h at 37°C (in a humidified atmosphere with 5% CO2) with different concentrations of flagellin diluted in RPMI or RPMI-10. IL-8 was determined in cell culture supernatants by ELISA.

**Serum does not affect expression of TLR5 or binding of flagellin to Jurkat cells.** (b) Cells were induced with flagellin (1 µg/ml) for 1 h in the presence (---) or absence of serum (----), washed and incubated with goat anti-TLR5 antibody followed by FITC-labeled rabbit anti-goat IgG antibody. Cells were analyzed by flow cytometry. Shaded curve shows staining of cells with an unrelated goat antibody. (c) Cells were incubated for 1 h with flagellin-FITC diluted in RPMI or RPMI-10 and analyzed in a flow cytometer. Flagellin-FITC in RPMI, 25 µg/ml (---) & 50 µg/ml (----); flagellin-FITC in RPMI-10, 25 µg/ml(-----) & 50 µg/ml (----). Shaded curve shows unrelated FITC antibody as a control.
abundant protein in serum and contains many biologically active lipids associated with it could also bring about this inhibition. The results showed that suppression mediated by serum could be reproduced with albumin (Fig. 3 a). To get further insights into the molecular identity of the molecule capable of inhibiting flagellin – mediated responses, we analyzed a number of lipids and saturated fatty acids for their ability to modulate TLR5 – triggered responses. Out of all the lipids tested, only sphingosine-1-phosphate suppressed flagellin – induced IL-8 secretion in a dose – dependent fashion (Fig. 3 b).

S1P is recognized by EDG family of G-protein coupled receptors, five of them are known to be present in human T cells (Ishii et al., 2004). Analysis by RT-PCR showed that Jurkat expressed mRNA for three of these receptors namely S1P1, S1P2 and S1P3 (Fig. 3 e). Although these cells also expressed mRNA for lysophosphatidylcholine (LPC) receptors, G2A and GPR4, LPC did not inhibit IL-8 secretion from Jurkat activated with flagellin (Fig. 3 d). To identify which one of the S1P receptors might be involved in the downregulation of flagellin – induced IL-8 secretion; cell stimulations were carried out in the presence of FTY720 which is an agonist of S1P1, S1P3, S1P4, S1P5 (Brinkmann et al., 2002) and with SEW2871 which is a specific agonist of S1P1 (Sanna et al., 2004). FTY720 downregulated flagellin - induced IL-8 secretion from Jurkat but SEW2871 did not (Fig. 4 a). Since Jurkat does not express S1P4 and S1P5 (Fig. 3 c), and FTY720 does not activate S1P2 (Brinkmann et al., 2002), these results suggested that S1P might inhibit flagellin-induced IL-8 secretion by engaging S1P3 receptor. This was supported by reversal of S1P – mediated inhibition with GPCR inhibitor suramin (Fig. 4 b) which has been shown to be a specific antagonist of S1P3 (Ancellin and Hla, 1999).

Importantly, the inhibition of flagellin – induced IL-8 secretion mediated by serum and S1P was also seen with ex-vivo T cells isolated from human peripheral blood (Fig. 5).
Figure 3: BSA and Sphingosine-1-phosphate (S1P) inhibit flagellin-mediated IL-8 secretion from Jurkat cells. (a) Cells ($10^5$ cells per well) were washed with RPMI and incubated for 16-18 h at 37°C with flagellin (10 ng/ml) diluted in RPMI, RPMI-10 or RPMI-BSA [RPMI supplemented with BSA (1 mg/ml)]. IL-8 was determined in culture supernatants by ELISA. (b) Jurkat cells were stimulated with flagellin (10 ng/ml) diluted in RPMI or RPMI containing 1 and 5 μM of S1P and IL-8 levels were determined in the supernatants by ELISA.
Figure 3 contd: (c) Expression of receptors for S1P and LPC on Jurkat. RNA was isolated from Jurkat cells (5 × 10⁶ cells) using TRIZOL reagent (Invitrogen) and cDNA was prepared using ThermoScript reverse transcriptase. PCR were performed with specific sets of primers for the indicated receptors in a Perkin-Elmer Lifesciences thermocycler. (d) Flagellin - mediated IL-8 secretion is inhibited by S1P but not by LPC. Jurkat cells were stimulated with flagellin (Figure 3 a) in RPMI or RPMI supplemented with S1P (5 μM) or LPC (5 μM) and IL-8 levels were determined in the supernatants by ELISA.
Figure 4: (a) Flagellin - induced IL-8 secretion from Jurkat is inhibited by FTY720 (an agonist of S1P1, S1P3, S1P4 and S1P5) but not by SEW2871 (an agonist of S1P1). Jurkat cells were stimulated with flagellin (10 ng/ml) diluted in RPMI-10, RPMI or RPMI containing 5 μM of S1P, FTY720 or SEW2871 and IL-8 levels were determined in the supernatants by ELISA.
Figure 4 contd: (b) Suramin reverses serum or S1P - mediated inhibition of IL-8 secretion from Jurkat activated with flagellin. Cells were washed extensively with serum - free RPMI and $10^5$ cells were incubated with suramin or the vehicle for half an h before stimulating with flagellin diluted in RPMI, RPMI-10 or RPMI containing S1P for 16-18 h at 37°C (in a humidified atmosphere with 5% CO$_2$). IL-8 was determined in cell culture supernatants by ELISA.
Figure 5: **S1P inhibits flagellin - mediated IL-8 secretion from ex-vivo T cells.**
Blood was collected by venipuncture from healthy human volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient. T cells were purified from PBMCs using Dynabeads from Invitrogen and incubated with different concentrations of flagellin in RPMI or RPMI supplemented with S1P (5 µM) for 24 h at 37°C. Culture supernatants were collected and analyzed for IL-8 by ELISA.
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demonstrating that the suppression mediated by the lipid was not a Jurkat cell line-specific phenomenon and it would be relevant *in vivo* as well.

5.2.3. *SIP inhibits inflammatory responses via calcium and targets flagellin – induced MAP-kinase signaling*

To understand the mechanism by which *SIP* might bring about inhibition of inflammatory responses produced through TLR5, experiments were carried out to look at the role of calcium that is induced following engagement of *SIP* receptor (Dorsam *et al.*, 2003). The inhibition mediated by serum or *SIP* was reversed in the presence of intracellular calcium chelator, BAPTA-AM, as well calcium channel blockers, nifedipine and verapamil, (Fig. 6 a & b) suggesting that calcium was involved in suppressing flagellin – induced IL-8 secretion. This reversal was almost complete with *SIP* but not with serum which suggests that there might be additional factors in serum involved in regulating IL-8 secretion through TLR5 in calcium – independent manner.

To identify the intracellular targets of *SIP* – mediated inhibition, Jurkat cells were activated with flagellin in the presence or absence of serum and activation of various intracellular signaling pathways was analyzed. Flagellin induced phosphorylation of ERK, p38 MAPK, CREB and JNK as well as degradation of *IkB* in a time – dependent manner (Fig. 7 a). The degradation of *IkB* was not altered upon stimulation with flagellin in the presence of serum. On the other hand, phosphorylation of p38 MAPK, CREB and JNK was reduced in the presence of serum (Fig. 7 a). The importance of p38 MAPK, ERK and JNK in flagellin – triggered IL-8 secretion was also revealed by reduced chemokine secretion in the presence of inhibitors targeting these three signaling pathways (Fig. 7 b).
Figure 6: Calcium is required for serum or S1P - mediated inhibition of flagellin – induced IL-8 secretion from T cells. Jurkat cells were washed extensively with serum - free RPMI and incubated at a density of 10^5 cells / well with BAPTA-AM (intracellular calcium chelator), or calcium – channel blockers, Verapamil and Nifedipine or vehicle (DMSO) for 30 min before stimulating with flagellin (100 ng/ml) in the presence of serum (10% FCS) or S1P (5 μM) for 16-18 h at 37°C (in a humidified atmosphere with 5% CO₂). IL-8 was analyzed in the supernatants by ELISA.
Figure 7: (a) Jurkat cells stimulated with flagellin in the presence of serum show reduced MAP kinase activation. Cells were washed with RPMI and stimulated with flagellin (100 ng/ml/10^6 cells) for different time points. Stimulation was stopped by adding ice cold PBS. Cells were washed with PBS and lysed with 50 μl lysis buffer containing 1% Triton X-100. The cell lysates were boiled with non-reducing Laemmli sample buffer and run in a 12% SDS-PAG. The proteins were transferred to a nitrocellulose membrane and probed with phospho-specific antibodies. The blot was developed using ECL reagent. Subsequently, the NC sheet was incubated with a solution containing 0.1 M acetic acid and 0.15 M NaCl to strip the blot and re-probed with different antibodies.
Figure 7 contd: (b) Flagellin-induced IL-8 secretion from Jurkat is reduced in the presence of MAP kinase inhibitors. Cells (10⁵ cells per well) were treated with MEK inhibitor (PD98059), p38 MAPK inhibitor (SB203580) or JNK inhibitor for half an h before incubating with flagellin in RPMI for 18 h at 37°C. Culture supernatants were collected and IL-8 was analyzed by ELISA.
5.2.4. Serum upregulates flagellin – induced IL-8 and flagellin – co-stimulated IL-2 secretion from activated T cells

Flagellin is known to co-stimulate cytokine secretion from activated human T cells (results presented in the previous section; Caron et al., 2005). To understand regulatory effects of serum on chemokine and cytokine secretion from activated T cells, Jurkat cells were stimulated with anti-CD3 antibody and flagellin in the presence or absence of serum. Activation of Jurkat with anti-CD3 antibody under serum-free or serum supplemented conditions did not induce secretion of IL-2 or IL-8 from these cells (Fig. 8 b & c). Stimulation of these cells with flagellin, as expected, led to secretion of IL-8 (Fig. 8 a) which was significantly upregulated in cells which were simultaneously activated with anti-CD3 antibody (Fig. 8 b). Serum inhibited IL-8 secretion from Jurkat whether stimulated with flagellin or flagellin and anti-CD3 antibody together (Fig. 8 a & b). On the other hand, however, secretion of IL-2 in response to stimulation with anti-CD3 antibody and flagellin was upregulated in the presence of serum (Fig. 8 c) indicating that IL-2 and IL-8 secretion might be regulated through different pathways in human T cells.

5.3. Discussion

The results presented with human IECs and monocytes showed that flagellin-mediated responses could be upregulated by serum-derived lysophospholipids. In this chapter, evidence has been presented which suggests that unlike what was observed with IECs and monocytes, IL-8 secretion following stimulation of human T cells with flagellin is downregulated by serum-derived factors and this downregulation can be in part reproduced with another albumin associated bioactive lipid S1P. Interestingly, lipids such as LPC which enhanced IL-8 secretion from human monocytes did not have any effect on...
Figure 8: (a) Jurkat cells were stimulated with flagellin (Figure 3 a) in the presence or absence of RPMI-10 and IL-8 levels were determined in the supernatants by ELISA. Serum downregulates IL-8 secretion (b) but upregulates IL-2 secretion (c) from activated Jurkat cells. Cells were incubated with different concentrations of flagellin in the presence or absence of plate-coated anti-CD3 antibody (10 μg/ml). Stimulations were carried out in serum - free or serum supplemented (10% FCS) RPMI for 18 h at 37°C. IL-2 and IL-8 levels in cell supernatants were determined by ELISA.
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flagellin - induced IL-8 secretion from human T cells. These results suggest that TLR5 might be differently coupled to intracellular signaling in different cell types. The degree of downregulation seen with S1P was lower as compared to that seen with serum suggesting that either serum has additional factors which can bring about this downregulation or S1P present in serum might be more active. Remarkably, while calcium mediated LPC – induced upregulation of IL-8 secretion from monocytes, intracellular calcium in T cells was involved in downregulating IL-8 secretion following treatment with S1P.

S1P is produced by many cell types of the immune system including macrophages, DCs, mast cells and platelets. It is involved in many immune cell functions such as cellular proliferation, survival and chemotactic processes (Rosen and Goetzl, 2005). The immune cells express different kinds of receptors for this lipid, which are regulated developmentally and by cellular activation (Ishii et al., 2004). In T cells, S1P1 is the receptor that predominates in modulating various T cell functional responses (Goetzl and Graeler, 2004; Dorsam et al., 2003). The signals transduced through S1P1 in T cells inhibit chemotactic responses to chemokines thereby limiting homing of blood and splenic T cells to secondary lymphoid organs (Graeler et al., 2002). In fact, agonists targeting this pathway have shown therapeutic potential in mouse models of autoimmune diseases such as Experimental Autoimmune Encephalomyelitis (EAE) (Webb et al., 2004). Dorsam et al. have reported that S1P can inhibit IFN-γ secretion from activated T cells and further demonstrated that intracellular calcium might play a major role in this inhibition (Dorsam et al., 2003). More recent studies suggest that S1P-S1P1 axis might be a major stimulus of terminal differentiation of CD4⁺ T cell subsets. Treatment of CD4⁺ T cells prior to IL-6 and TGF-β enhances differentiation of Th17 cells (Liao et al., 2007). However, the results presented in this study suggest that S1P3 and not S1P1 might be involved in regulating chemokine
secretion from T cells. Therefore different S1P receptors might be modulating different kinds of responses from T cells. It is intriguing that calcium has totally opposite effects in regulating IL-8 secretion from monocytes and T cells. It is likely that different GPCRs target different intracellular signaling pathways in different cell types which might in turn determine the positive vs negative regulatory effects of calcium. It will be important to identify the intracellular targets of S1P – mediated calcium – dependent inhibition of innate responses in future studies. The results presented here strengthen the notion that T cells can contribute to innate immunity by a mechanism that is independent of their effects on cells of the innate immune system. Furthermore, their ability to secrete inflammatory mediators like chemokine IL-8 in this study seems to be under a regulatory control that might be different from the one seen in innate immune cells. This kind of a regulation would limit excessive inflammatory responses which might be mediated via recruitment of neutrophils to the site of T cell activation without affecting other important functions of activated T cells including T cell proliferation and secretion of other cytokines such as IL-2. S1P might therefore play an important role in ensuring an optimal immune response without much tissue damage.

Together with the findings discussed in the previous chapter, this study suggests that host – derived lipids might be critical regulators of immune responses induced through TLR5 from IECs, innate immune cells as well as T lymphocytes. The levels of these lipids might change in the course of an infection and that could have a determining effect on the magnitude of innate immunity. Flagellin has radioprotective activity in mouse and primate models, and has also been shown to protect animals against chemicals, bacteria and viruses (Burdelya et al., 2008; Vijay-Kumar et al., 2008). It is also under development as a vaccine adjuvant. It has been suggested from studies in mice that both radioresistant and
hepatopoietic cells promote innate and adaptive immune responses to this molecule (Feuillet et al., 2006). Our data suggest that in humans, its adjuvant effect could be partly mediated by T cells. More importantly, the therapeutic effects and the adjuvanticity effects of flagellin might be regulated \textit{in vivo} by the levels of different kinds of lysophospholipids. Further investigations are clearly needed to understand molecular basis of the cross talk between different GPCRs and TLR5. The use of GPCR agonists as adjuncts with flagellin for various therapeutic purposes may also be explored.