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Immunomodulatory role of flagellin in antigen-presenting cells

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Immunomodulatory Role of Flagellin in Antigen-Presenting Cells

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC	Antigen presenting cells
BCR	B cell receptor
BM	Bone marrow
C/EBP	CCAAT enhancer-binding protein
CFA	Complete Freund Adjuvant
DD	Death domain
ds	Double-stranded
FOXP3	Forkhead box p3
HPLC	High-performance liquid chromatography
IKK	Inhibitor of kappaB factor kinases
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
JAK	Janus kinase
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
LTA	Lipoteichoic acid
MAL	Myeloid Differentiation Protein 88 Adapter-Like
MAL/TIRAP	MyD88-adaptor-like/TIR-associated protein

MAPK	Mitogen-activated protein kinase
MHC II	Major histocompatibility complex II
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa B
PAMPs	Pathogen-associated molecular patterns
PG	Peptidoglycan
PGE2	Prostaglandin E2
PRRs	Pattern-recognition receptors
PS	Phosphatidylserine
Sp	Specificity protein 1
ss	Single-stranded
STAT	Signal transducer and activator of transcription
TAB	TAK-1-binding protein
TAK	Transforming growth factor β–activated kinase
TBK	TANK-binding kinase
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Th	T helper
TIDCs	Tumor infiltrating DCs
TIR	Toll/IL-1R homology
TIRAP	TIR domain–containing adaptor protein
TLRs	Toll-like receptors
Tr1	Type 1 regulatory T cells

TRAF	TNF receptor associated factor
TRAM	Toll-receptor-associated molecule
TRAM	Toll-receptor-associated molecule
TRIF	Toll-receptor-associated activator of interferon

Immunomodulatory properties of flagellin in antigen presenting cells

Ildefonso Vicente-Suarez

ABSTRACT

Toll-like receptors (TLRs) expressed by cells of the immune system play a central role in the generation of immune responses against pathogens. Following TLR ligation, both pro-inflammatory and anti-inflammatory mediators are produced in order to elicit an immune response that controls the microbial infection while limiting tissue damage. Among these mediators, the proinflammatory cytokine IL-12 and the anti-inflammatory cytokine IL-10 are known to play major roles. Here, we show that *in vitro* or *in vivo* stimulation with flagellin, the TLR5 ligand, does not result in IL-10 production. Furthermore flagellin inhibits IL-10 production by other specific TLR ligands at the protein and mRNA levels while increasing IL-12p70 production. Several studies have linked the activation of extracellular signal regulated kinases (ERKs) with IL-10 induction by TLRs. Our findings that LPS-induced ERK activation is significantly decreased in flagellin-treated macrophages suggest that this pathway might play a role in the inhibition of IL-10 production by flagellin. Flagellin-mediated IL-10 inhibition was not observed in cells that do not express TLR5 supporting that this effect is TLR5-dependent.

Flagellin used as an adjuvant is capable of priming antigen specific T cell responses in an *in vivo* model of tolerance using high dose peptide. Furthermore, DCs differentiated in tolerogenic conditions (tolerogenic-DCs) express higher levels of TLR5 mRNA than

standard BM-DCs and respond more vigorously to flagellin stimulation. Antigen presentation by LPS-matured tolerogenic-DCs results in the differentiation of IL-10 producing T cells with a Tr1-like phenotype. On the contrary, antigen presentation by tolerogenic-DCs that have been stimulated with flagellin results in the differentiation of a typical Th1 response

This study provides a new insight of the role of flagellin recognition by TLR5 in shaping the immune response elicited by flagellated microorganisms.

INTRODUCTION

T cell tolerance and antigen presenting cells

Bone marrow-derived antigen presenting cells (APCs) and in particular dendritic cells (DCs) play a central role in the generation of antigen-specific T-cell responses (1). However, these same cells are also required for the induction of T-cell tolerance (2). This seemingly dual function of APCs was attributed initially to the existence of specific APC subpopulation(s) that induce T-cell priming while other subpopulations induce T-cell anergy (3-5). The demonstration that priming and tolerance can be induced by a single APC subpopulation (6) led to the alternative explanation that the functional status of the APC would be the major factor determining T-cell activation versus T-cell tolerance. Antigen encounter in the presence of inflammatory mediators and/or microbial products triggers the maturation of the APC to a functional status capable of priming T cell responses (Figure 1A). In contrast, antigen capture by these same APCs in the absence of inflammatory signals, or in the presence of inhibitory mediators, leads to the development of antigen specific T-cell tolerance (Figure 1B) (2).

Dendritic cells (DCs), macrophages and B-cells are all bone marrow (BM)-derived cells that express major histocompatibility complex class II (MHC II) as well as costimulatory molecules and, as such, can potentially present antigens to the T-cells. Although it is plausible that under particular conditions each subpopulation might induce

T-cell tolerance (7-12), several lines of evidence have pointed to the DCs as the main cell type involved in the development of peripheral tolerance (13-16). Immature DCs constantly migrate within tissues and secondary lymphoid organs (17). Under steady-state conditions, immature DCs capture apoptotic bodies arising from cell turnover and migrate to the draining lymph nodes where antigen presentation takes place. Phagocytosis of apoptotic bodies does not cause DC maturation, and therefore antigen presentation of tissue antigens phagocytosed by immature DCs might represent a common mechanism of tolerance-induction to self antigens (17). Tumor growth especially at early stages when the tissue architecture has not been yet disrupted does not induce the release of inflammatory mediators. Therefore, it was proposed that uptake of tumor antigens occurs in an identical manner as uptake of tissue self-antigens and the same mechanisms that establish T-cell tolerance against self will also prevent the immune response against tumors (18). In the mid 90's, the demonstration by the Bogen's and Levitsky's groups that antigen-specific $CD4^+$ T-cells were rendered tolerant during tumor growth *in vivo* provided the first experimental evidence supporting the tumor-induced tolerance hypothesis (19-20).

Since then, several studies have confirmed that antigen specific CD4+ as well as CD8+ T cell tolerance occurs during the progression of both hematologic and solid tumors expressing model or true tumor antigens (21-28). Furthermore, data from our laboratory and others have demonstrated that bone marrow (BM)-derived APCs are required for the induction of tolerance to antigens expressed by tumor cells (21) (29). These studies also provided evidence that the intrinsic antigen presenting capacity of

tumor cells has little influence over T cell priming versus tolerance, a critical decision that is regulated at the level of the APC.

Antigen presentation by immature DCs, self-antigens or tumor antigens alike, induces a modest T cell proliferation but not polarization into T helper 1 (Th1) or Th2 subsets. Instead, after several rounds of cell division almost all the antigen specific T-cells are deleted. The remaining T cells are functionally anergic upon antigen restimulation even when the antigen is administered with a strong adjuvant such as Complete Freund Adjuvant (CFA) (30-31). Furthermore, antigen presentation by immature DCs can also result in the development of regulatory T cells with the ability to suppress effector responses by other T cells (32) (Figure 1B).

In the tumor bearing host, antigen encounter by DCs might occur not only in the absence of inflammatory signals, but also in the presence of inhibitory factors such as interleukin (IL)-10, transforming growth factor-beta (TGF-β) (33), phosphatidylserine (PS) and prostaglandin E2 (PGE2) (34-36) that further suppress the APC's function. In this adverse environment, DCs acquire features as the production of IDO, and IL-10 that result in strong suppression of immune responses (37) (38). IL-10 production by DCs induces the differentiation of regulatory T cells that themselves produce high levels of IL-10 and further suppress inflammatory responses (38). The goal of immunotherapy is to disrupt this vicious cycle in order to elicit an effective immune response against the tumor. Microbial products recognized by Toll-like receptors (TLRs) are considered the strongest stimulus to induce APC maturation and as such, in this proposal we focus in the better understanding of their inflammatory properties as a potential strategy to effectively harness antitumor immune responses (39).

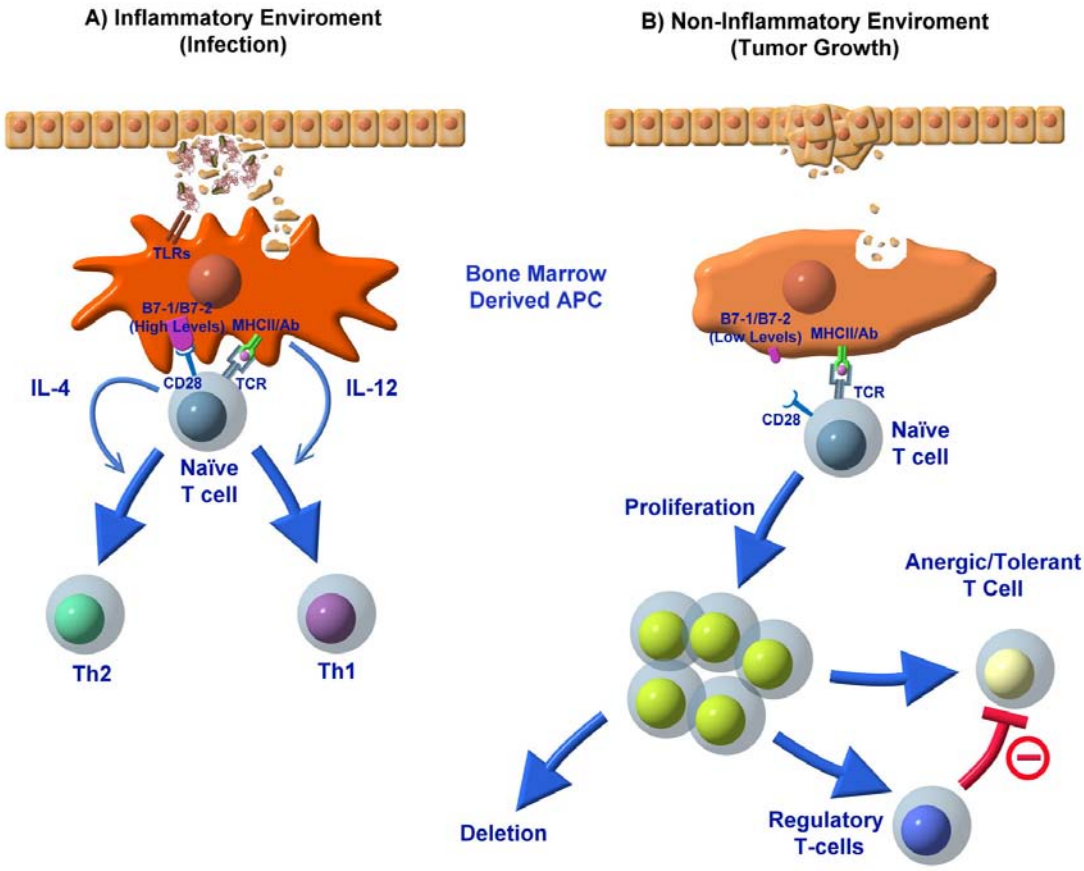


FIGURE 1. T cell priming versus tolerance is determined by the state of activation of the APC

(A) In the course of an infection, microbial products are recognized by the APC through TLRs. TLR stimulation results in APC maturation, a process that increases the levels of costimulatory molecules and cytokine production. Antigen presentation by mature APCs leads to efficient priming of naïve T cells that proliferate and differentiate into effector cells. (B) Phagocytosis of self-antigens or tumor antigens in a non-inflammatory environment does not cause APC maturation. Immature APCs display low levels of costimulatory molecules and cytokine production. Antigen presentation by immature APCs to naïve T cells results in transient proliferation followed by induction of anergic and/or regulatory T-cells.

TLRs

The immune system has evolved to fight off microbial invasions that represent a constant thread to any multicellular organism. The mammalian immune system is divided in two broad categories, innate and acquired immunity. The acquired branch of immunity is only present in vertebrates and is characterized by a specialized cell type known as lymphocytes. There is two major lineages of lymphocytes called T (for thymus-derived) and B (for bursa- or bone-marrow-derived). The attribute that makes lymphocytes different from other cells of the immune system is the presence of antigen receptors in their cellular surface. Engagement of these receptors results in clonal amplification, cellular differentiation, and in B cells, production of antibodies with the same antigen binding specificity. Somatic diversification of the antigen-receptor genes generates a vast repertoire of cells, each of which expresses a different antigen receptor. This feature provides the acquired immune system with the ability to recognize an unlimited array of ligands. The T cell receptors (TCRs) bind peptide fragments presented by other cells within cell-surface molecules encoded by the MHC class I and class II genes. Therefore, T lymphocytes typically recognize antigens that have been partially digested by the antigen-presenting cells, primarily dendritic cells. In contrast, the B cell receptor (BCR) and antibodies recognize exposed determinants (epitopes) of intact molecules.

Innate immunity is ancient, characterized by the presence of phagocytic cells and found in every organism studied. Innate immune cells express receptors that are present on all cells of a given type and are not subject to rearrangement. These receptors recognize microbial products that are not present in the host, and therefore discriminate "non-infectious self" from "infectious non-self" (40). Microorganisms have the ability to

rapidly change under environmental pressure; consequently innate immune receptors have evolved to target molecules essential for the microorganism survival and therefore less susceptible to be altered. These molecules have been denominated pathogen-associated molecular patterns (PAMPs) (41) and the receptors that sense them, pattern-recognition receptors (PRRs) (42). Toll-like receptors are the most studied family of PRRs and received their name from the Drosophila receptor Toll (43), a receptor involved in embryonic development but also in the defense against fungal infection (44). The first indication that mammalian TLRs may function as pattern recognition receptors came with the description of a human homologue of Drosophila Toll, now known as TLR4 (45). So far 11 members of the TLR family have been identified based on the homology in the cytoplasmic region that exists among all of them. The TLR intracellular domain is homologous to the IL-1R domain and for that reason it has been named Toll/IL-1R homology (TIR) domain. The TLR extracellular domains contain a varying numbers of leucine-rich-repeat (LRR) motifs. These motifs form a horseshoe structure, and it is thought that the concave surface of this structure is directly involved in the recognition of PAMPs (46). The TLR family members identified so far and their ligands are summarized in Table 1.

Microbial Components	Species	TLR/s
BACTERIA		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/ TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/ TLR2
LTA (Lipoteichoic acid)	Group B *Streptococcus*	TLR6/ TLR2
PG (Peptidoglycan)	Gram-positive bacteria	TLR2
Porins	*Neisseria*	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
Non-defined	Uropathogenic bacteria	TLR11
FUNGUS		
Zymosan	*Saccharomyces cerevisiae*	TLR6/ TLR2
Phospholipomannan	*Candida albicans*	TLR2
Mannan	*Candida albicans*	TLR4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
PARASITES		
tGPI-mutin	*Trypanosoma*	TLR2
Glycoinositolphospholipids	*Trypanosoma*	TLR4
Hemozoin	*Plasmodium*	TLR9
Profilin-like molecule	*Toxoplasma gondii*	TLR11
VIRUSES		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV,MMTV	TLR4
Hemagglutinin protein	Measles virus	TLR2
Non-defined	HCMV,HSV1	TLR2

Modified from Akira S 2006 Cell (46)

TABLE 1. Toll-like receptors and their ligands

Certain TLRs (TLRs 1, 2, 4, 5, and 6) are expressed on the cell surface while others (TLRs 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as endosomes (46-48) (Figure 2). Intracellular TLRs recognize nucleic acids, suggesting that degradation of the viral particles or the bacterial cell walls might be needed to release the microbial nucleic acids and allow their recognition by TLRs.

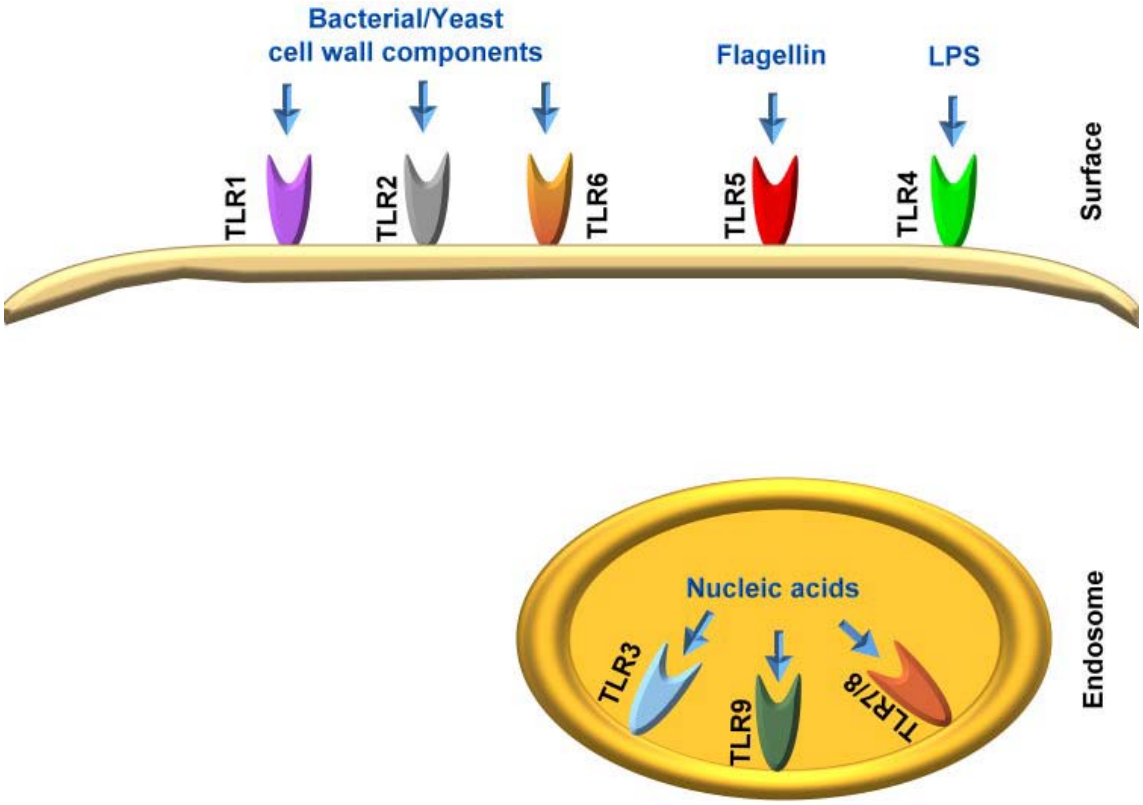


FIGURE 2. TLR localization

TLR common signaling pathway (MyD88-dependent pathway)

Ligand binding by TLRs results in receptor dimerization and conformational changes required for the recruitment of TIR-domain-containing adaptor molecules to the TIR domain of the TLR. Four adaptor proteins, MyD88 (myeloid differentiation factor 88) (49-50), MAL/TIRAP (MyD88-adaptor-like/TIR-associated protein) (51-54), TRIF (Toll-receptor-associated activator of interferon) (55-56) and TRAM (Toll-receptor-associated molecule) (56-57) have been identified. MyD88 is essential for responses against a broad range of microbial components since MyD88-deficient mice display impaired cytokine production when exposed to IL-1 (58) or the TLR2-4-5-7or 9 ligands (59-62). MyD88 is characterized by the presence of a TIR domain that interacts with the cytoplasmic domain of the TLRs and IL-1R, and a death domain (DD) that binds DD containing proteins situated downstream in the signaling pathway. Serine-threonine kinases that belong to the interleukin-1 receptor-associated kinase (IRAK) family contain an N-terminal DD. MyD88 functions as an adaptor recruiting IRAK4 and IRAK1 proteins to the TLRs/IL-1Rs through interaction with their DD domain. Activated IRAK1 auto-phosphorylates residues in its N terminus and recruits TNF receptor associated factor 6 (TRAF6) to the TIR signaling complex. The IRAK1–TRAF6 complex then uncouples from the receptor and interacts at the plasma membrane with members of the mitogen-activated protein kinase kinase kinase (MAPKKK) family transforming growth factor β–activated kinase (TAK) and TAK-1-binding protein (TAB). This interaction induces phosphorylation of TAB2/TAB3 and TAK1, which then translocate together with TRAF6 and TAB1 to the cytoplasm. TAK1 is subsequently activated in the cytoplasm, leading to the activation of inhibitor of kappaB factor kinases (IKKs) and MAPKs.

MAPKs activate the transcription factor activating protein-1 (AP-1) while activated IKKs phosphorylate the inhibitors of kappaB (IκBs) leading to their degradation and consequently the release of nuclear factor kappa B (NFκB) (Figure 3).

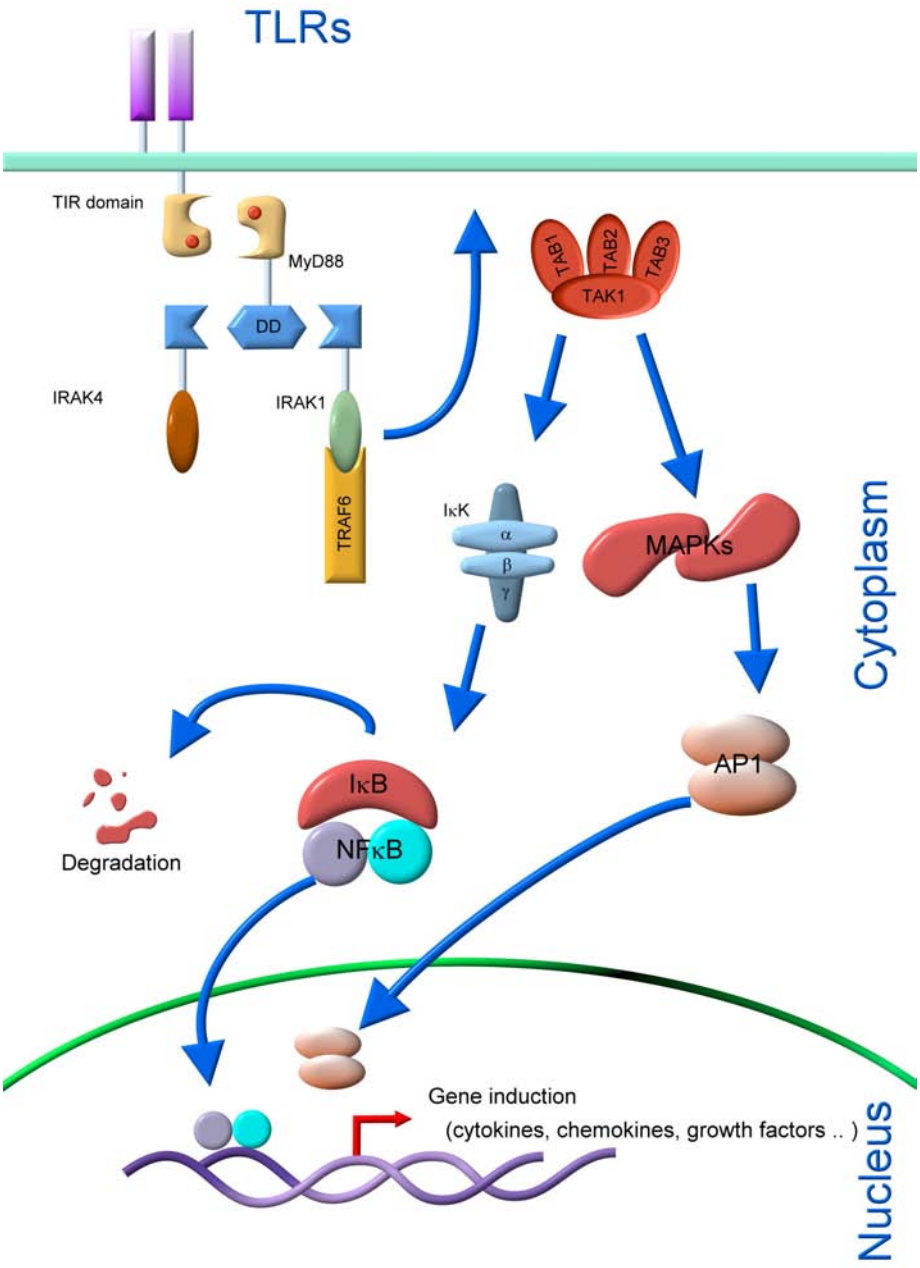


FIGURE 3. TLR common signaling pathway

The activated NFκB complex translocates into the nucleus and binds the DNA at kappa-B-binding motifs. As a result NFκB and AP-1 (46) activate genes that code for cytokines, chemokines, growth factors and cell adhesion molecules.

TRIF dependent pathway (MyD88-independent pathway)

LPS stimulation results in MAPK activation and recruitment of NFκB to the nucleus in MyD88-deficient macrophages even when cytokine production was impaired (59). Furthermore, LPS-treatment induces MyD88-deficient DC maturation as demonstrated by upregulation of costimulatory molecules (CD80, CD86 and CD40) and increased ability to induce T cell proliferation (55-56). These data demonstrates the existence of a MyD88-independent pathway/s that can be activated by specific TLR ligands but not all since TLR2-5-7 and 9 signaling is completely abrogated in MyD88-deficient cells (60-62). Analysis of the gene expression pattern elicited by LPS in MyD88- deficient macrophages identified IFN-β and IFN-inducible genes (63).

TRIF (also known as TICAM1) was identified as an interacting partner with TLR3 by yeast two-hybrid screening (64). Following generation of TRIF-deficient mice, it was revealed that they were impaired in the induction of IFN-β and IFN-inducible genes by the TLR3 and TLR4 ligands. Furthermore, activation of IRF3, a transcription factor involved in the induction and response to type I IFNs, was also impaired (56). The downstream events of TRIF activation that result in NFκB and IRF3 activation are still unclear. NFκB activation by TRIF might depend on TRAF6 meanwhile TANK binding kinase (TBK)-1 has been shown to be involved in IRF3 activation (65).

TLR7 and TLR9 also induce type I IFN production in addition to proinflammatory signals. Interestingly, TLR7- and TLR9-mediated IFN-α secretion occurs in a MyD88-dependent manner (66-68), in contrast to TLR3- or TLR4-mediated IFN responses, which are dependent on TRIF but not on MyD88.

Other TIR-domain containing proteins

TRAM

TRAM associates with TLR4 and TRIF suggesting that its function is to bring them together (57). LPS induced production of IFN-β and IRF3 activation are impaired in TRAM-deficient mice indicating that TRAM is involved in the activation of the MyD88-independent pathway by TLR4 (69).

TRAM's role as an adaptor might be specific for TLR4 signaling since cytokine production by TLR2 -3-7 and 9 ligands remained intact in TRAM-deficient mice (69).

MAL/TIRAP

Expression of dominant-negative TIRAP, which encodes only the TIR domain, blocked NFκB activation by TLR4 (51). Furthermore TIRAP-deficient mice are impaired in inflammatory cytokine production and activation of the NFκB and MAPK pathways induced by TLR1-2-4 and -6 ligands. In contrast, the TLR4-mediated maturation of DCs and activation of IRF3 were intact in TIRAP-deficient mice (52). As explained above IRF3 activation and DC maturation can be achieved by the TRIF-dependent pathway alone, suggesting that TIRAP participates in the MyD88-dependent pathway of specific TLRs.

TLR5 and flagellin

Flagella are complex organelles that play a major role in bacterial motility and chemotaxis. The body of the flagellum consists of a mass of protofilaments, each of which is a long, end-to-end polymer of a single protein, flagellin. Many important human pathogens are flagellated and for a long time expression of flagella has been examined as a virulence trait, but this examination has been largely in the context of motility rather than immune stimulation (70-72). As mentioned before TLRs are characterized by the presence of an intracellular TIR domain shared by them and the IL-1R. TLR5 was identified in human and mice based on the presence of the TIR domain (73). TLR5 is expressed in epithelial cells, endothelial cells, macrophages, DCs, and T cells (72). Initial identification of flagellin as the ligand for TLR5 came from a study that isolated stimulatory components from *Listeria* culture supernatant proteins by high-performance liquid chromatography (HPLC) (61). Parallel studies examining the activation of epithelial cells in response to *Salmonella* also identified flagellin as a stimulatory ligand for TLR5 (74). Analysis of the flagellin amino acid sequences from Gram-positive and -negative bacteria revealed the presence of highly conserved sequences at the amino and carboxyl termini (75). TLRs target conserved sequences making the carboxy- and amino-terminus the predictable candidates for TLR5 recognition. Studies from Eaves-Pyles et al initially confirmed this hypothesis. In their study constructs consisting of only the amino, central or carboxy regions of flagellin lacked stimulatory capacity, as evaluated by *in vitro* activation of NFκB and IL-8 production in intestinal epithelial cells. However, a fusion protein composed of the conserved amino and carboxyl termini, induced a response that was identical to the response induced by wild-type flagellin suggesting that

both ends of the protein but not the central variable region are needed for flagellin recognition by TLR5 (76). Further and deeper studies by Aderem's group targeted the specific location of the TLR5-flagellin interaction to a group of amino acids from both ends brought together in the folded protein (77). Interestingly, mutation of those specific residues greatly diminished the bacteria's motility (77) supporting the paradigm that TLRs target molecules that are conserved since their mutation would affect functions that are vital for the microorganism.

TLR5's binding site is buried within the flagella since it acts as part of the anchorage region that binds one monomer to another in order to form the flagellum filament (Figure 4). Accordingly, it has been shown by cross-linking studies that flagellin monomers are much more potent than polymers to elicit TLR5 activity (77). It is not yet clear how the internal structure of the flagellum filament would come in contact with TLR5. One possibility is that flagellin monomers are released in the phagosome or may be exposed during the flagellum assembly. A surprising finding is that lysophospholipids produced by epithelial cells stimulate the synthesis and release of monomeric flagellin from Salmonella (78). It is unclear how this evidence affects our understanding of Salmonella infection but it could be that through induction of inflammatory mediators and consequently the recruitment of inflammatory cells to the site of infection, recognition of flagellin promote systemic dissemination of the pathogen. Supporting this conclusion TLR5-/- mice survived oral *Salmonella* infection better than wild-type mice as shown in a recent work from Uematsu et al (79).

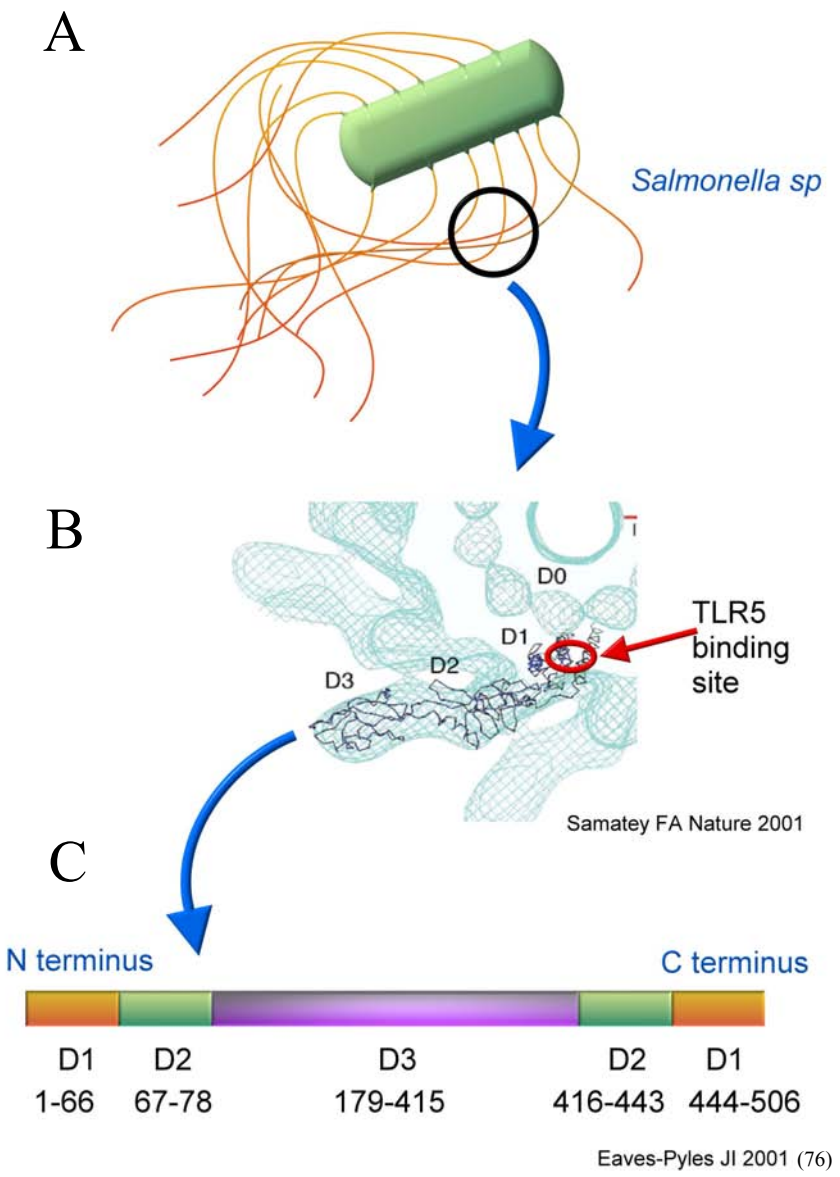


FIGURE 4. TLR5 binds the conserved domains in flagellin
The flagella present in the different species of Salmonella (A) are polymers of the protein flagellin. The flagellin monomers are assembled leaving the D3 and D2 domains exposed while the D1 domain is enclosed within the filament structure (B). The exposed region of the flagella will act as a paddle to facilitate the bacterial movement while the internal part is necessary for its assembly. TLR5 binds a group of aminoacids from the carboxy and amino terminus that are brought together in the folded protein as part of the D1 domain (C). Probably due to their structural role aminoacids in the D1 domain are highly conserved among flagellated bacteria while the other domains show a much higher variability.

TLR5 in the epithelia

The epithelial surfaces of the host represent principal entry sites for many pathogens and the expression of TLR5 by epithelium allows for rapid recognition of flagellated organisms. Studies by Gewirtz et al. identified flagellin as the bacterial factor inducing IL-8 production following the interaction of *Salmonella* with human intestinal epithelial cells (74). The effects of flagellin on epithelial gene expression are not restricted to interleukin-8, but rather flagellin has been shown to be Salmonella's dominant proinflammatory determinant, being necessary and sufficient for nearly all induction of epithelial proinflammatory gene expression induced by *Salmonella typhimurium* (80). Interestingly, the activation of this inflammatory response was dependent upon the translocation of flagellin to the basolateral surface of the epithelial cells, where TLR5 is expressed (81). Presumably, this requirement limits epithelial cell activation to pathogens that can translocate flagellin, and therefore, avoids TLR5 ligation by the normal intestinal flora since flagellin expression is not restricted to pathogens but rather is expressed by all motile bacteria. TLR5 is also highly expressed in the airway epithelia and it is a potent inducer of inflammation in the lung (82). Flagellin detection plays an important role in the defense against pathogens of the respiratory tract since suppressing TLR5 signaling increases susceptibility to infection by Legionella (83).

TLR5 and innate immunity

Flagellin has been shown to induce production of cytokines and upregulation of costimulatory molecules from human DCs (84). The results with mouse DCs have been more controversial and while TLR5 expression and flagellin-induced maturation of mouse splenic and BM derived DCs have been reported by several groups (85-87), others

found murine DCs not to be responsive to flagellin stimulation due to very low or absent TLR5 expression (79) (84). The cause for these discrepancies is not clear since the purity of the flagellin preparation is well documented in the majority of these studies. One possibility is that these discrepancies are due to the plasticity of TLR expression on APCs that is rapidly modulated in response to cytokines and environmental stresses. Uematsu et al. recently demonstrated that high levels of TLR5 are expressed by murine lamina propria DCs. These DCs when stimulated with flagellin produced high levels of IL-6, IL-12 but not IL-10 (79). Interestingly, DCs from the gut stimulated with microbial products or CD40L have been shown to produce higher levels of IL-10 than those from the spleen (88). IL-10 production by intestinal DCs probably plays an important role in the establishment of tolerance towards commensal bacteria and food antigens through the development of regulatory T cells. In contrast, the cytokine profile seen in flagellin stimulated lamina propria DCs (production of proinflammatory cytokines in the absence of IL-10) suggests that these cells would induce inflammatory responses rather than tolerance. These data supports the prior notion that flagellin plays an important role in eliciting inflammatory responses in the gut.

TLR5 and adaptive immunity

Flagellin's ability to work as an adjuvant for T cell responses has been evaluated by several studies. The first report came from Gewirtz group demonstrating that *Salmonella*'s flagellin was capable of enhancing antigen specific CD4+ T cell expansion and memory development *in vivo*. Antigen-specific transgenic T cells primed *in vivo* with antigen in the presence of flagellin produced IFN-γ but not IL-4 upon *in vitro* restimulation suggesting that flagellin induces Th1-biased responses *in vivo* (89). These

data is at odds with reports from other groups supporting that flagellin induces a Th2 response *in vivo.* In the latter a Th2 antibody response was observed in flagellin immunized animals followed by the production of IL-4 and IL-13 by lymph node cells restimulated *in vitro* (85).

In addition to flagellin's ability to prime the adaptive immune response through its effects on APCs, recent evidence supports that flagellin could also directly stimulate lymphocytes. Expression of mRNA from different TLRs has been detected in human and murine T cells among them TLR5. In addition, flagellin has been shown to increase proliferation and IFN-γ production by memory-effector T cells and also to directly enhance IL-2 production and proliferation by naïve T cells (90-91). In murine and human T cells, TLR5 expression has been shown to be selectively enhanced in CD4+CD25+ T cells as compared with their CD4+CD25- naïve counterparts (91-92). Furthermore, treatment of human CD4+CD25+ T cells with flagellin increased their ability to suppress the proliferation of naïve T cells stimulated with anti-TCR and anti-CD28 antibodies (91). Forkhead box p3 (FOXP3) is a transcription factor directly related with regulatory T cell development as FOXP3-transfected naïve T cells suppressed inflammatory responses *in vitro* and *in vivo* (93). Interestingly, treatment of CD4+CD25+ T cells with flagellin increased FOXP3 expression which could explain the enhancement of the suppressive phenotype observed in flagellin-treated regulatory T cells (91).

IL-10 and IL-12

Recognition of viral or bacterial products through TLRs induces profound changes in APCs, many of them directed towards cytokine production (45). Cytokines act as cellular messengers shaping and expanding the immune response, furthermore they are responsible to terminate an ongoing response before tissue damage might occur. The cytokines IL-12 and IL-10 play key roles in expanding and limiting inflammation usually acting in opposition.

IL-12

Macrophages, monocytes, neutrophils, and DCs, are the main sources of IL-12 (94). The biologically active form of IL-12, IL-12p70, is a heterodimer formed by a 35-kDa light chain (known as p35 or IL-12α) and a 40-kDa heavy chain (known as p40 or IL-12β) (95). IL-12p40 has been shown to be produced in excess over IL-12p35 suggesting that IL-12p35 acts as a limiting factor for IL-12p70 formation (96). IL-12p40 can be secreted as a monomer but not IL-12 p35 that is secreted only when associated with p40 (96). IL-12p40 homodimers have been observed in mice *in vivo* but their function has not been completely elucidated (97-99).

Regulation of IL-12 production

IL-12 p35 and IL-12p40 are encoded by two genes located in different chromosomes and regulated independently. Regulation of IL-12p40 expression has been reported to occur only at the transcriptional level while IL-12p35 might be regulated by transcriptional and translational mechanisms (100). The endogenous p40 promoter is assembled in four tightly positioned nucleosomes (101) and therefore is not accessible to

the transcriptional machinery. LPS stimulation has been shown to result in chromatin remodeling that allows binding of the transcription factor CCAAT enhancer-binding protein (C/EBP) (102). However, C/EBP by itself is not enough to induce IL-12p40 transcription and cooperation with other elements as NFκB is necessary (94).

The low levels of IL-12p35 expression even in activated cells have complicated the study of the mechanisms that regulate its transcription. Nonetheless, cloning of the IL-12p35 promoter region from murine and human cells have served to identify binding sites for the transcription factors specificity protein 1 (SP1), IFN-γ response element (γ-IRE) and C/EBP (103-104).

TLR activation by microbial products is the strongest stimulus to induce IL-12 production that can be further enhanced by the cytokines IFN-γ and IL-4 (105-106). On the contrary, IL-10 plays a major role in the negative regulation of IL-12 production by blocking transcription of the p40 and p35 genes (107-108).

IL-12 biological activity

The IL-12 receptor (IL-12R) is mainly expressed in activated T and NK cells making them the main target for this cytokine (94). Engagement of the IL-12R, as other cytokine receptors, activates the Janus kinase (JAK) family of protein tyrosine kinases, which in turn activate cytoplasmic signal transducer and activator of transcription (STAT) proteins. Activated STATs dimerize and translocate to the nucleus, where they induce expression of STAT-regulated genes (109). Although STAT1-3-4 and 5 have been reported to be activated by the IL12 receptor (110), STAT4 appears to be the major player in the IL-12-induced response (111-112). The proinflammatory nature of IL-12 is mainly due to its strong ability to induce IFN-γ production by T cells and NK cells. IL-12

is required for differentiation of CD4+ T cells into IFN-γ producing T helper cells that are denominated T helper 1 (Th1) cells. Th1 cells are fundamental in the development of cellular immune responses as the differentiation of cytotoxic CD8+ T cells (CTLs) and increase phagocytosis and microbial killing by members of the innate immune system (94). Treatment with IL-12 has been shown to have a marked anti-tumor effect on mouse models by inhibiting the establishment or inducing the regression of tumors (113). The anti-tumor effect of IL-12 is complex and involves not only antigen-specific responses against the tumor but also the production of inflammatory mediators that block angiogenesis and/or have a direct toxic effect over the tumor (113). Finally, the powerful proinflammatory nature of IL-12 plays a central role not only in the immune responses against tumors and pathogens but also in the development of organ-specific autoimmunity (114).

IL-10

IL-10 was discovered in the early 1990s by Mossman's group using an assay to isolate factors that could suppress T cell activity (115). Later on, the same group and others showed that IL-10 acted indirectly, via APCs, to block T cell function (116-117). The current understanding is that IL-10 primarily acts by inhibiting a broad spectrum of activated functions in macrophages and dendritic cells. The functions inhibited include cytokine and chemokine production and expression of MHC class II and costimulatory molecules such as CD80 and CD86 (118-121).

Regulation of IL-10 production

The molecular mechanisms that regulate expression of the IL-10 gene are poorly understood. The transcription factors specificity protein (Sp) 1 (122-123), Sp3 (123),

CCAAT/enhancer-binding protein(C/EBP)-β (124), IFN regulatory factor 1 (IRF-1), and STAT3 (122) have been reported to be involved in IL-10 transcription and among them Sp1 seems to play a central role in the induction of IL-10 by LPS in macrophages (122). Sp1 is ubiquitously expressed suggesting that other mechanisms of control exist to ensure IL-10 production upon stimulation and not in a constitutive manner. One mechanism would be through restricted accessibility to the promoter. Supporting this possibility ERK activation by LPS and other stimuli results in histone 3 (H3) phosphorylation at specific regions of the IL-10 promoter and enhanced chromatin accessibility (126). Furthermore mRNA stability might also play an important role in IL-10 regulation as the presence of AU-rich elements that increase mRNA degradation have been described in the IL-10 mRNA (127).

IL-10 biological activity

Macrophages, DCs and certain T cell subsets are the main source of IL-10. The main targets seem to be the antigen presenting cells even when the IL-10 receptor (IL-10R) is expressed in most hematopoietic cells (128-129). IL-10R signaling results in the activation of STAT3 (121). STAT3 activation is essential for the anti-inflammatory properties of IL-10 signaling since blockade of the STAT3 function abrogates IL-10 mediated suppression of LPS-induced cytokines and costimulatory molecules (130-132). Conversely, constitutively activated STAT3 recreates the anti-inflammatory effects induced by IL-10 (133). The anti-inflammatory response induced by IL-10 has been shown to require the synthesis of new proteins suggesting that STAT3 induces one or more genes that execute the anti-inflammatory response but the identity of these proteins is still unknown (134).

IL-10 deficient mice spontaneously develop chronic intestinal inflammation as a result of aberrant immune responses to the microbiota of the gastrointestinal tract (135). Disease development depends on IL-12 and requires the presence of resident enteric bacteria (136). Furthermore upon bacterial and fungal infection these mice develop strong Th1 responses that results in enhanced clearance of the pathogen but also in toxicity and death due to massive inflammatory responses (137-138). Disease development in IL-10 deficient mice demonstrates that IL-10 is essential to limit immune responses and other factors can not compensate for its loss.

Elevated IL-10 serum levels have been reported as a negative prognostic factor for survival in lymphoma, lung cancer, hepatocellular, renal or gastric carcinoma and other solid tumors (121). How IL-10 contributes to a poor prognosis for some cancers is not completely elucidated and probably its role might vary for different malignancies. Nonetheless, it is clear that the presence of IL-10 will blunt the induction of an immune response against tumor cells by inhibiting DC function. Furthermore, IL-10 might serve as a growth factor for certain cell types as B cells (139).

The main objective of tumor immunotherapy is to induce productive and specific immune responses against malignant cells. The powerful proinflammatory nature of TLR ligands led us to explore the possibility of using them to achieve this goal. Among all TLR ligands, flagellin is the only protein, this characteristic facilitates its use for vaccination purposes as it can be cloned or modified easily. Therefore, we decided to focus our research in flagellin and its receptor (TLR5). The goals of our project were basically two. First, characterize the response elicited by TLR5 in APCs. Second, evaluate the ability of flagellin to prime immune responses in tolerogenic conditions.

Furthermore, we also investigated the translation of these studies into a suitable anti-tumor vaccination strategy.

MATERIALS AND METHODS

TLR ligands.

Salmonella typhimurium flagellin was isolated as previously described (140). Briefly, *Salmonella typhimurium* is grown in a chemically defined media, in order to avoid the contamination of the flagellin preparation with other proteins. The dissociation of flagella from the bacterial cells was achieved by reducing the pH to 2.0 with HCl for only 30 min. The reduction in pH results in the detachment and breakdown of flagella into its monomeric form, but it does not cause the death or disruption of the bacteria. The monomeric form of the flagella, flagellin protein, is no longer centrifugable. This property is exploited to remove pH 2.0 insoluble contaminants before protein precipitation. Protein precipitation was achieved by saturating the preparation with ammonium sulfate (2.67 M final concentration). After which the protein precipitate is re-suspended in an appropriate volume of endotoxin free water and dialyzed overnight in distilled water plus activated charcoal. Even after all these purification steps the presence of small amounts of endotoxin in the protein preparation were detectable using the Limulus Amebocyte Lysate method (Cambrex, Rutherford, NJ). Further purification steps combining filtration through 100 Kd pore size centricon columns (Millipore, Billerica, MA) and polymixin B columns, Detoxi-Gel AffinityPak columns (Pierce, Rockford, IL) were used to remove the remaining LPS.

SDS-PAGE analysis revealed no contaminating proteins accompanying the expected flagellin doublet at around 50kDa. In addition western blot using a monoclonal antibody against flagellin further confirmed the protein's identity (Igen international,Gaithersburg, MA). In those experiments in which flagellin was digested with proteinase K (Sigma Aldrich, St Louis ,MO) 100 μg/ml proteinase K were used, digestion carried for 4 hours at 37°C and the protease was inactivated at 100°C for 1 hour. Experiments were also performed with commercially available *S. typhimurium* flagellin (InVivoGen, San Diego, CA) which yielded identical results. LPS was obtained from Sigma (Sigma, St Louis, MO). CpG, and Zymosan were obtained from InVivoGen, (InVivoGen,San Diego, CA) and they were used as indicated in the text.

Isolation of peritoneal elicited macrophages (PEM) and generation of bone marrow (BM)-derived macrophages.

BALB/c mice were injected intraperitoneally with 1 mL of thioglycollate (DIFCO Laboratories, Detroit, MI). Four days later, PEMs were obtained by peritoneal lavage as previously described (141). For CK analysis by ELISA 10^5 cells per well are plated in a 96 well plate, for RNA analysis 1 x 10^6 cells per well are plated in a 24 well plate. After 2 hours those cells that did not adhere to the plate are washed off using ice-cold HBSS (2 washes).

BM-derived macrophages were differentiated from bone marrow cells by harvesting the hind legs and flushing the bone marrow with RPMI. Red blood cells were lysed using ACK lysis buffer and the remaining cells extensively washed. Differentiation of bone marrow cells into macrophages was achieved by 5-6 days incubation of the cells

in complete media (20% FBS, 100μM β-mercapto-ethanol in DMEM) supplemented with L-929 cells supernatants as a source of M-CSF (142). At the end of the incubation period the plates are washed vigorously with ice-cold HBSS and the strongly attached cells are macrophages. FACS analysis of these cells showed that they are CD11b high Gr1 low/+

Quantification of cytokines

Mice were treated with flagellin (10 μg/animal), LPS (10 μg/animal) or HBSS and the volume injected was 0.1 ml via tail vein. Ninety minutes later the animals were sacrificed and blood obtained by heart puncture. Blood was spun down at maximum speed in a minicentrifuge to separate the serum from the cellular components of the blood. Cytokine levels were quantified by sandwich ELISA following manufacturer's instructions (BD Pharmingen, San Diego, CA). For *in vitro* determination of cytokine production, $1x10^5$ PEMs were plated by triplicate in 96 well-plates and treated for 24 hours unless otherwise specified. Supernatants were harvested and kept at -70°C until ELISA for IL-10; IL12p40-70 (BD Pharmingen, San Diego, CA) or IL-12 p70 (Ebioscience San Diego, CA) was performed following manufacturer's instructions

Real time (RT)-PCR analysis.

Two million PEMs or BM-derived macrophages were plated per well in a 24 well plate. After 2 hours non-adherent cells were washed off with media and attached cells were then treated as indicated. Total RNA was extracted using TriZol reagent (Qiagen,Valencia,CA) and cDNA obtained with the iScript cDNA synthesis kit (Bio-Rad,Hercules,CA). Target mRNA was quantified using MyIQ single color real time PCR detection system (Bio-Rad) and iQ SYBR green Supermix (Bio-Rad,Hercules,CA). IL-

12p35 primers (left oligo ATGGTGAAGACGGCCAGAG, right oligo CAGGTCTTCAATGTGCTGGTT) IL-12p40 primers, (left oligo GCAACGTTGGAAAGGAAAGA, right oligo AAAGCCAACCAAGCAGAAGA), IL-10 primers (left oligo CAGGGATCTTAGCTAACGGAAA, right oligo GCTCAGTGAATAAATAGAATGGGAAC), TLR5 primers (left oligo GCATAGCCTGAGCCTGTTTC, right oligo AAGTTCCGGGGAATCTGTTT) and GAPDH primers (left oligo ATGGCCTTCCGTGTTCCTAC, right oligo CAGATGCCTGCTTCACCAC) were used for PCR amplification (cycling parameters 3 min 95°C, 15 secs 95 °C, 30 secs 60°C 40 reps, 1 min 95°C). Single product amplification was confirmed by melting curve analysis and primer efficiency was near to 100% in all the experiments performed. Quantification is expressed in arbitrary units and target mRNA levels were normalized to GAPDH expression.

Preparation of flagellin-coated beads

Carboxyl (COOH) microparticles (Fluoresbrite YG carboxylate microspheres 1 μm Polysciences Inc, Warrington, PA) were used for covalent coupling of flagellin by activating the carboxyl groups with water-soluble carbodiimide. The carbodiimide reacts with the carboxyl group to create an active ester that is reactive toward primary amines on the protein of interest (PolyLink Protein Coupling Kit for COOH Microspheres Polysciences Inc, Warrington, PA)

Antigen-specific T-cell tolerance model.

Single cell suspensions were made from peripheral lymph nodes and spleen collected from anti-HA TCR transgenic donors (BALB/c HA+/- mice). The percentage of

lymphocytes double positive for CD4 and the clonotypic TCR was determined by flow cytometry. Cells were washed three times in sterile Hanks balanced salt solution (HBSS), and injected into the tail vein of male recipients such that a total of $2.5x10^6$ CD4+ anti-HA TCR^+ T cells were transferred into BALB/c mice. One day later, mice were injected intravenously (i.v) with a tolerogenic dose of HA-$peptide_{110-120}$ (200 μg) in combination - or not- with 10 μg of flagellin. Two weeks later the animals were sacrificed and the spleen harvested. Transgenic T cells numbers in the spleen were assessed by Flow Cytometry with FITC conjugated rat anti mouse CD4 antibodies (Pharmingen) and byotinilated rat anti-clonotypic TCR antibody MAb 6.5 (kindly provided by Dr Hyam Levitsky, John Hopkins University) and analyzed by Flow-Jo software (Treestar Inc). For *in vitro* restimulation analysis, splenocytes were plated by triplicate in a 96 well plate at a final concentration of $1x10^6$ cells per well, in the presence or not of synthetic HA-$peptide_{110-120}$. After 48 hours stimulation supernatants were collected and the production of IFN-γ and IL-2 was assessed by ELISA. Values for cells cultured in media alone are less than 10% of the values for antigen-stimulated cells. Data are expressed as the amount of cytokine produced by 100 clonotype+ T cells/well.

Cell lysates and western blot

Total cell lysates were prepared in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, pH8.0, 0.1% SDS, 1% deoxycholate, 1% Triton X-100) containing protease inhibitors cocktail (Sigma Aldrich, St Louis ,MO) and phosphatase inhibitors cocktail (I and II) (Sigma Aldrich, St Louis ,MO) and subjected to SDS-PAGE-immunoblot analysis with anti-phospho-p44/42 MAPKinase (Thr202/Tyr204) polyclonal antibody (Cell Signaling), phospho-IkappaB-alpha (Ser32) (14D4) rabbit monoclonal

antibody (Cell Signaling) and anti-MAPKinase (ERK1+ERK2) monoclonal antibody (Invitrogen)

Generation of DCs.

Dendritic cells were differentiated as in (146). Briefly bone marrow was collected from the femur and tibia of 6-10 week old BALB/c mice. Red blood cells were lysed in ACK lysis buffer and CD4+, CD8+ cells depleted using complement-mediated lysis followed by extensive washing. Cells were cultured for 7 days in media supplemented with 20 ng/ml GM-CSF plus 20 ng/ml IL-4 (BM-DCs) or 20 ng/ml GM-CSF plus 40 ng/ml IL-10 plus 2.5 ng/ml TNF-α (as already documented (144) we noticed that the addition of TNF-α did not alter the DCs phenotype or ability to produce cytokines but it helped to expand the yield of these cells.). Every 2 days old media was removed from the plate and fresh media added. After 7 days in culture cells were harvested. Around 90% of the cells harvested were CD11c + as determined by FACS analysis.

Naïve T cell preparation and *in vitro* differentiation

Naïve T cells were purified from HA +/- BALB/c mice using magnetic column separation. The phenotype of these T cells (CD4+ CD62L+) was confirmed by FACS analysis before culture and cells plated with BM-DCs or tolerogenic-DCs and antigen for 7 days. After this incubation period cells were washed twice with ice cold HBSS and transferred to a new plate where they were restimulated using fresh splenocytes as APCs plus antigen (HA peptide). After 48 hours restimulation supernatants were collected and CK production was quantified by ELISA. T cell proliferation was assessed after 72 hours restimulation by pulsing the cells with [3H]thymidine (1 μCi/well, NEN Life Science,

MA). Eighteen hours later, cells were harvested with a Packard Micromate cell harvester and thymidine incorporation into DNA was measured as counts per minute (cpm).

FliC expressing tumor cells

The ATTCAGTGCCGATACCAAGG (left primer) and ACGTGTCGGTGAATCAATC (right primer) oligos were designed to amplify the whole fliC coding sequence from *Salmonella typhimurium* (Genbank accession number D13689). The PCR product was identified as a unique band running at the expected size of 1.6 Kb and purified from the agarose gel using the Qiaquick gel extraction kit (Qiagen). The PCR product obtained was initially cloned into the pCR 2.1 vector (Invitrogen TA cloning kit). The proper orientation of the insert was verified by sequencing using an oligo for the T7 promoter present in the plasmid. Following this step, the construct was digested with the restriction enzymes XhoI and HindIII and subcloned into the pcDNA 3.1 (-)Hygro vector (Invitrogen) for IL-10 protein expression in mammalian cells. The inserted fragment was sequenced and identified as the fliC gene of *Salmonella typhimurium*.

The pcDNA 3.1/fliC construct was then used to transfect the murine melanoma cell line B78H1 using the Lipofectamine plus reagent (Gibco). Transfected cells were initially selected in complete media plus 400 μg/ml of Hygromicin. Cells that survived were further selected in media containing 1200 μg/ml of Hygromicin. Flagellin expression by these cells was confirmed by western blot analysis of cell lysates using an anti-flagellin specific monoclonal antibody (Igen International).

RESULTS

Flagellin preparation

Flagellin was purified from *Salmonella typhimurium* as explained in the Materials and Methods section. Briefly, the dissociation of flagella from the bacterial cells was achieved by reducing the pH to 2.0 with HCl for 30 min. The reduction in pH results in the detachment and breakdown of flagella into its monomeric form, but it does not cause the death or disruption of the bacteria. Soluble flagellin is precipitated using ammonium sulfate at saturating conditions and further purified using dialysis, ultrafiltration and polymyxin B beads to eliminate endotoxin contamination.

SDS-PAGE analysis of the flagellin preparation revealed no contaminating proteins accompanying the expected flagellin doublet at around 50kDa (Figure 5A).

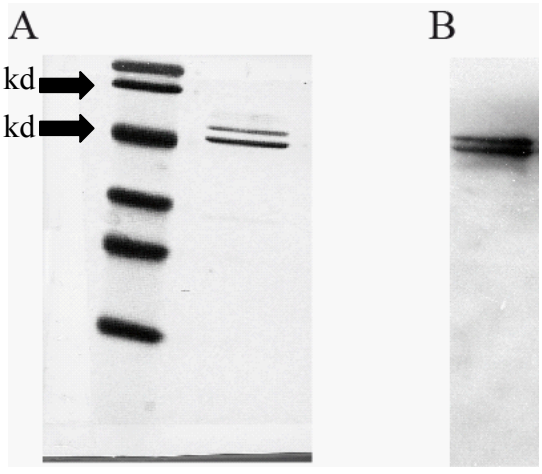


FIGURE 5. Flagellin protein preparation
(A) SDS PAGE followed by Coomasie staining of a Salmonella flagellin preparation. (B)Western blot analysis of the same protein preparation using monoclonal antibodies against flagellin

The presence of two bands is due to the expression of two different flagellin proteins by wild type Salmonella known as FliC (flagellin phase type 1/H1) and FljB (flagellin phase type 2/H2) (145). The FliC and FljB Salmonella flagellin proteins display 78% sequence identity and seem to activate TLR5 signaling with similar potency (145). The identity of both proteins was verified by western blot using a monoclonal antibody against flagellin (Figure 5B).

Flagellin-stimulated PEM produce IL-12 but not IL-10

Peritoneal elicited macrophages (PEM) were treated *in vitro* with increasing concentrations of purified flagellin or with LPS for 24 hours. At this time the supernatants were collected and production of IL-12 p40/p70 and IL-10 was determined by ELISA. Reminiscent of previous studies (121), LPS-treated PEM produce both the proinflammatory cytokine IL-12 as well as the anti-inflammatory cytokine IL-10 in a dose-dependent manner (Figure 6A). Treatment of PEM with flagellin also resulted in a dose dependent production of IL12p40/p70 (Figure 6B). The magnitude of this response was however not as potent as the one induced by LPS (Figure 6A versus 6B, black squares). IL-10 is commonly produced by macrophages upon stimulation with different TLR ligands, but to our surprise we did not detect any IL-10 production in flagellin-stimulated PEM (Figure 6B-open squares). The lack of IL-10 production was not the result of delayed production of this cytokine since kinetic studies showed that even after 72 hours of treatment IL-10 was still not detected in the supernatants of flagellin-treated PEM (Figure 6C-open squares). In contrast, when PEM were stimulated with LPS, IL-10

was detected at early time points and its levels remained elevated throughout the duration of the incubation period (Figure 6C-open diamonds).

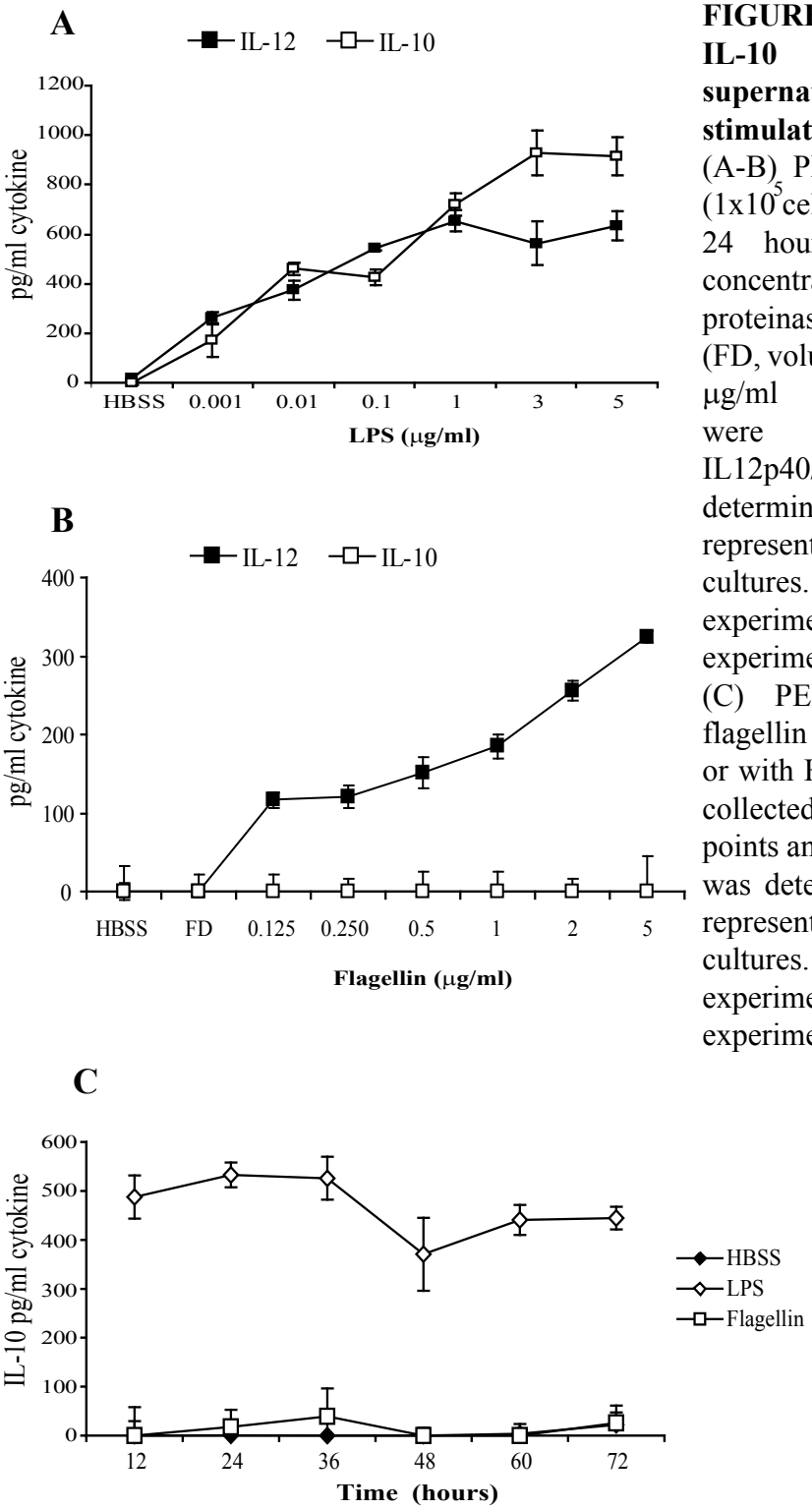


FIGURE 6. IL-12p40-70 but not IL-10 is detected in the supernatants of flagellin-stimulated PEM.
(A-B) PEM from BALB/c mice ($1x10^5$ cells/well) were cultured for 24 hours with the indicated concentrations of LPS, flagellin or proteinase K digested flagellin (FD, volume added equivalent to 5 μg/ml flagellin) Supernatants were then collected and IL12p40/70 or IL-10 were determined by ELISA. Data represent mean ± s.d. of triplicate cultures. Shown is a representative experiment of three independent experiments with similar results. (C) PEM were treated with flagellin (5 μg/ml), LPS (1 μg/ml) or with HBSS. Supernatants were collected at the indicated time points and the production of IL-10 was determined by ELISA. Data represent mean ± s.d. of triplicate cultures. Shown is a representative experiment of two independent experiments with similar results.

One concern in the interpretation of the results described above is the possibility that the flagellin preparation would be contaminated with other bacterial products that could activate macrophages, especially lypopolysaccharide (LPS). However we have used several controls to rule out this possibility. First, we tested the flagellin preparation with the Limulus amebocyte lysate test (LAL). This chromogenic method is based on the activation of a proenzyme on the lysate when in contact with endotoxin and it is of standard use in pharmaceutical and medical facilities. Using this test we could not detect LPS contamination in our flagellin preparation. Second, digestion of the flagellin preparation with proteinase K resulted in loss of its ability to induce IL-12 production in PEM (Figure 6B-FD, and Figure 7A) indicating that this effect is induced by a protein and not by other non-proteinaceous potential contaminants in the preparation. Finally, given the previous demonstration that the inflammatory effect of flagellin requires binding to TLR5 (61) (79), we treated the macrophage cell line RAW 264.7, which lacks TLR5 (146 and Figure 7B), with flagellin or LPS. While treatment with LPS resulted in IL-12 production by these cells, treatment with flagellin failed to induce such a response, indicating that induction of IL-12 by flagellin requires expression of TLR5 in the target cell (Figure 7C). The absence of IL-12 production in RAW 264.7 cells also provides additional support to our claim that the flagellin preparation used in our experiments was LPS-free, since some LPS contamination might have otherwise resulted in IL-12 production by RAW 264.7 cells. To conclude, our data shows that the observed effects induced by flagellin on macrophages are not due to LPS contamination and require expression of TLR5 in the responding cells.

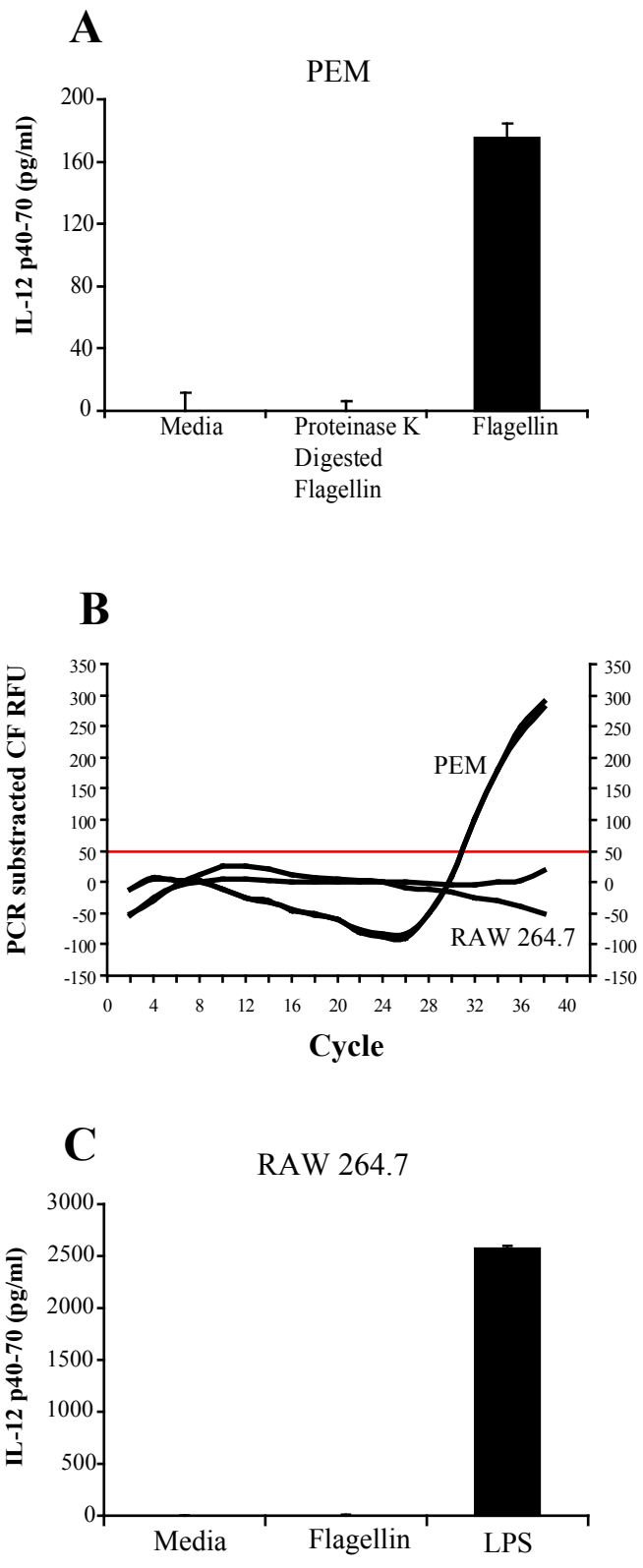


FIGURE 7. IL-12 p40-70 production by flagellin-stimulated macrophages is not caused by endotoxin contamination

(A) Proteinase K digested flagellin does not induce IL-12 production by PEM. Peritoneal macrophages were cultured in media alone or in the presence of flagellin (5 μg/ml) digested or not with proteinase K. Supernatants were collected after 24 hours and IL-12 p40-70 was quantified by ELISA. Shown is one of at least three experiments made. (B) and (C) RAW 264.7 do not express detectable TLR5 mRNA levels and do not respond to flagellin stimulation. (B) TLR5 mRNA level from PEM or RAW 264.7 cells was determined by quantitative real-time RT-PCR. GAPDH mRNA levels were similar among the samples compared. Shown is a representative experiment of two independent experiments with similar results. (C) RAW 264.7 cells were cultured for 24 hours with flagellin (5μg/ml), LPS (1 μg/ml) or media alone. Supernatants were collected and the levels of IL12p40/70 were measured by ELISA. Data represent mean ± s.d. of triplicate cultures from a representative experiment of three with similar results.

IL-12 but not IL-10 is detected in the serum of flagellin-treated mice.

To confirm the above *in vitro* results, we assessed next the production of IL-10 and IL-12 in mice that received a single intravenous (iv) injection of either flagellin or LPS. As shown in Figure 8A, similar levels of IL-12 p40-70 were detected in the serum of LPS or flagellin treated mice. In contrast, IL-10 production showed a completely divergent outcome upon treatment with either TLR ligand. While significant levels of IL-10 were detected in the serum of LPS-treated mice, this cytokine could not be detected in the serum of flagellin-treated mice (Figure 8B).

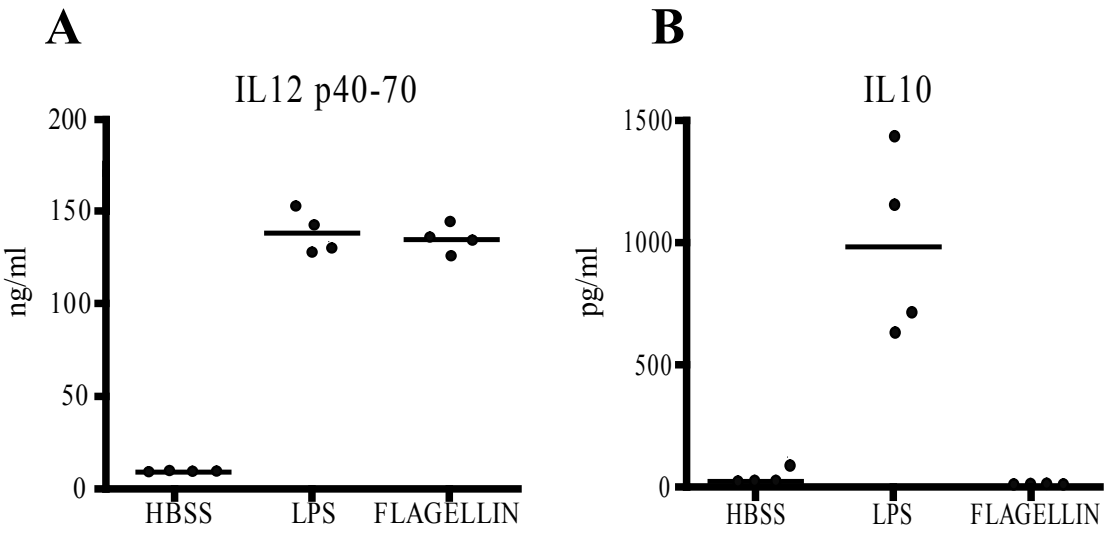


FIGURE 8. Serum levels of IL-12p40-70 and IL-10 in mice treated with LPS or flagellin.

BALB/c mice were injected iv with either flagellin (10 μg), LPS (10 μg) or an equal volume of HBSS (0.1 ml). Blood was collected 1.5 hours later and serum levels of IL-12p40-70 (A) or IL-10 (B) were determined by ELISA. Shown is a representative experiment with four animals per group of two independent experiments with similar results. Each dot represents an animal and the line indicates the group's mean.

Other proinflammatory cytokines as IL-6 were also detected in flagellin-treated mice. Of notice, TNF-α was absent from the serum of these animals (Figure 9). Several lines of evidence indicate that TNF-α is the principal mediator in the pathogenesis of septic shock. First, neutralizing anti-TNF-α antibodies can prevent the pulmonary failure and death associated with administration of endotoxin or *Escherichia coli* in mice (147). Second, intravenous infusion of TNF-α leads to a toxic syndrome indistinguishable from that caused by endotoxemia and gramnegative sepsis (147-148). These observations might indicate that the low toxicity observed by previous studies and also in our own experiments in flagellin-treated as compared with LPS-treated mice might be due to the absence of TNF-α in the serum of flagellin-treated animals.

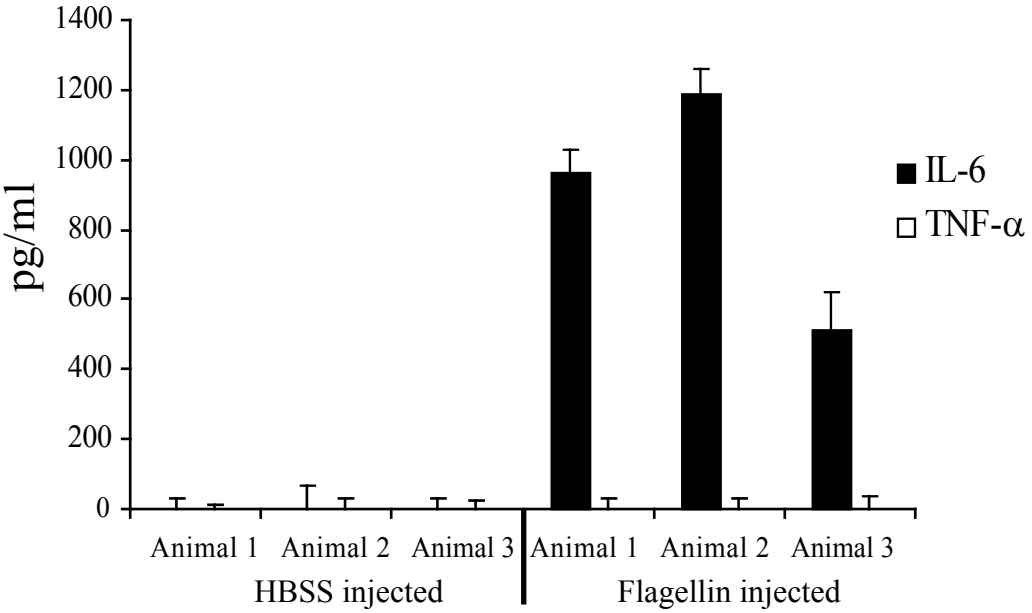


FIGURE 9. IL-6 and TNF-α blood serum levels in flagellin-treated animals

BALB/c mice were injected iv with either flagellin (10 μg) or an equal volume of HBSS (0.1 ml). Blood was collected 1.5 hours later and serum levels of IL6 or TNFα were determined by ELISA. Shown is a representative experiment with three animals per group

IL-10 mRNA dynamics in flagellin-stimulated PEM

In order to assess if flagellin stimulation of macrophages results or not in the induction of IL-10 mRNA we determined the IL-10 mRNA kinetics in flagellin treated macrophages and compare them to those obtained in LPS-treated macrophages (Figure 10).

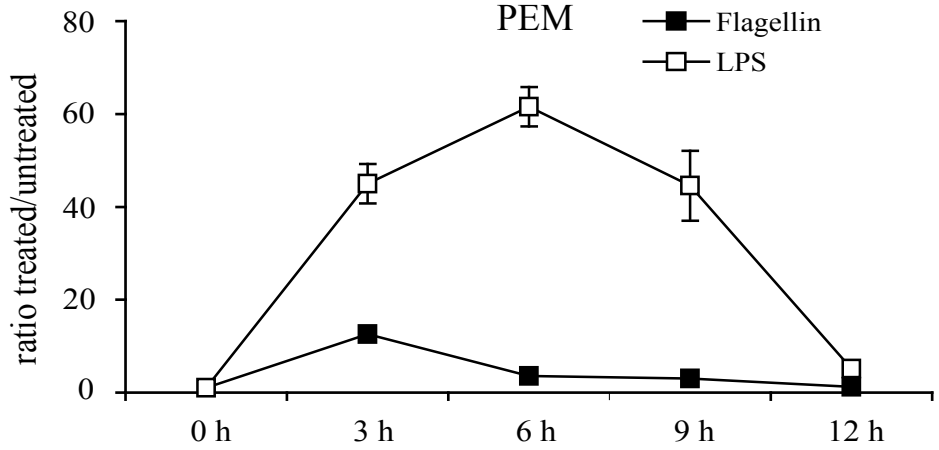


FIGURE 10. IL-10 mRNA dynamics in flagellin-stimulated macrophages. PEM were cultured in media or stimulated with LPS (1 μg/ml) or flagellin (5 μg/ml) and the cells harvested in TriZol at the indicated time points. RNA was extracted and IL-10 relative to GAPDH mRNA was determined by quantitative RT-PCR. Data shows the ratio expressed in arbitrary units of treated versus untreated cells obtained at each of the time points. Shown is one of three independent experiments with similar results.

In response to LPS stimulation, a rapid increase in IL-10 mRNA was observed by 3 hours, followed by a peak response at 6 hours after treatment. This induction is followed by a progressive decline and, after 12 hours of exposure to LPS, the IL-10 mRNA levels were back to baseline (Figure 10-white squares). The magnitude and kinetics of IL-10 mRNA were significantly different in flagellin-treated PEM. Following three hours of incubation, only a weak induction of IL-10 mRNA was detected by RT-PCR. This initial response was followed by a rapid decline and by 6 hours, IL-10 mRNA levels in flagellin-treated PEM were equivalent to those found in untreated cells (Figure 10-black squares).

IL-10 mRNA dynamics in bone marrow-derived macrophages

PEM are elicited using a mild inflammatory agent (thyoglicollate). Therefore they have been exposed to maturating signals that might have affected their ability to respond to flagellin. In other to assess this possibility we generated macrophages from bone marrow (BM-macrophages) and exposed them to either LPS or flagellin. As shown in Figure 11 a similar pattern of IL-10 mRNA expression was observed in flagellin-treated BM-derived macrophages as compared to PEM (black squares).

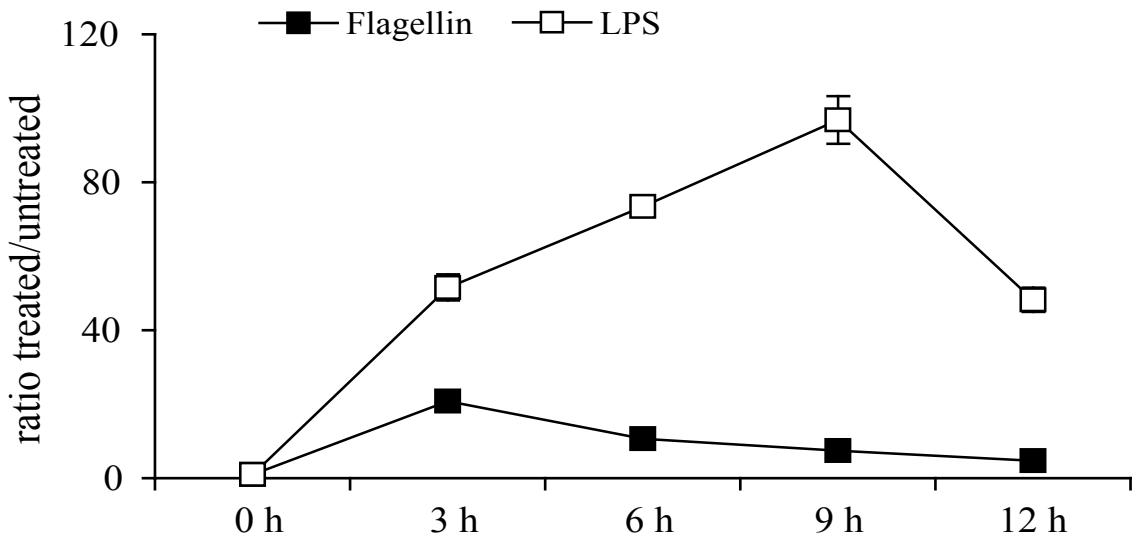


FIGURE 11. IL-10 mRNA dynamics in flagellin stimulated BM-derived macrophages.
(A) BM-derived macrophages were cultured in complete media and stimulated with either LPS (1 μg/ml) or flagellin (5 μg/ml). Cells were then harvested in TriZol at the indicated time points. RNA was extracted from these cells and IL-10 mRNA relative to GAPDH mRNA was determined by quantitative RT-PCR. Data shows the ratio expressed in arbitrary units of treated cells versus untreated cells obtained at each of the indicated time points. Shown is a representative experiment of three independent experiments with similar results.

BM-derived macrophages express TLR5 (Figure 12A). Reminiscent of our findings with PEM (Figure 6B), no IL-10 protein was detected in the supernatants of BM-derived macrophages stimulated with flagellin (Figure 12B). The lack of IL-10

production is not due to the unresponsiveness of these cells since they produce significant levels of IL-12 p40-70 when stimulated with flagellin (Figure 12C)

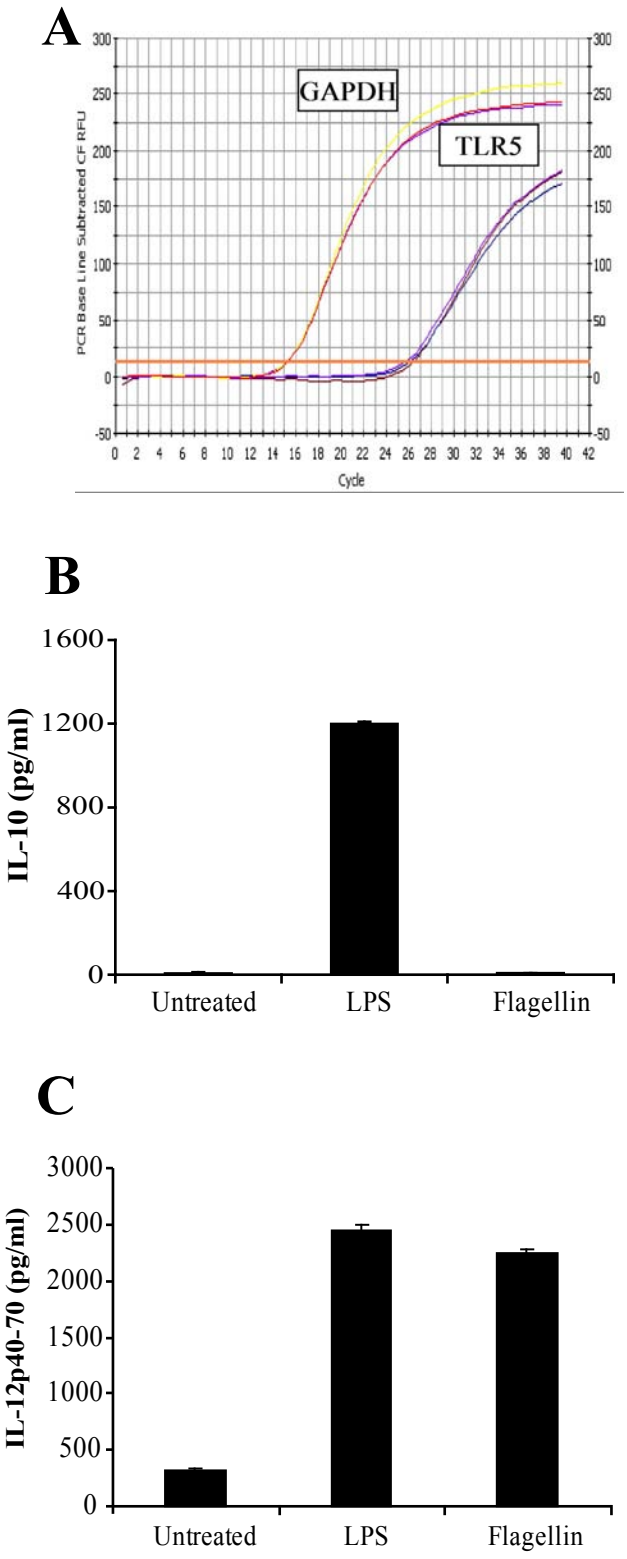


FIGURE 12. BM-derived macrophages express TLR5 and produce IL-12p40-70 but not IL-10 when stimulated with flagellin.

(A) RNA from BM-derived macrophages was analyzed for TLR5 mRNA expression relative to GAPDH using realt-time RT-PCR. (B) and (C) BM-derived macrophages were cultured for 24 hours with LPS (1 μg/ml) or flagellin (5 μg/ml). Supernatants were collected and the levels of IL-10 and IL-12p40-70 were measured by ELISA. Data represent mean ± s.d. of triplicate cultures. Shown is a representative experiment of two independent experiments

Taken together, our data indicates that flagellin induces a short-lived expression of IL-10 mRNA in macrophages. The transient induction of IL-10 expression by flagellin might not be enough to result in detectable levels of IL-10 protein production

LPS-induced IL-10 mRNA and protein are inhibited by flagellin

Several TLR ligands can be found in any microbial organism; furthermore APCs and other immune cells express more than one TLR. Therefore during an ongoing infection, flagellin and other TLR ligands expressed in flagellated bacteria are likely to be recognized by cells of the immune system either simultaneously or sequentially. In order to find whether flagellin could influence the production of IL-10 in response to other TLR ligands, we treated PEM with flagellin and the TLR4 ligand (LPS) either simultaneously (Figure 13) or sequentially (Figures 14 and 15). As shown in Figure 13A, no differences in IL-10 mRNA levels were observed in peritoneal macrophages after 2 hours of exposure to either LPS alone (white squares) or LPS in the presence of flagellin (F/LPS: black squares). However, unlike stimulation with LPS alone, IL-10 mRNA levels declined rapidly by 4 and 6 hours of simultaneous engagement of TLR4 and TLR5. The inhibition of IL-10 by flagellin was also reflected at the protein level. As displayed in Figure 13B, a dose dependent inhibition of IL-10 production was observed when PEM were treated with LPS (1 μg/ml) in the presence of increasing concentrations of flagellin. Inhibition of IL-10 was not observed when the TLR5-deficient cell line RAW 264.7 was treated with LPS and flagellin, indicating that flagellin-induced IL-10 inhibition in response to LPS requires TLR5 expression (Figure 13C).

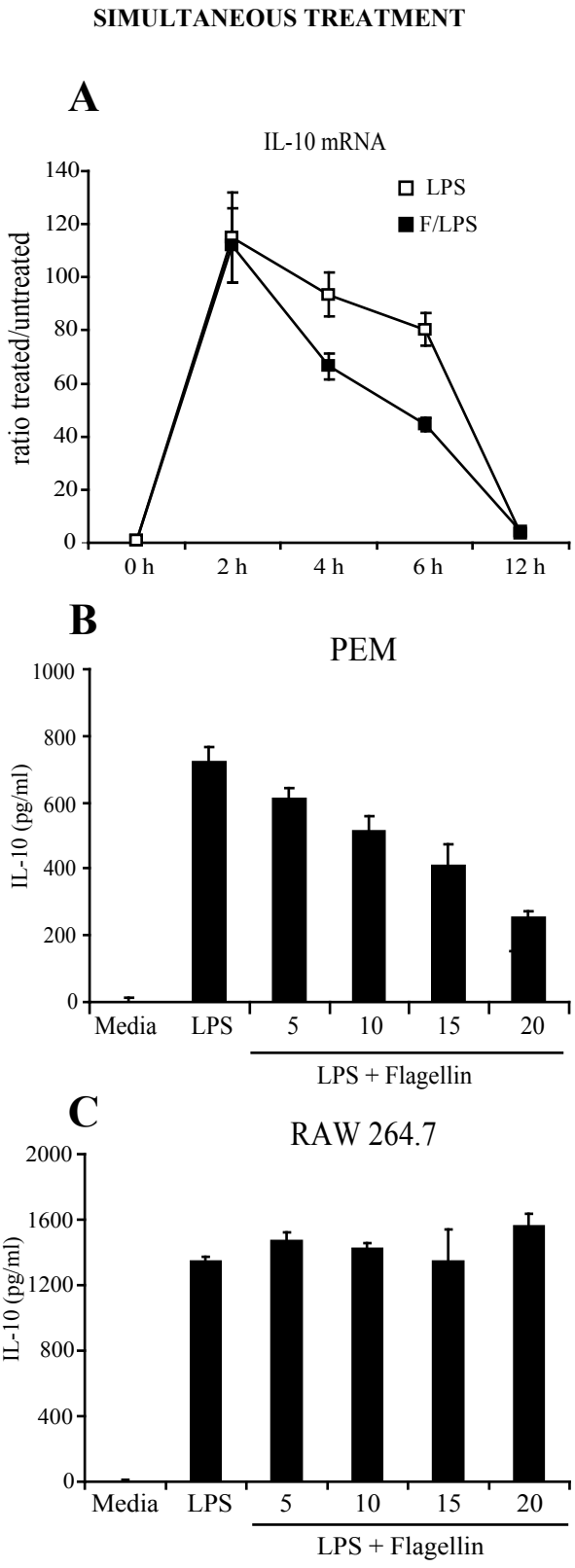


FIGURE 13. Flagellin inhibits LPS-induced IL-10 protein and mRNA
(A) PEM were stimulated with 1 μg/ml LPS alone (open squares) or with LPS (1μg/ml) in the presence of 20 μg/ml flagellin (F/LPS, black squares). Cells were then harvested at the indicated time points. IL-10 mRNA levels were determined as in Figure 10. Data shows the ratio expressed in arbitrary units of treated cells versus untreated cells obtained at each of the indicated time points. Shown is a representative experiment of two independent experiments with similar results. (B, C) PEM or RAW 264.7 cells were stimulated with LPS alone (1 μg/ml) or LPS plus increasing concentrations of flagellin for 24 hours as indicated. Supernatants were then collected and the levels of IL-10 were determined by ELISA. Data represent mean ± s.d. of triplicate cultures. Shown is a representative experiment of two independent experiments with similar results.

Pretreatment of macrophages with flagellin results in stronger inhibition of LPS-induced IL-10

Flagellin treatment decreased LPS-induced IL-10 mRNA levels at later times when macrophages are simultaneously stimulated with flagellin and LPS. This result prompted us to investigate how LPS induced IL-10 mRNA would behave if the cells are exposed to flagellin before LPS stimulation (Figure 14).

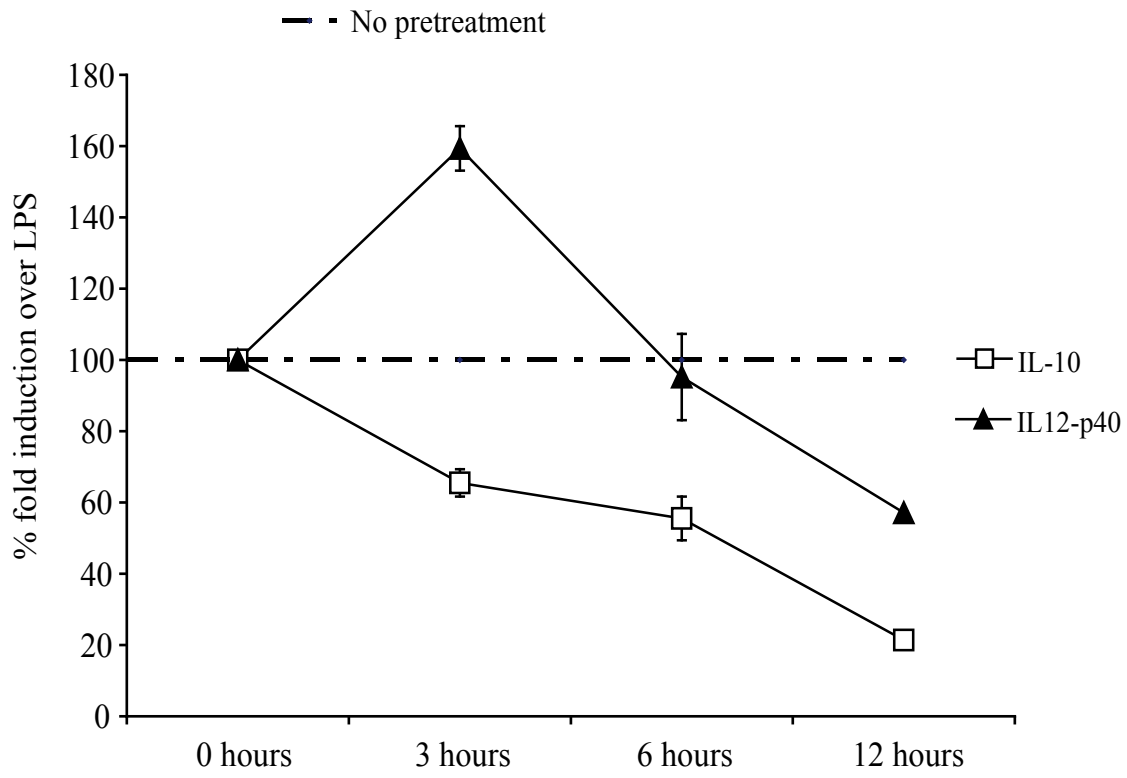


FIGURE 14. A short pretreatment with flagellin affects IL-10 and IL-12 induction in an opposite way
PEM were cultured with flagellin (5 μg/ml) or just media for the indicated time length after which LPS (final concentration 1μg/ml) was added. Cells were harvested 2 hours after LPS addition and the levels of IL-10 (white squares) and IL-12 p40 (black squares) mRNA relative to GAPDH quantified using real-time RT-PCR.

Figure 14 shows IL-10 (white squares) and IL-12p40 (black triangles) mRNA after 2 hours of treatment with LPS in cells that have received or not flagellin pretreatment. IL-10 mRNA inhibition by flagellin was substantially increased when the cells were exposed to flagellin 3 hours before LPS treatment. Increasing the time in contact with flagellin to 6 hours before LPS stimulation resulted in similar levels of inhibition to those observed after 3 hours treatment.

Interestingly, IL-12p40 mRNA production by LPS was enhanced by pretreatment with flagellin for 3 hours. However the observed enhancement was gone when flagellin-pretreatment was increased to 6 hours. Treating the cells for 12 hours with flagellin prior to LPS stimulation made the cells refractory to further stimulation with LPS (149-150).

Given these results, in our next experiments we used macrophages that have been stimulated with flagellin for a short period of time (3 hours) before adding LPS to the media. A profound inhibition of IL-10 mRNA expression was observed when PEM were exposed to flagellin (5 μg/ml) for 3 hours prior to LPS stimulation (Figure 15A). In addition decreased levels of IL-10 protein were observed in PEM pretreated with flagellin (5μg/ml) and then stimulated with increasing concentrations of LPS (Figure 15B). In the pretreatment setting, flagellin at a dose of 5μg/ml, which has minimal inhibitory effect when used simultaneously with LPS (Figure 13B), displayed stronger IL-10 inhibitory properties. Conversely, the inhibitory effect of flagellin on IL-10 production was not observed when the TLR5-deficient cells RAW 264.7 were pretreated with flagellin (Figure 15C).

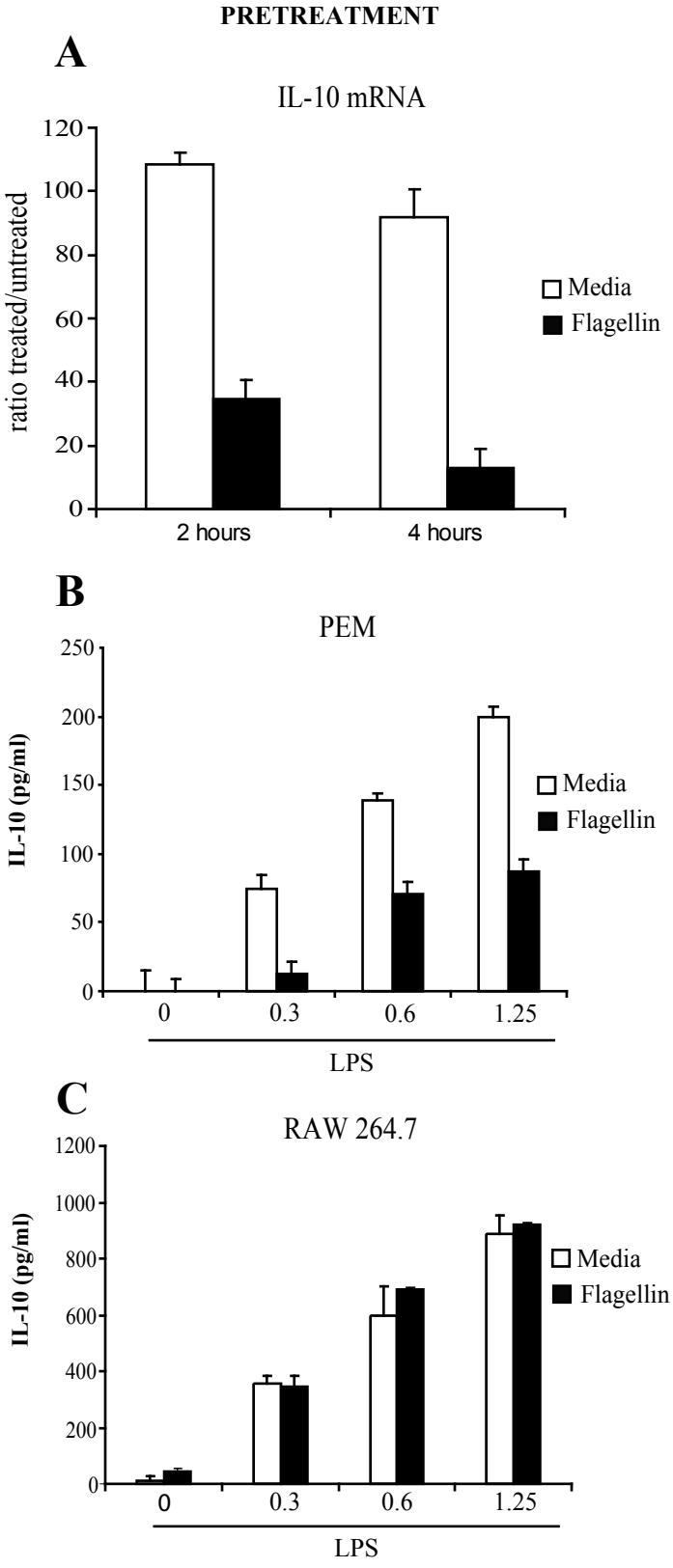


FIGURE 15. Inhibition of IL-10 by flagellin is enhanced by a short pretreatment

(A) PEM were treated with 5 μg/ml flagellin (black bars) or with media alone (white bars) for 3 hours, followed by the addition of LPS (1μg/ml). Cells were harvested at the indicated time points after the addition of LPS. IL-10 mRNA relative to GAPDH mRNA was determined by quantitative real-time RT-PCR. Shown is a representative experiment of four independent experiments with similar results. (B, C) PEM or RAW 264.7 cells were cultured with flagellin (5 μg/ml) or media alone for 3 hours, followed by stimulation with LPS (1μg/ml). Supernatants were collected 24 hours after LPS addition and the levels of IL-10 were determined by ELISA. Data represents mean ± s.d. of triplicate cultures. Shown is a representative experiment of three independent experiments.

IL-10 inhibition by flagellin is restricted to specific TLRs

To determine whether a similar inhibitory effect over IL-10 production occurs when PEM are exposed to TLR-ligands other than LPS, we treated PEM with flagellin followed by stimulation with either zymosan (TLR2/6 ligand) or CpG (TLR9 ligand). As shown in Figure 16A, treatment of PEM with increasing concentrations of zymosan results in IL-10 production in a dose dependent manner (white bars). However, when these cells were treated with flagellin and then zymosan, a dramatic decrease in IL-10 production was observed (Figure 16A-black bars). In contrast, such an inhibitory effect on IL-10 protein production was not observed when PEM were pretreated with flagellin followed by CpG (Figure 16B). In order to assess if flagellin pretreatment decreased CpG-induced IL-10 at the mRNA but not at the protein levels we quantified the levels of IL-10 mRNA in PEM pretreated with flagellin or media followed by CpG. In agreement with the results obtained at the protein level, flagellin did not affect the IL-10 mRNA levels induced by CpG in PEM (Figure 16C). To summarize, our results have unveiled a previously unknown effect of flagellin in the negative regulation of IL-10 production in response to specific TLR ligands.

Interestingly zymosan, flagellin and LPS engage TLRs that localize in the cellular membrane while CpG is detected by an intracellular receptor. IL-10 production by surface receptors but not by intracellular receptors is affected by flagellin stimulation suggesting that receptor colocalization might be required for TLR5-mediated IL-10 repression.

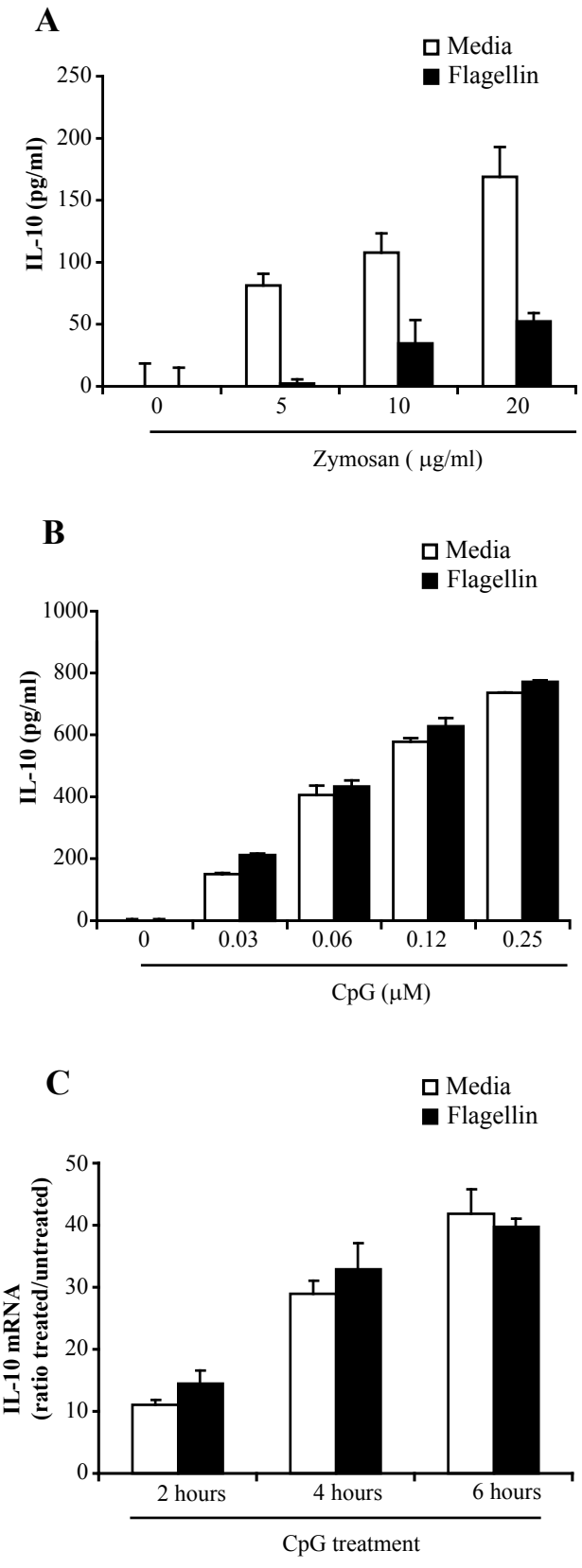


FIGURE 16. IL-10 production by macrophages treated with flagellin and other TLR ligands.

PEM were cultured in media alone (white bars) or with 5 μg/ml of flagellin (black bars) for 3 hours, followed by stimulation with either Zymosan (A) or CpG (B). Supernatants were collected 24 hours later and the production of IL-10 protein was determined by ELISA. Data represent +/- s.d. of triplicate cultures. Shown is a representative experiment of two independent experiments with similar results. (C) Cells were harvested at the indicated time points after the addition of CpG. IL-10 mRNA relative to GAPDH mRNA was determined by real-time RT-PCR. Shown is a representative experiment of two independent experiments with similar results.

IL-10 inhibition might depend on receptor colocalization

In unstimulated macrophages and DCs TLR5 and TLR9 have been shown to be expressed in different cellular compartments. TLR5 localizes to the cellular membrane (47) while TLR9 has been detected in the ER (endoplasmic reticulum) (48). Nonetheless, TLRs present in the cellular membrane have been shown to be recruited to phagosomes (151). In addition, phagosomes contain ER-resident proteins since ER membranes are a source of phagocytic membranes in macrophages and other cells (152). Therefore, in the context of phagocytosis it would be possible that TLR5 and TLR9 could localize in the phagosomal/lysosomal compartment. To test this hypothesis we used beads as a vehicle to introduce flagellin into the phagosomal/lysomal compartment (Figure 17).

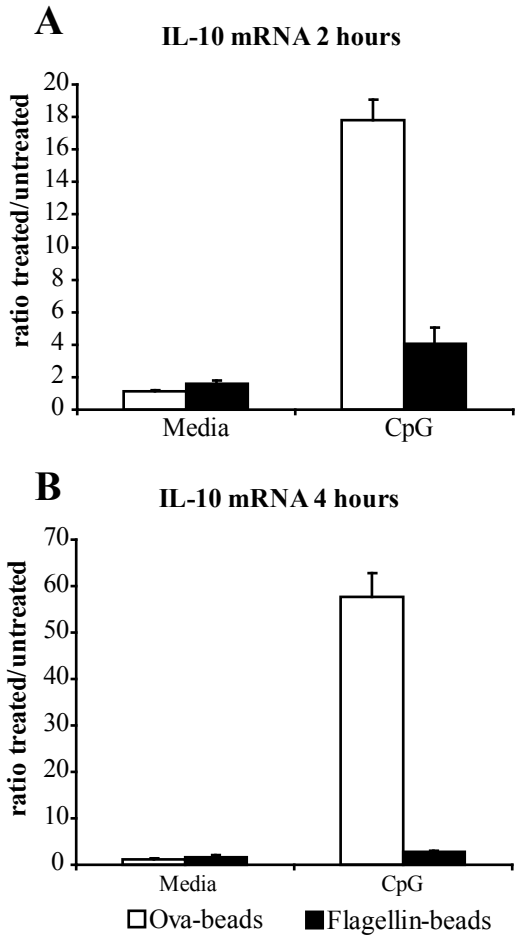


FIGURE 17. Macrophages stimulated with flagellin-coated beads showed impair IL-10 induction by CpG
PEM were cultured with flagellin coated beads (equivalent to 5μg/ml protein) (black bars) or beads coated with an irrelevant protein (OVA) (white bars). After 3 hours cells were washed and fresh media or media with CpG (final concentration 0.25 μM) added. Cells were harvested at the indicated time points after the addition of CpG. IL-10 mRNA relative to GAPDH mRNA was determined by quantitative real-time RT-PCR.

As shown in Figure 17 CpG stimulation of macrophages that have phagocyted flagellin-coated beads resulted in inhibition of IL-10 mRNA as compared to those that uptook control protein (ova)-coated beads. These data could support that signaling from the same compartment is required for TLR5 negative regulation of IL-10 induction by other TLRs.

Flagellin treatment does not affect LPS-induced IL-10 mRNA stability

Stimulation of macrophages with LPS causes a dramatic increase in the IL-10 mRNA levels that might be due to an increase in the IL-10 gene transcription and also in the IL-10 mRNA stability (126-127), (153). In order to determine whether suppression of LPS-induced IL-10 by flagellin was caused by an increase in IL-10 mRNA degradation, we treated macrophages with actinomycin D, an inhibitor of mRNA synthesis, and followed the changes induced by flagellin in the stability of LPS-induced IL-10 mRNA. Figure 18A shows that actinomycin D suppresses mRNA synthesis. For this control experiment, cells were treated with LPS plus actinomycin D or LPS plus media for two hours. Cells were then harvested and the levels of IL-10 mRNA were measured by RT-PCR. Actinomycin D (5 μg/ml) completely blocked the synthesis of new mRNA (LPS + ActD). In our next set of experiments, PEM were cultured for 3 hours with either flagellin or media and then LPS was added. Actinomycin D was added to the cultures 2 hours after LPS treatment and cells were harvested at the indicated time points after actinomycin D treatment. As shown in Figure 18B, an almost identical rate of IL-10 disappearance was observed; suggesting that pretreatment with flagellin does not affect IL-10 mRNA stability in response to LPS stimulation.

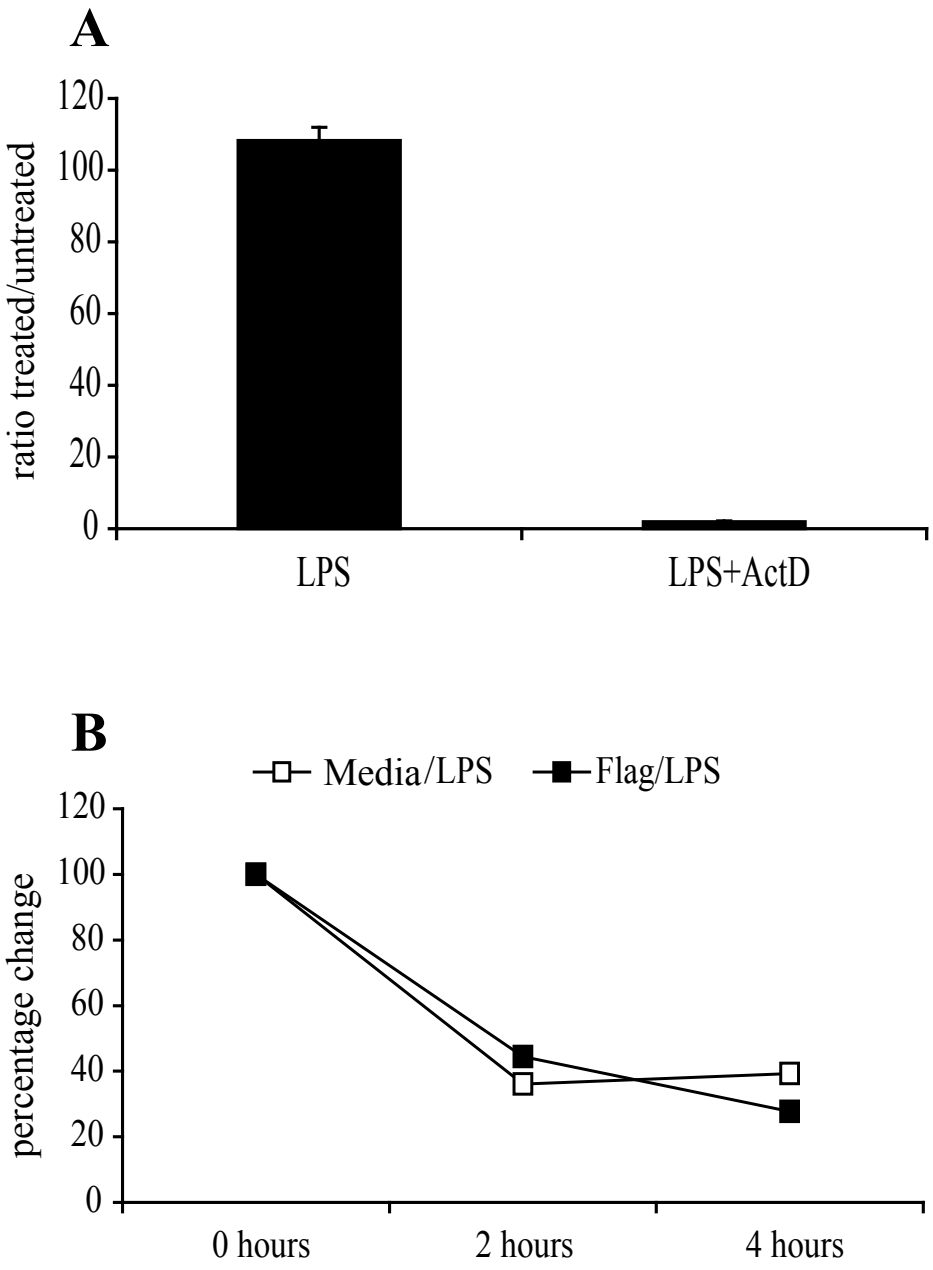


FIGURE 18. IL-10 repression by flagellin is not caused by a decrease in IL-10 mRNA stability

(A) PEM were cultured with LPS (1 μg/ml) plus actinomycin D (5 μg/ml) or vehicle. After 2 hours treatment the cells were harvested and the IL-10 mRNA levels relative to GAPDH mRNA were determined by RT-PCR. (B) Actinomycin D or vehicle were added to the media 2 hours after LPS treatment of PEM that had been cultured with (black squares)or without flagellin (without squares). Cells were harvested 2,4 and 6 hours after actinomycin D addition and the IL-10 mRNA levels relative to GAPDH mRNA were determined by RT-PCR. Shown is a representative experiment of two independent experiments with similar results

Flagellin stimulation decreases ERK activation by LPS in macrophages

A general outcome upon TLR engagement is the activation of the NFκB and the mitogen-activated protein kinase (MAPK) signaling pathways. Among the MAPKs, several studies have linked the extracellular signal regulated kinases (ERKs) with IL-10 regulation by TLRs. These studies found that IL-10 induction by different TLRs depends on ERK activation (154-158). In order to assess if flagellin stimulation would affect LPS-induced ERK phosphorylation we treated PEM with flagellin or media followed by LPS stimulation. Since significant changes in the LPS-induced IL-10 mRNA levels are detected in flagellin-treated macrophages at 2 hours stimulation with LPS (Figure 15A) we decided to quantify ERK phosphorylation at earlier time points (30 and 60 minutes). Interestingly, LPS-induced phosphorylated ERK 1/2 levels were significantly reduced in flagellin-treated macrophages as compared with media-treated macrophages (Figure 19). This result suggests that decreased ERK activation could play a role in flagellin-mediated IL-10 inhibition.

NFκB activation plays a central role in inducing the expression of proinflammatory mediators by TLRs. In resting cells NFκB is sequestered in the cytoplasm by molecules known as inhibitor of nuclear factor-κB (IκB). Phosphorylation of IκB results in its degradation and in the release of NFκB that now can translocate to the nucleus and activate its target genes (62). Macrophages treated with flagellin or LPS alone showed increased IκB phosphorylation suggesting that treatment with these TLR ligands results in NFκB activation. As seen in Figure 19, flagellin treatment in combination with LPS did not result in decreased p-IκB levels as compared with cells treated with LPS alone. These data suggest that the NFκB activation by LPS is not

affected by flagellin treatment and therefore is not involved in flagellin-induced IL-10 inhibition

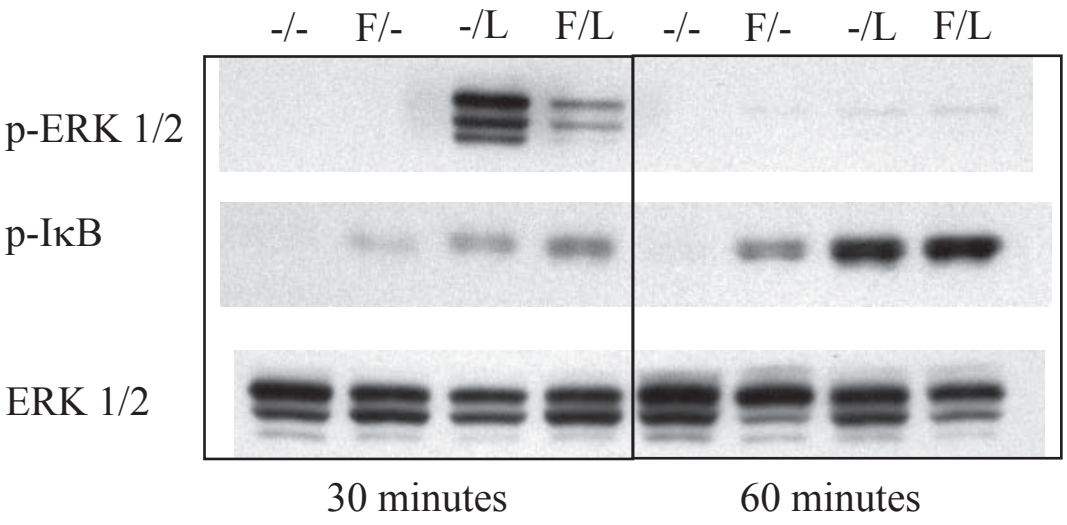


FIGURE 19. Flagellin treatment decreases ERK activation by LPS in macrophages

PEM were cultured with 5 μg/ml flagellin (F/) or just media for 3 hours (-/) followed by the addition of 1 μg/ml LPS (/L). Cells were harvested 30 minutes and 1 hour after LPS addition. Whole-cell lysates were obtained and subjected to SDS-PAGE immunoblotting with the indicated antibodies. Unphosphorylated ERK 1/2 was used as loading control. Shown is a representative experiment of two independent experiments with similar results.

Flagellin enhances IL-12 production in response LPS

To further confirm the data suggesting that a short pretreatment with flagellin results in enhanced IL-12 production by LPS (Figure 14), we determined the IL-12 p40/ IL-12 p35 mRNA levels in macrophages treated with flagellin and LPS (Figure 20).

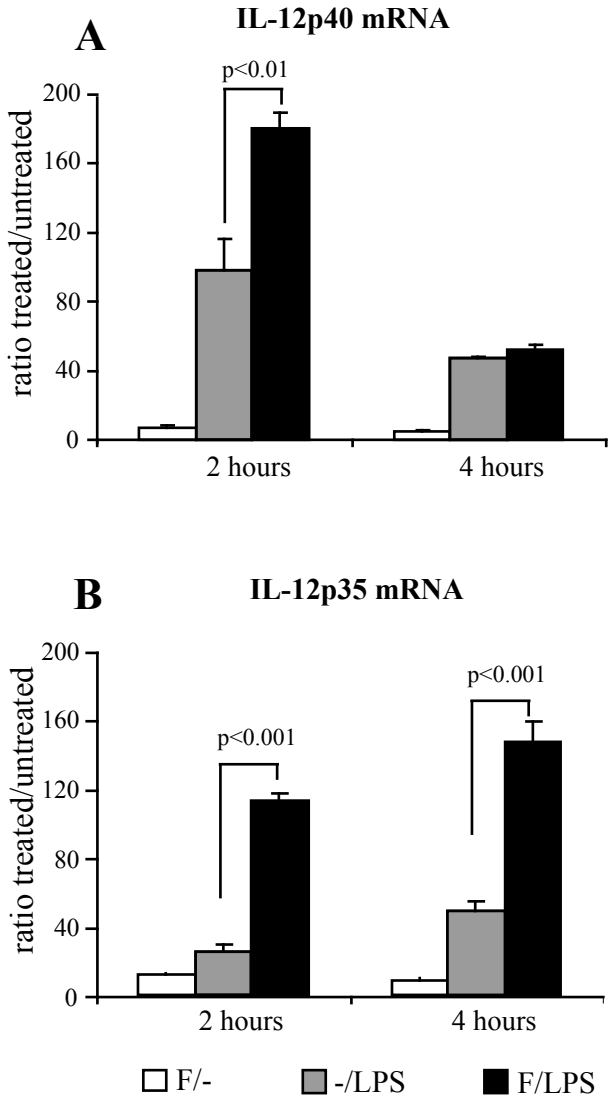


FIGURE 20. Flagellin-stimulated PEM produce higher levels of IL-12p35 and IL-12p40 mRNAs in response to LPS.
(A-B) PEM were cultured with 5 μg/ml flagellin (F) for three hours. Then supernatants were removed and media (-) was added (F/-, white bars). In parallel, PEM were cultured with media alone (-) for three hours and then LPS was added (-/LPS, gray bar). A third group consisted of PEM pretreated with flagellin (F) for three hours and then stimulated with LPS (F/LPS, black bar). Cells were harvested at the indicated time points after the addition of LPS and the levels of IL-12p40 (A) and IL-12p35 mRNA (B) relative to GAPDH were determined by quantitative RT-PCR. Shown is a representative experiment of four independent experiments with similar results.
Statistical analysis was performed using t-test.

Flagellin pretreatment increased the level of IL-12p40 mRNA in response to LPS stimulation for two hours (Figure 20A, black bar-2 hours). This increase is short-lived since at 4 hours IL-12p40 mRNA levels were back to those observed in PEM treated with LPS alone (Figure 20A- 4 hours). In addition, an increase in IL-12p35 mRNA levels was observed in PEM treated with flagellin and subsequently stimulated with LPS (F/LPS: black bars) (Figure 14B). The response duration was longer than the one observed for IL-12p40 mRNA induction, since enhanced levels of IL-12p35 mRNA were still observed after 4 hours of exposure to LPS (Figure 20B-4 hours).

The biologically active form of the cytokine IL-12 is a heterodimer composed of the IL-12 p40 and p35 proteins and known as IL-12 p70 (94). IL-12 p40 has been reported to be produced in excess over IL-12p35 which acts as a limiting factor for IL-12 p70 production (96). Therefore, an increase in IL-12 p35 might result in elevated IL-12 p70 protein production that would not be detected using standard ELISA methods that do not discriminate between IL-12p40 and IL-12p70 production. For this reason, we specifically analyzed IL-12p70 production by PEM pretreated with flagellin followed by LPS. As shown in Figure 21, when PEM are pretreated with flagellin and then exposed to increased concentrations of LPS (black bars) they produced higher levels of IL-12p70 relative to PEM treated with LPS alone (white bars). In summary, PEM treated with flagellin and then stimulated with LPS display enhanced expression of both IL-12p40 mRNA (Figure 20A) and IL-12p35 mRNA (Figure 20B) as well as increased production of IL-12p70 protein (Figure 21).

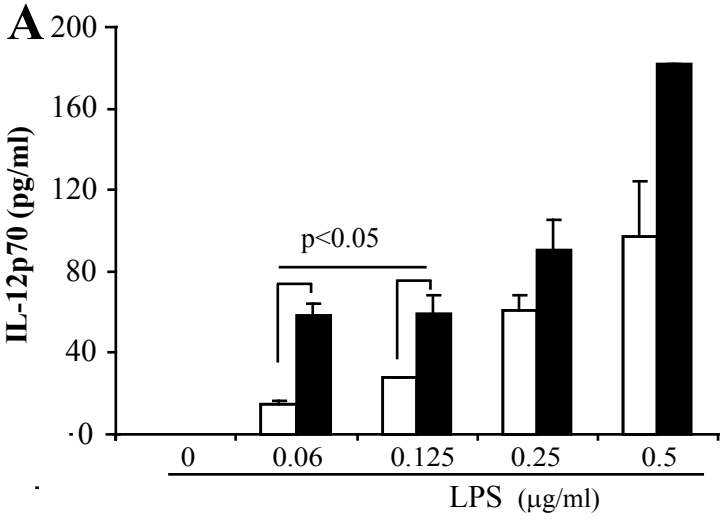


FIGURE 21 Flagellin-stimulated PEM produce higher levels of IL-12p70 in response to TLR4 ligands. PEM were cultured with media alone (open bar) or with flagellin 5 μg/ml (black bar) for 3 hours. Then, PEM were treated with increasing concentrations of LPS. IL-12 p70 was quantified as in Figure 22. Shown is a representative experiment of three with similar results

Flagellin enhances IL-12p70 production in response to CpG

To determine whether flagellin-mediated enhancement of IL-12p70 production occurs only in response to LPS-stimulation or could also be observed in response to other TLR ligands, PEM were treated with flagellin in combination with either zymosan or CpG. While treatment with flagellin did not enhance IL-12p70 production in response to zymosan that remained undetectable (data not shown), a significant increase in IL-12p70 production was observed when PEM were treated with flagellin and CpG (Figure 22).

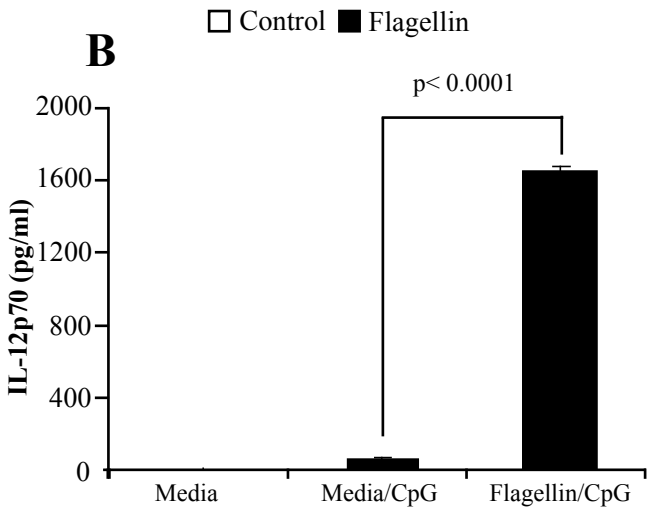


FIGURE 22 Flagellin-stimulated PEM produce higher levels of IL-12p70 in response to TLR9 ligands.

PEM were cultured with media alone (open bar) or with flagellin 5 μg/ml (black bar) for 3 hours. Then, PEM were treated with 0.25 μM CpG. Supernatants were collected after 24 hours and IL-12 p70 levels were determined by ELISA. Data represent mean ± s.d. of triplicate cultures. Shown is a representative experiment of three with similar results.
Statistical analysis used was t-test

These results (Figure 21 and 22) indicate that ligation of TLR5 in macrophages influences the inflammatory response of these cells to TLR4 and TLR9 ligands (LPS and CpG) resulting in enhanced production of the proinflammatory mediator IL-12p70.

Blocking IL-10 increases IL-12p70 production by LPS-stimulated macrophages

IL-10 has been shown to act as a powerful inhibitor of IL-12 production (107-108). Therefore, in the next experiment we assessed if blocking IL-10 signaling would enhance IL-12p70 production by LPS-treated PEM (Figure 23).

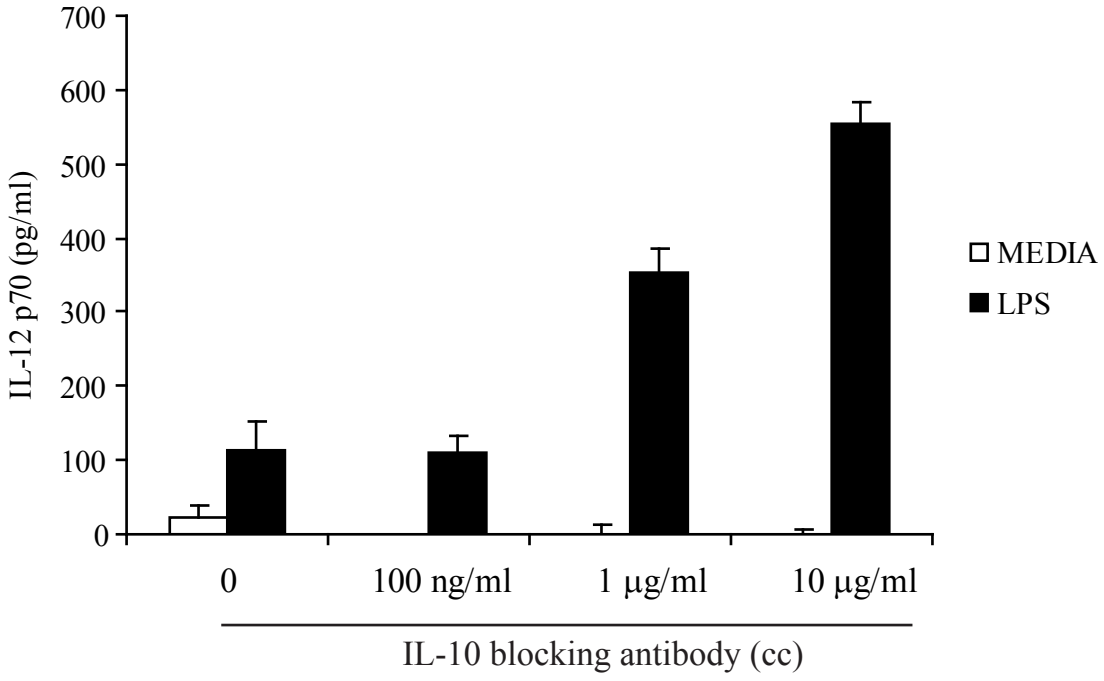


FIGURE 23. IL-10 blockage increases IL-12p70 production by LPS-stimulated macrophages
Macrophages were cultured with LPS (1 μg/ml) (black squares) or media alone (white bars) plus IL-10 blocking antibody at the indicated concentration. After 24 hours culture the supernatants were harvested and IL-12 p70 production quantified by ELISA.

Incubating the cells with increasing amounts of IL-10 blocking antibody increases IL-12 p70 production in a dose dependent manner (Figure 23). This result indicates that IL-10 repression by flagellin could be, at least in part, responsible for the increase in IL-12 p70 production observed in macrophages treated with flagellin in combination with LPS. Nonetheless, other mechanisms must be also playing a role since enhanced IL-12 p70 production was observed in macrophages treated with a combination of flagellin and CpG even when on those cells IL-10 production was not affected by flagellin stimulation (Figure 16).

Flagellin elicits CD4+ T-cell activation in a tolerogenic setting *in vivo*

In previous studies we have shown that APCs that produce significant levels of IL-12 in the absence of the inhibitory cytokine IL-10 effectively prime naïve antigen-specific CD4+ T-cells and are capable of restoring the responsiveness of tolerized CD4+ T-cells (141). This finding, along with our data demonstrating that flagellin-treated APCs produce IL-12 but not IL-10, led us to explore whether treatment with this TLR-ligand could overcome antigen-specific CD4+ T-cell tolerance *in vivo*.

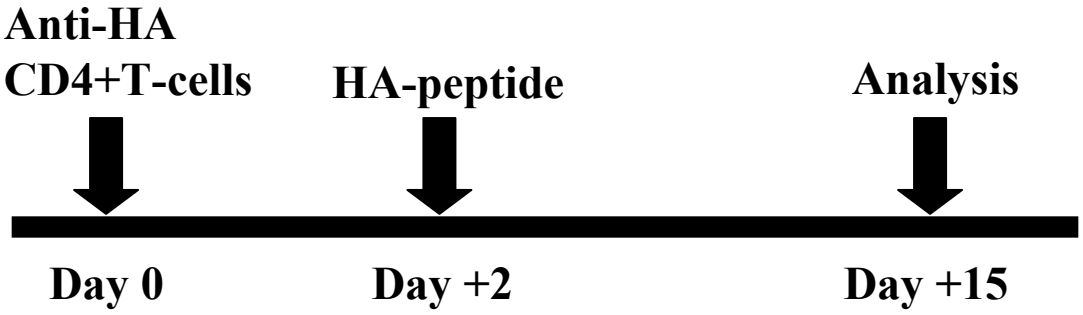


FIGURE 24. High dose peptide model of antigen-specific CD4+ Tcell tolerance (I)

To answer this question, we followed the experimental design outlined in Figure 24. Briefly, $2.5x10^6$ naïve $CD4^+$ T-cells specific for a MHC class II-restricted epitope of influenza hemagglutinin (HA) were adoptively transferred intravenously (iv) into BALB/c mice. Twenty-four hours later, mice were given iv a tolerogenic dose of HA-peptide (200 μg), a combination of HA-peptide together with flagellin (10 μg), or left untreated. Two weeks later, all the mice were sacrificed and the splenocytes harvested. Previous studies have shown that antigen encounter by clonotypic $CD4^+$ T-cells in mice treated with a high dose of their cognate antigen leads to progressive T-cell deletion (159-160). Indeed, as shown in Figure 25, in those mice treated with high dose HA-peptide, we observed a dramatic decrease in the percent of clonotypic $CD4^+$ T-cells (0.34% in HA-peptide treated mice versus 1.02% in untreated mice). Such a decrease in clonotypic T-cells was prevented however in mice treated with tolerogenic dose of HA-peptide plus flagellin (Figure 25A). Assessment of cytokine production by clonotypic T-cells isolated from the different experimental groups also showed important differences. While treatment with high dose HA-peptide resulted in slight decrease in the production of IFN-γ by clonotypic T-cells isolated from these mice relative to the untreated group, the opposite outcome was observed in mice treated with a high dose of peptide together with flagellin. Clonotypic T-cells from these mice produced higher levels of IFN-γ in response to restimulation with cognate antigen, indicating that they have been primed *in vivo* in response to an otherwise tolerogenic dose of peptide (Figure 25B)

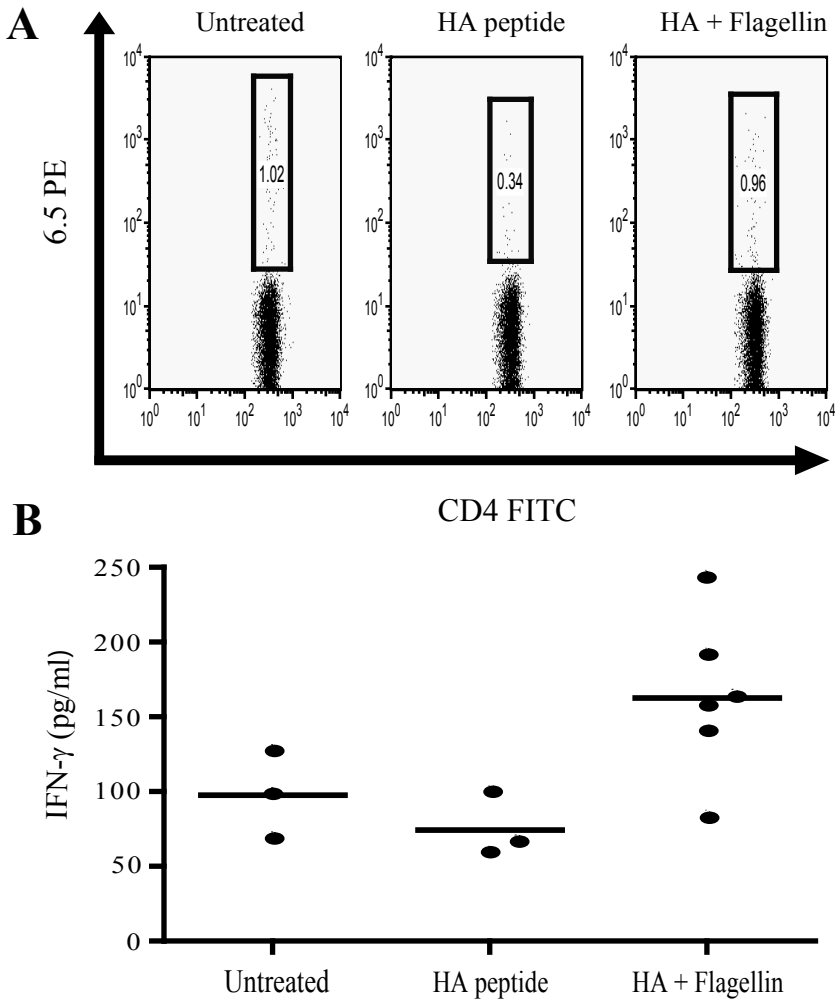


FIGURE 25. Flagellin elicits CD4+ T-cell activation in a tolerogenic setting (I).

BALB/c mice received 2.5 x 10^6 anti-HA TCR+ transgenic CD4+ T cells iv Twenty-four hours later mice were left untreated, given HA-peptide (200 μg/iv) alone or HA peptide (200 μg/iv) in combination with flagellin (10 μg/iv). Two weeks later mice were sacrificed and T cells were purified from their spleens as described in Methods. (A) Shows two-color FACS analysis of splenocytes from one animal in each experimental group (each consisted of four animals). Upper right quadrant numbers represent the percentage of double positive CD4+ T- cells. (B) Purified T-cells from untreated mice or mice treated with high dose HA-peptide *in vivo* or HA-peptide plus flagellin were restimulated with HA-peptide (12.5 μg/ml) *in vitro* for 48 hours. Supernatants were collected and assayed for IFN-γ production by ELISA. Each dot represents an animal and the bar represents the group's average. Shown is a representative experiment of three independent experiments with similar results. Data is expressed as the amount of cytokine produced by 100 clonotype+ T cells/well.

In this model of antigen-specific T-cell tolerance we have also demonstrated that anergic anti-HA CD4+ T-cells are unable to respond to inflammatory stimuli provided by a recombinant vaccinia virus encoding HA (vaccHA). Therefore, in our next experiment we asked whether flagellin treatment could change this outcome and preserve antigen-specific T-cell responsiveness to the model antigen expressed by vaccinia virus. For this experiment we followed the esperimental design outlined in Figure 26.

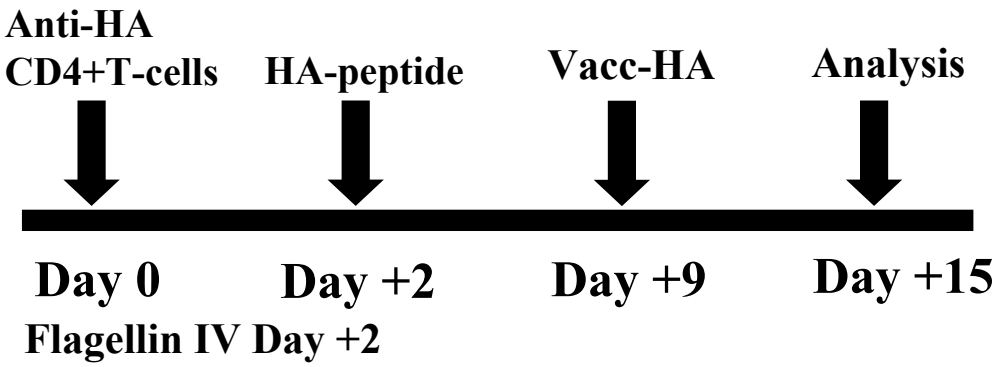


FIGURE 26. High dose peptide model of antigen-specific CD4+ T cell tolerance (II)

First, antigen-specific T-cells reisolated from vaccinia HA-infected mice produced significant levels of IL-2 in response to *in vitro* stimulation with cognate HA-peptide (Figure 27, Untreated). Clonotypic T-cells from mice treated with a tolerogenic dose of HA-peptide failed to respond to vaccHA immunization *in vivo* as determined by their lack of IL-2 production in response to cognate HA-peptide (Figure 27, HA peptide). In sharp contrast, flagellin treatment preserved the response to vaccination in this tolerogenic setting, since clonotypic T-cells isolated from these mice produce levels of IL-2 that are equivalent to those produced by antigen-specific T-cells from control mice (Figure 27, HA + flagellin).

Summarizing, *in vivo* treatment with flagellin converts a tolerogenic environment into an inflammatory one in which antigen-specific CD4+ T-cells not only are not deleted but are efficiently activated and capable of responding to a subsequent immunization with recombinant vaccinia encoding HA.

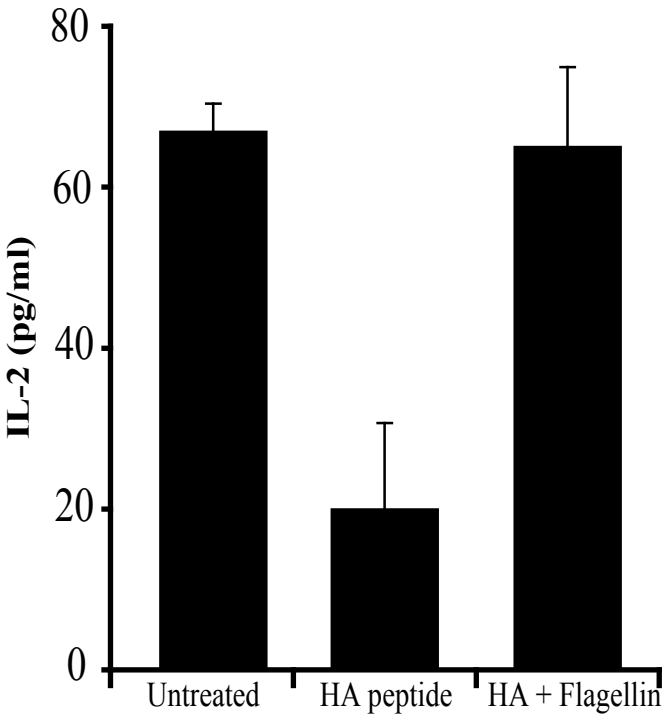


FIGURE 27. Flagellin elicits CD4+ T-cell activation in a tolerogenic setting (II). Untreated mice, mice treated with high dose HA peptide or mice that received high dose HA-peptide plus flagellin *in vivo* were immunized with $1x10^7$ pfu of recombinant vaccinia encoding HA (vaccHA) on day +9 after T-cell adoptive transfer. Six days later animals were sacrificed and T-cells isolated from their spleens. Purified T cells were stimulated with HA-peptide plus fresh splenocytes for 48 hr. Supernatants were collected and assayed for IL-2 production by ELISA. Data represent mean ± s.d. of triplicate cultures from three mice in each group.

DC differentiation in the presence of IL-10

Several studies have shown that DC differentiation in the presence of IL-10 results in the development of DCs that produce high levels of IL-10 and induce T cell anergy and regulatory T cell differentiation (143-144). DCs differentiated in these conditions have been shown to induce tolerance (144), therefore to avoid confusion between BM-DCs differentiated using different protocols, DCs differentiated with GM-CSF plus IL-4 will be called just BM-DCs and those differentiated in the presence of IL-10 will be called tolerogenic-DCs.

Our first goal for the series of experiments described bellow, was to generate DCs in the presence of IL-10 and evaluate their ability to present antigen. Differentiation of DCs in the presence of the anti-inflammatory cytokine IL-10 rendered similar yield of CD11c+ cells to those obtained by standard procedures (around 90%) even when these cells are characterized by a lower expression of this marker (Figure 28).

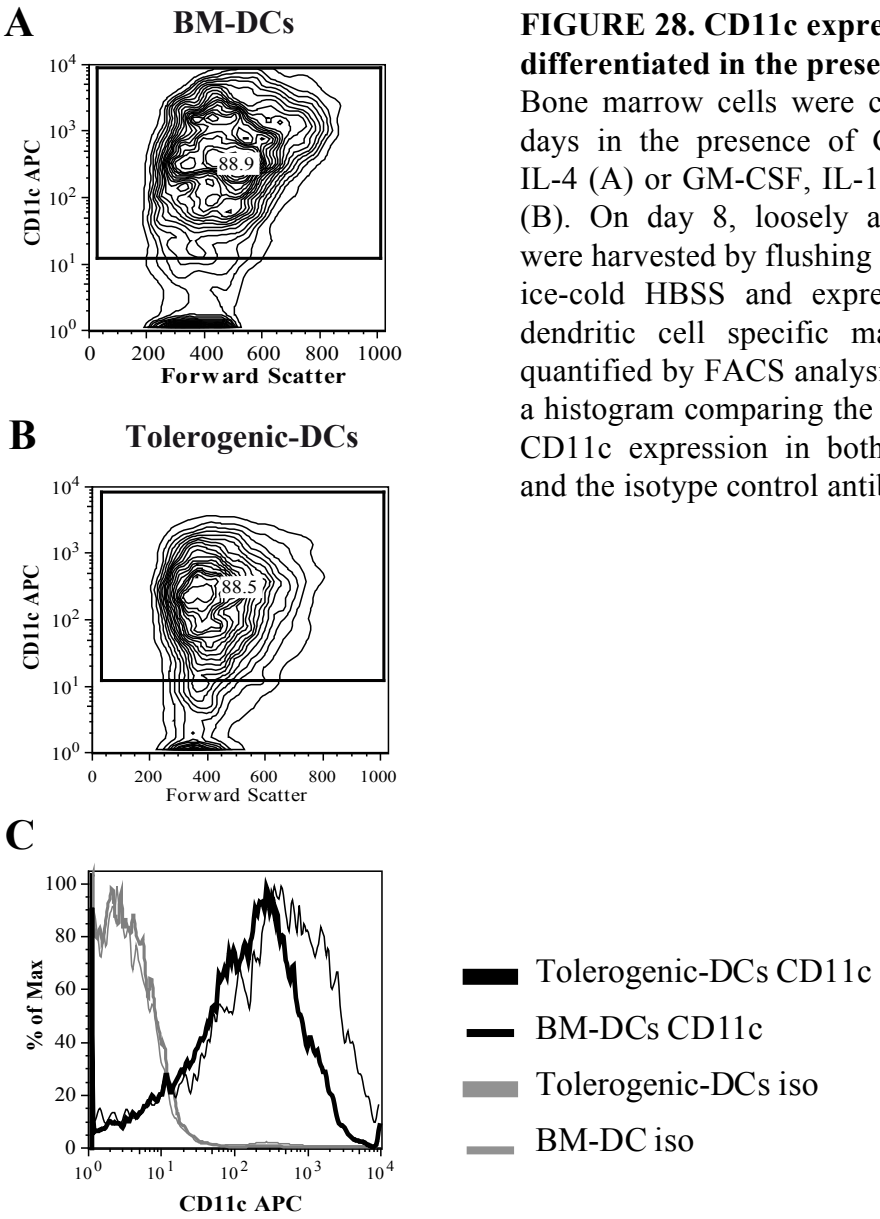


FIGURE 28. CD11c expression in DCs differentiated in the presence of IL-10 Bone marrow cells were cultured for 7 days in the presence of GM-CSF and IL-4 (A) or GM-CSF, IL-10 and TNF-α (B). On day 8, loosely adherent cells were harvested by flushing the plate with ice-cold HBSS and expression of the dendritic cell specific marker CD11c quantified by FACS analysis. (C) Shows a histogram comparing the magnitude of CD11c expression in both DC subsets and the isotype control antibodies.

As previously shown by others, expression of costimulatory molecules as MHCII or B7.2 was reduced in those cells that have been differentiated in the presence of IL-10 as compared with those differentiated following a standard protocol (Figure 29).

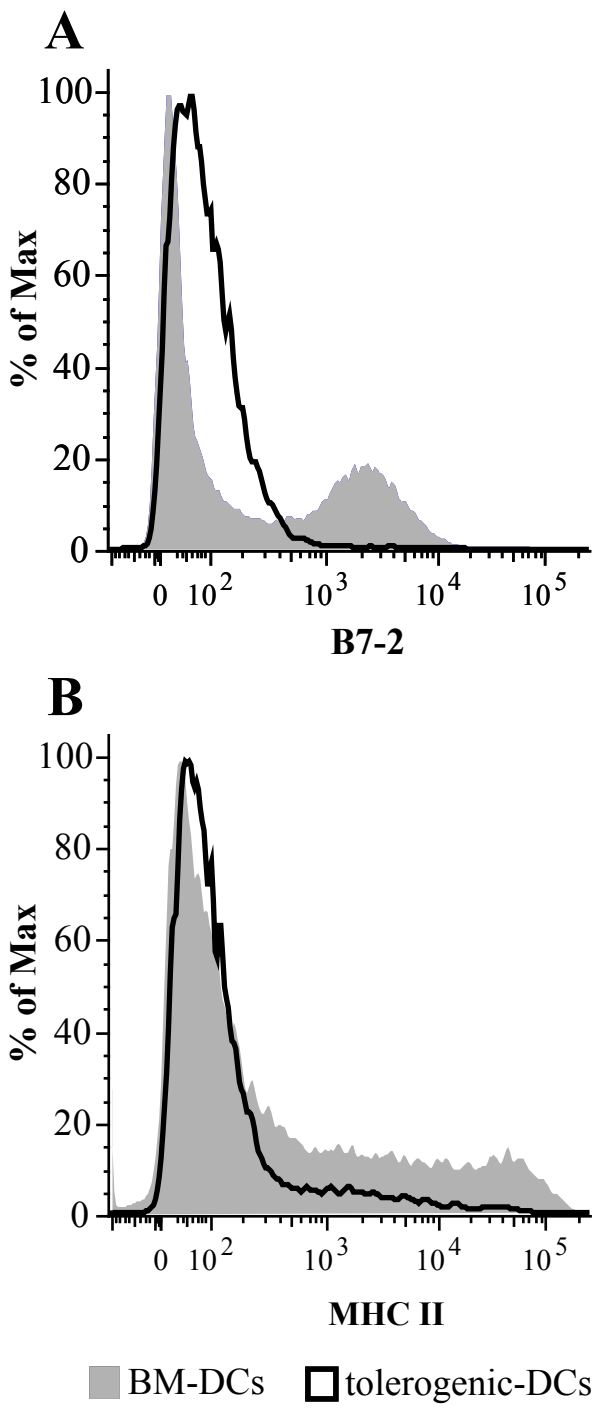


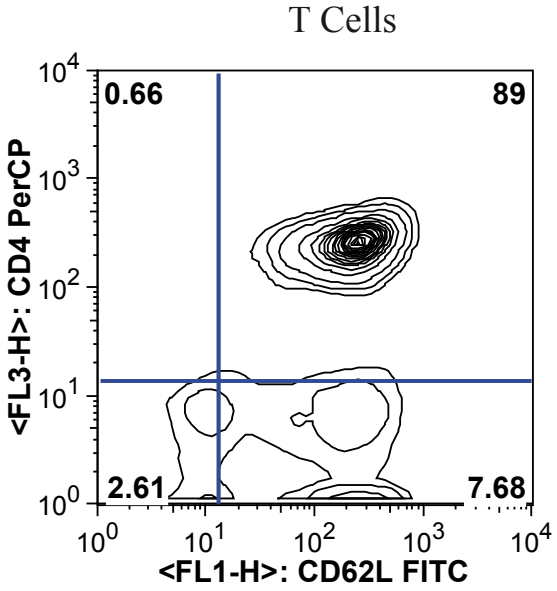
FIGURE 29. Tolerogenic-DCs express lower levels of costimulatory molecules than BM-DCs

Bone marrow cells were cultured for 7 days in the presence of GM-CSF and IL-4 (BM-DCs) (shaded area) or GM-CSF, IL-10 and TNF-α (tolerogenic-DCs) (solid line). On day 8, loosely adherent cells were harvested by flushing the plate with ice-cold HBSS and expression of the costimulatory molecules B7-2 (A) and MHC class II (B) were quantified by FACS analysis.

Tolerogenic-DCs induce T cell unresponsiveness *in vitro*.

In order to compare T cell priming by tolerogenic-DCs and BM-DCs we developed a protocol for T cell priming and differentiation *in vitro*, summarized in Figure 30

BM-DCs or tolerogenic-DCs + Purified CD4+ CD62L+ T cells (from HA+/- mice)



7 days

Re-stimulation splenocytes +/- HA peptide

48h / 72h

CK analysis/ Proliferation analysis

FIGURE 30. T cell differentiation diagram

Briefly, naïve CD4+ T cells (CD4+ CD62L high) from HA +/- mice were cultured with DCs and antigen (HA peptide) for 7 days. This period of time is enough to allow T cell differentiation. After 7 days T cells were washed and transferred to a second plate were they were restimulated with antigen and fresh splenocytes used as antigen presenting cells. Culture of naïve CD4+ T cells with tolerogenic-DCs resulted in significantly lower levels of IFN-γ production and proliferation relative to T cells that have been cultured with BM-DCs (Figure 31) indicating that the antigen presenting function in tolerogenic-DCs is impaired. Lower expression of MHCII and costimulatory molecules by tolerogenic-DCs could account for the deficient priming of antigen specific CD4+ T cell responses.

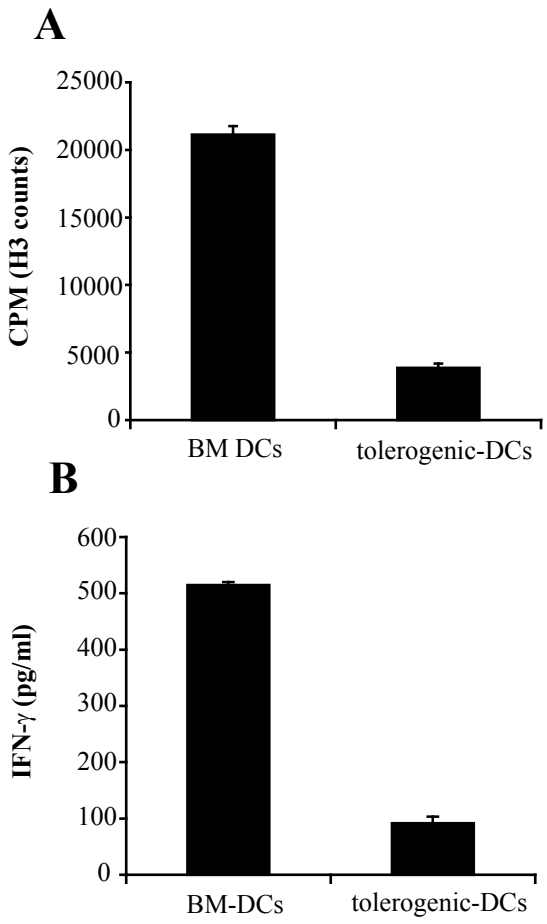


FIGURE 31. T cells differentiated using tolerogenic-DCs showed impaired responses upon antigen restimulation
T cells were differentiated as in Figure 30 and their responses upon antigen restimulation evaluated in terms of (A) proliferation (H3 incorporation) and IFN-γ production (B).

LPS stimulation of tolerogenic-DCs results in enhanced production of IL-10

Cytokine induction in tolerogenic-DCs by TLR ligands has not been fully characterized but evidence in the literature suggests an increase in IL-10 production associated with a decrease in proinflammatory cytokine production (144). In Figure 32 tolerogenic-DCs or BM-DCs were treated with LPS and the production of IL-10 or IL-12p40-70 quantified after 24 hours treatment.

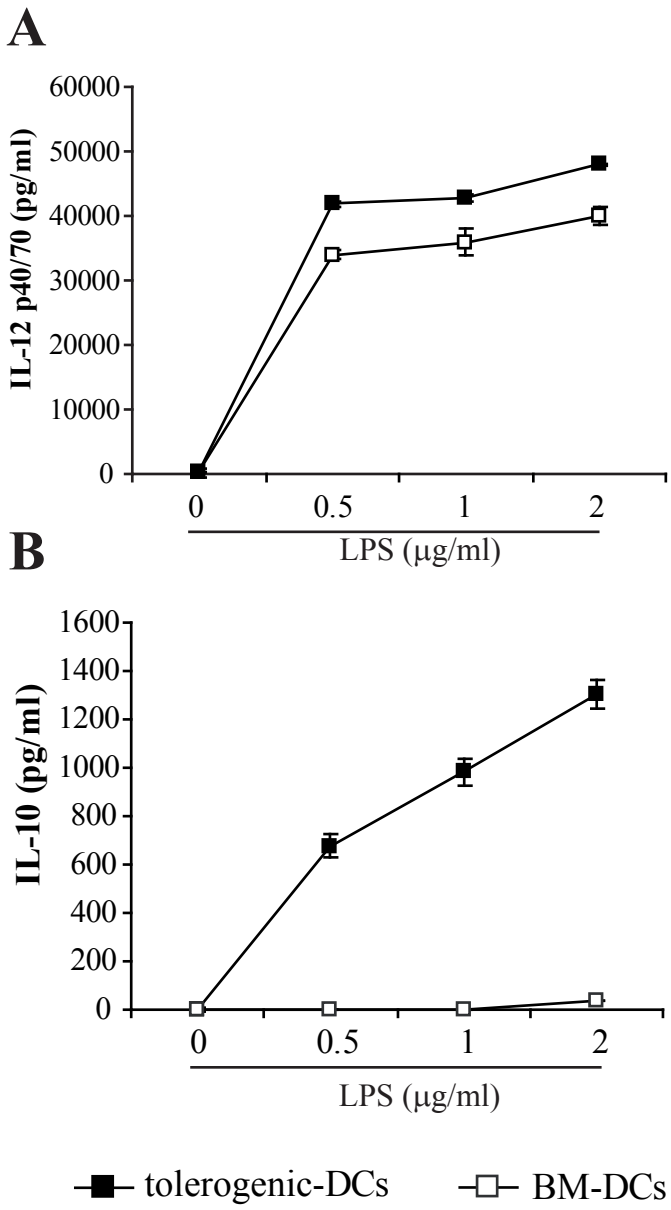


FIGURE 32. LPS stimulation of tolerogenic-DCs results in enhanced production of IL-10 BM-DCs (white squares) or tolerogenic-DCs (black squares) were cultured in the presence of increasing concentrations of LPS (0.5, 1 and 2 μg/ml). After 24 hours the supernatants were removed and IL-10 and IL-12 p40-70 quantified by ELISA

In agreement with others (165), Figure 32B shows that tolerogenic-DCs produce much higher levels of IL-10 than BM-DCs. Nonetheless, IL-12 p40-70 production by both DC subsets was similar (Figure 32A) suggesting that the capacity to produce IL-12p40-70 was not diminished in DCs that have been exposed to IL-10 during differentiation.

TLR5 expression is specifically enhanced in tolerogenic-DCs

The increased capacity to produce IL-10 by tolerogenic-DCs prompted us to investigate whether they would produce this cytokine in response to flagellin. First, we evaluated their ability to respond to flagellin stimulation by measuring TLR5 expression in BM-DCs and tolerogenic-DCs using quantitative RT PCR (Figure 33).

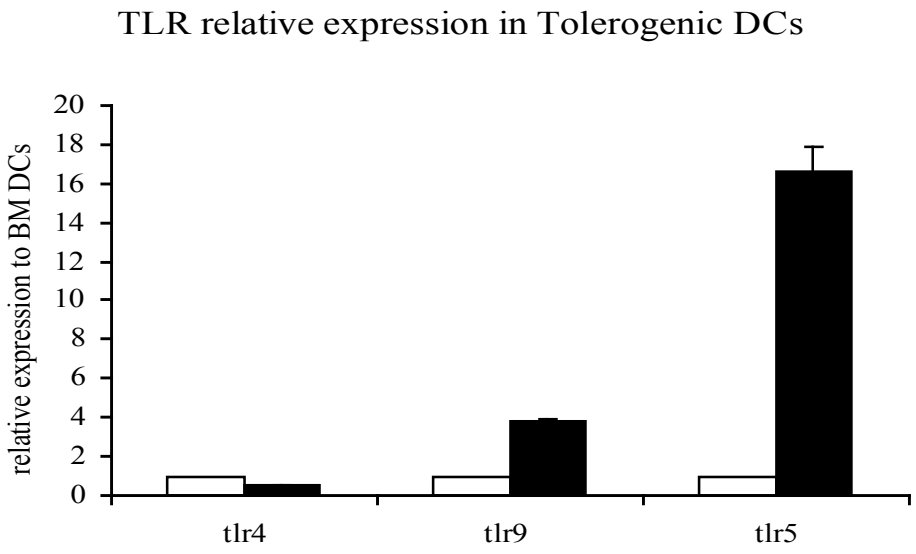


FIGURE 33. TLR5 expression is specifically enhanced in tolerogenic-DCs

BM-DCs (white bars) and tolerogenic-DCs (black bars) were obtained as detailed in the materials and methods section. After harvesting the cells, RNA was isolated using Trizol reagent and the levels of TLR5, TLR4 and TLR9 were quantified by real time RT-PCR using GAPDH as an internal control. In the graph the levels of TLR expression in tolerogenic-DCs are quantified relative to the levels of expression in BM-DCs.

Surprisingly, TLR5 expression was greatly enhanced in DCs differentiated in the presence of IL-10. To assess if increased TLR expression in tolerogenic-DCs was specific for TLR5 or also occurred with other TLRs we quantified TLR4 and TLR9 expression in both DCs subsets. As shown in Figure 33, TLR4 and TLR9 expression levels are similar between BM-DCs and tolerogenic-DCs suggesting that IL-10 signaling might specifically enhance TLR5 expression in DCs.

IL-10 treatment of BM-DCs does not result in TLR5 upregulation

DCs differentiated in the presence of IL-10 display higher levels of TLR5 expression than regular BM-DCs. To test whether a similar effect would occur when the DCs are already differentiated, we cultured BM-DCs in media alone or supplemented with IL-10. DCs were harvested 24 hours after treatment and TLR5 mRNA expression analyzed using quantitative RT-PCR (Figure 34).

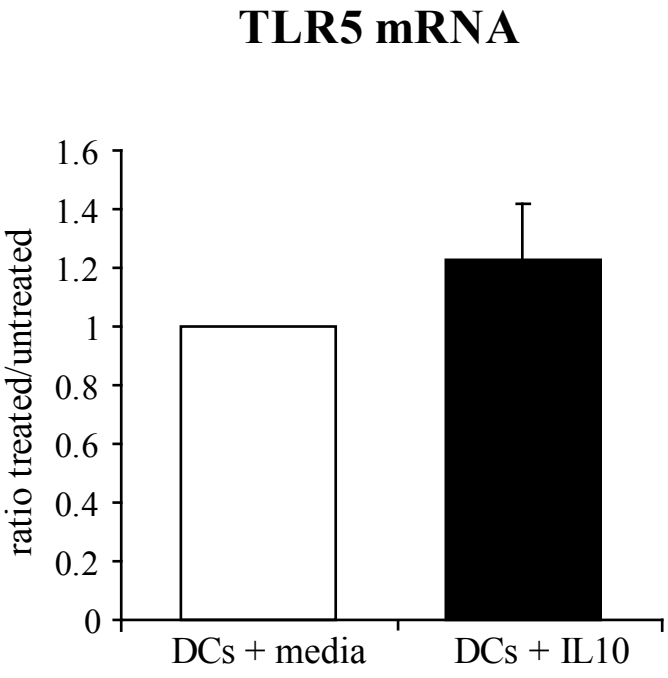


FIGURE 34. IL-10 treatment of BM-DCs does not result in TLR5 upregulation

BM-DCs were differentiated as described in the materials and methods section. DCs were harvested and cultured for 24 hours in media alone (white bar) or media plus IL-10 (10 ng/ml) (black bar). After the treatment period RNA was extracted using Trizol and TLR5 expression relative to GAPDH quantified using real-time RT PCR

TLR5 expression levels were virtually identical in DCs treated with IL-10 or those treated with media suggesting that TLR5 expression is not enhanced by IL-10 in DCs that are completely differentiated.

Flagellin stimulation of tolerogenic-DCs results in enhanced production of IL-12p40-70 in the absence of IL-10

Tolerogenic-DCs express higher levels of TLR5 mRNA than BM-DCs. In agreement flagellin-treated tolerogenic DCs produced much higher levels of IL-12p40-70 than flagellin-treated BM-DCs (Figure 35A).

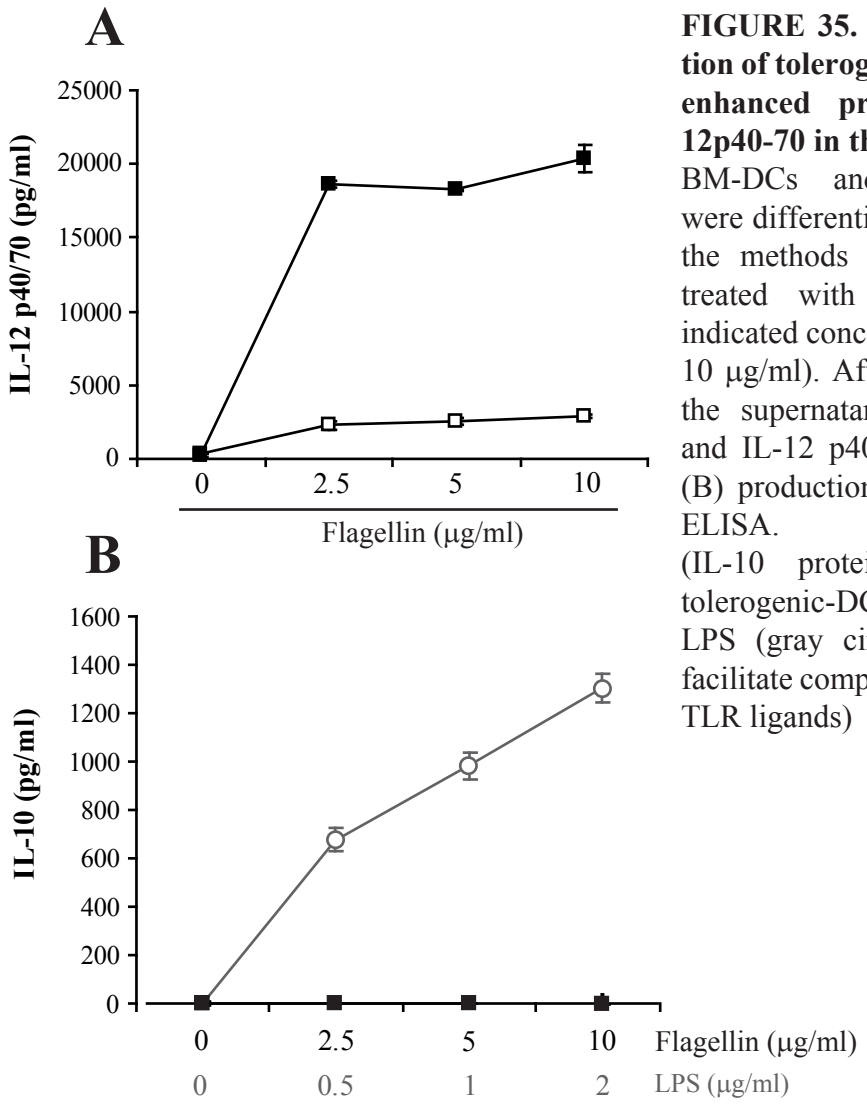
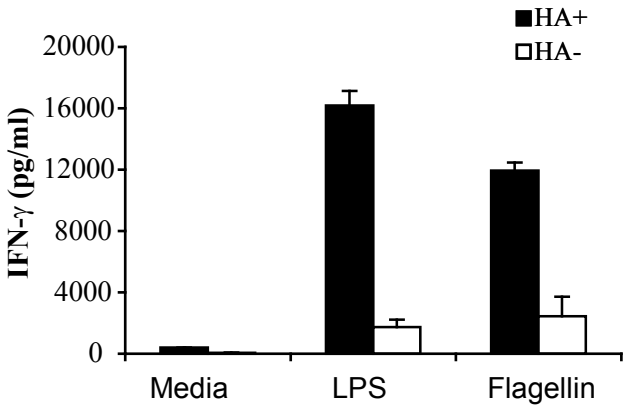


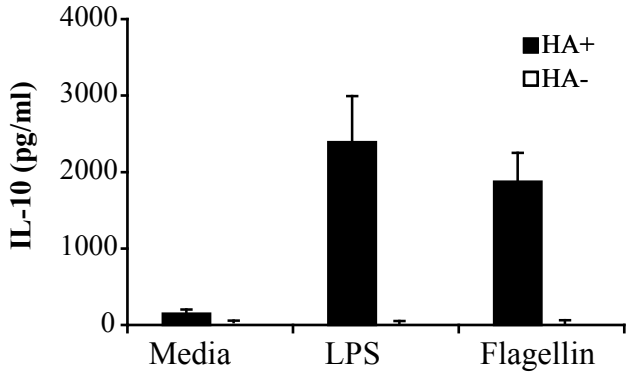
FIGURE 35. Flagellin stimulation of tolerogenic-DCs results in enhanced production of IL-12p40-70 in the absence of IL-10 BM-DCs and tolerogenic-DCs were differentiated as described in the methods section. DCs were treated with flagellin (at the indicated concentrations 2.5, 5 and 10 μg/ml). After 24 hours culture the supernatants were harvested and IL-12 p40-70 (A) and IL-10 (B) production were analyzed by ELISA.
(IL-10 protein production by tolerogenic-DCs stimulated with LPS (gray circles) is shown to facilitate comparison between both TLR ligands)

Flagellin-stimulated DCs, both BM and tolerogenic, did not produce IL-10. IL-10 production by tolerogenic-DCs plays a fundamental role in regulatory T cell differentiation and inhibition of antigen-specific T cells (38) (144). Therefore, regulatory T cell differentiation and inhibition of antigen-specific T cells should not occur when tolerogenic-DCs are activated with flagellin since there is no production of IL-10.

LPS-treated tolerogenic-DCs but not flagellin-treated tolerogenic-DCs induce T cells that produce high levels of IL-10

The differences in cytokine production encountered among BM-DCs and tolerogenic-DCs prompted us to investigate how they could affect CD4+ T cell priming and differentiation. Dendritic cell maturation and T cell priming and restimulation were carried out as detailed in Figure 30. Briefly, BM-DCs or tolerogenic-DCs were cultured for 24 hours in the presence of LPS, flagellin or just media. At this time supernatants were collected and the cells were washed before adding fresh media, antigen and naïve antigen-specific transgenic T cells. Antigen restimulation of T cells that have been differentiated in the presence of BM-DCs resulted in production of IFN-γ, IL-10 and IL-4. Effector T cells with a Th1 phenotype produce IFN-γ while IL-10 and IL-4 are produced by Th2 cells. Therefore, our data suggest that Th1 as well as Th2 cells are obtained when the APCs used are LPS- or flagellin-matured BM-DCs (Figure 36)





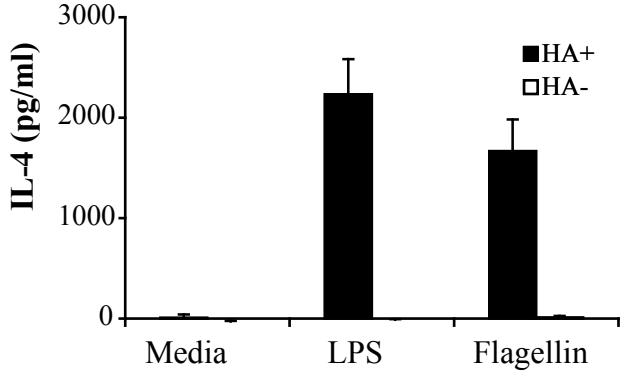


FIGURE 36. T cells cultured with BM-DCs produce Th1 and Th2 cytokines

BM-DCs were matured with LPS (1μg/ml) or flagellin (5μg/ml) and used to prime naïve CD4+ T cells as indicated in Figure 30.

Supernatants were collected after 48 hours of T cell restimulation and analyzed by ELISA to detect IFN-γ, IL-4 or IL-10 production.

Interestingly, antigen presentation by LPS-activated tolerogenic-DCs resulted in the differentiation of T cells that produce IFN-γ, very low levels of IL-4 and high levels of IL-10 (Figure 37).

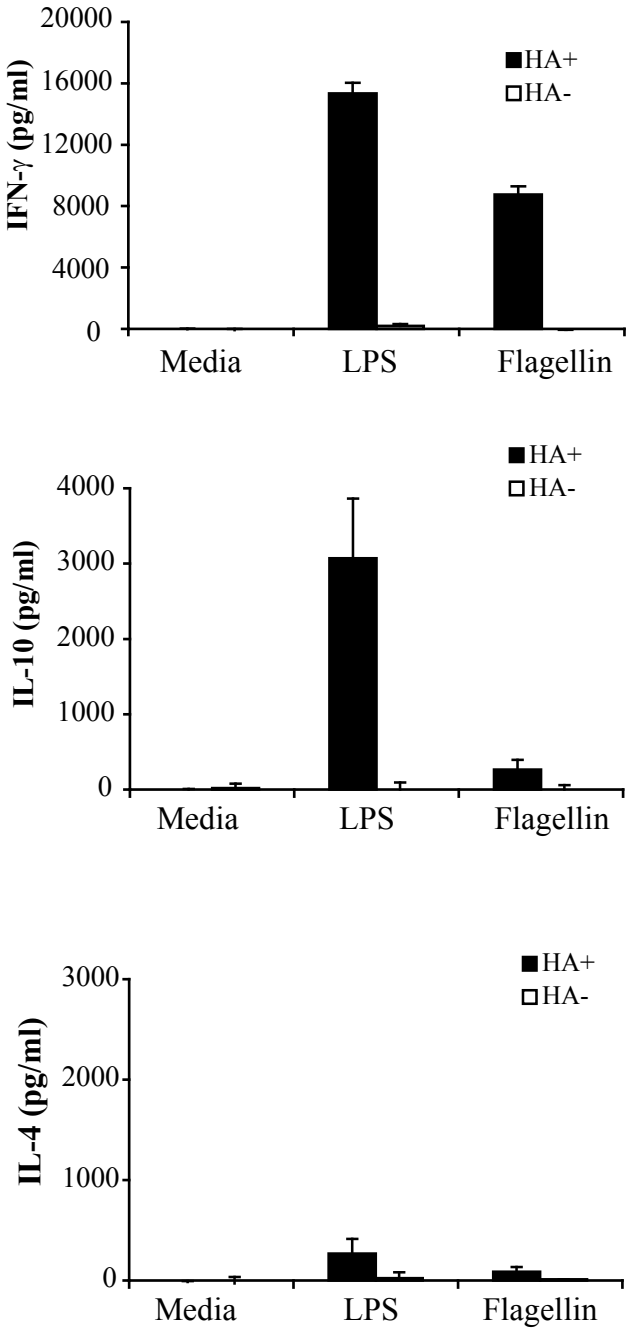


FIGURE 37. LPS-treated but not flagellin-treated tolerogenic-DCs induce T cells that produce high levels of IL-10

BM-DCs or tolerogenic-DCs were matured with LPS (1μg/ml) or flagellin (5μg/ml) and used to prime naïve CD4+ T cells as indicated in Figure 30.

Supernatants were collected after 48 hours of T cell restimulation and analyzed by ELISA to detect IFN-γ, IL-4 or IL-10 production.

On the contrary, T cells that encounter antigen in flagellin-activated tolerogenic-DCs are primed to produce IFN-γ but not IL-10 or IL-4. Taken together, our data indicates that antigen presentation by LPS-matured tolerogenic-DCs results in the differentiation of T cells with the potential to suppress the immune response as they produce high levels of IL-10. This outcome was not observed when T cells were primed with flagellin-matured tolerogenic-DCs as T cells differentiated this way seem to have a typical Th1 phenotype.

Flagellin-expressing B78-H1 cells induce IL-12p40-70 production by PEM

As shown by our data and by others flagellin might function as an adjuvant to induce inflammation in tolerogenic environments as the gut (79), lungs (83) and of important relevance for our studies in the tumor microenvironment. A good strategy to administer flagellin with a therapeutic purpose would be flagellin-expressing tumor cells. Using these cells as a vaccine would ensure a continuous release of flagellin (as long as the cells would live inside the host). Furthermore, the flagellin produced by these cells would be free of bacterial products such as LPS that are highly toxic. To explore this possibility, we constructed a flagellin-expressing cell using the murine melanoma cell line B78-H1. This cell line does not express MHC class I antigens or class II molecules (163) so it does not raise strong alloresponses against the MHC class antigens. A strong alloresponse would divert the immune system from the antigens of therapeutic interest, and it may also rapidly eliminate the flagellin-expressing cell from the host. We transfected B78-H1 cells with a construct expressing the fliC gene from *S.typhimurium* as described in the methods section. Figure 38A shows that flagellin production can be detected in fliC transfected cells using a monoclonal antibody. Furthermore lysates from

fliC expressing cells induce IL-12p40-70 production by PEM more than twice as effectively as the wild-type, untransfected cell (Figure 38B).

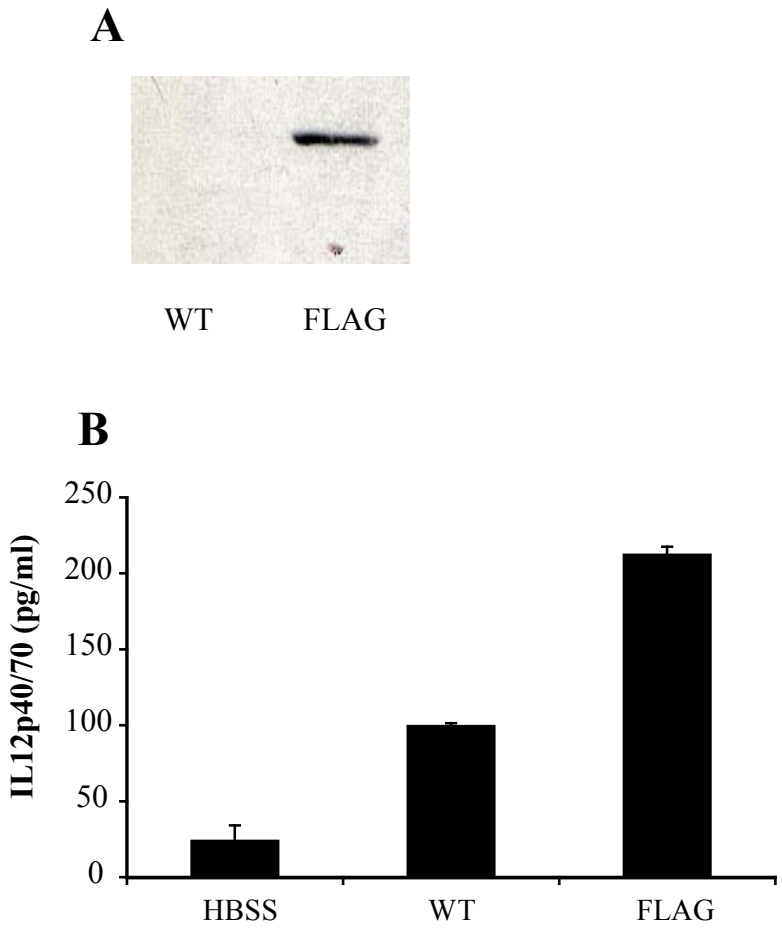


FIGURE 38. Flagellin-expressing cells stimulate production of IL-12p40-70 by PEM

(A) shows the production of flagellin by B78-H1cells transfected with a construct expressing the fliC gene from *S.typhimurium* as assessed by western blot using a monoclonal antibody against flagellin. (B) shows the production of ILp40-p70 cytokine by PEM upon 24 hours of treatment with lysates from B78-H1cells transfected (FLAG) or untransfected (WT) with the fliC expressing construct. HBSS shows the results from vehicle-treated PEMs.

These results provide the basis for the future development of a tumor-cell based vaccine to be used first in experimental models (to assess efficacy and side effects) and ultimately as an immunotherapeutic strategy to treat cancer patients.

DISCUSSION

In this study we found that flagellin, the TLR5 ligand does not induce IL-10 protein production either *in vitro* or *in vivo.* This occurs in contrast to other TLR ligands tested by us and others that induced production of this anti-inflammatory cytokine. Therefore, our results unveil a distinctive feature of flagellin relative to other TLR ligands. In addition, flagellin modifies the response elicited by specific TLR ligands by inhibiting IL-10 and/or increasing IL-12p70 production in macrophages.

Immune cells specifically macrophages and dendritic cells display a broad repertoire of TLRs. Furthermore, different TLR ligands (PAMPs) are often expressed within one microorganism. These features suggest that pathogen recognition *in vivo* involves more than one TLR and imply that the final cellular output will arise from the combination of several specific responses. Combined activation of APCs with different PAMPs has shown that specific TLRs synergize resulting in increased production of specific cytokines, mainly IL-12p70 and IL-23 (164-165). The mechanism by which stimulation with selected TLR pairs results in increase induction of certain genes remains to be established. One possibility could be that this increase is due to the activation of a novel signaling pathway/s not activated by engagement of a single TLR. Other explanation would be that more sustained signaling occurs when TLRs act in combination.

Making the portrayal of pathogen recognition even more complex certain TLRs (TLRs 1, 2, 4, 5 and 6) are present on the cell surface while others (TLRs 3, 7, 8 and 9) are restricted to intracellular compartments (47-48). Recognition of PAMPs by TLRs in different cellular compartments probably affects the dynamics of engagement of the different ligands resulting in stimulation of surface receptors earlier than intracellular receptors. In summary, detection of microorganisms by APCs probably involves activation of several TLRs that will occur simultaneously of sequentially depending on their cellular location

Little is known whether signaling through a TLR may result in the suppression of a specific cytokine or signaling pathway. To our knowledge, this work represents the first evidence that flagellin specifically suppresses IL-10 production by macrophages in response to other TLR ligands. IL-10 inhibition by flagellin does not affect all TLR ligands and seems to be rather selective. Our results show that flagellin's engagement to the membrane receptor TLR5 decreases IL-10 production in response to ligands whose receptors are also in the cellular membrane such as TLR4 or TLR2/6 (47). In contrast, flagellin does not decrease IL-10 production in response to a ligand (CpG) that binds to an intracellular receptor (TLR9) (48). These findings suggest that some degree of TLR colocalization might be needed for flagellin-mediated IL-10 suppression. Also our data showing that CpG-induced IL-10 production is dramatically reduced in macrophages treated with flagellin-coated beads as compared to those treated with control beads provide some support to this possibility.

After ligand binding, TLRs dimerize and recruit TIR-containing adaptor molecules to the TIR domain of the TLR (62). Although TLRs have been fundamentally

described to work as homodimers, the existence of TLR heterodimers has also been reported (46). TLR2 has been shown to interact with TLR1 and TLR6 to form heterodimers that play an important role in the recognition of bacterial and yeast wall components (166). Recently, TLR5 has been shown to dimerize with TLR4 in flagellin-treated macrophages (167). If TLR5 is able to form heterodimers with TLRs that co-localize with it, then specific adaptor molecules recruited to the TLR5 intracellular domain might influence their responses. However, if TLR colocalization and/or dimerization with TLR5 are required for IL-10 inhibition in response to flagellin remains undetermined.

The mechanism(s) by which flagellin inhibits IL-10 production are not elucidated yet. However, the finding that flagellin-treated macrophages display a transient and short-lived expression of IL-10 mRNA along with the decrease in IL-10 mRNA observed when macrophages are treated with flagellin and LPS suggest that the suppression occurs at the IL-10 gene transcriptional level and/or IL-10 mRNA stability. We did not find differences in the stability of IL-10mRNA in macrophages treated with LPS alone or with LPS plus flagellin, what might indicate that the mechanism of suppression operates at the transcriptional level of the IL-10 gene.

The use of specific MAPKs inhibitors and studies in mice deficient in MAPK phosphatases have shown significant alterations on the cytokine profile induced by different TLRs (168-169) indicating that MAPKs activity regulates TLR-induced cytokine production. ERK, a MAPK family member, have been shown to play an important role in the induction of IL-10 by LPS (154) and other TLR ligands (155-157) but not in the production of other cytokines such as IL-12 (158). Recent evidence

indicates that ERK might be involved in chromatin remodeling of the IL-10 promoter in macrophages in order to allow binding of transcription factors and gene expression (126). In our study we report that ERK activation by LPS is significantly impaired in flagellin-treated macrophages. These data suggests that flagellin might inhibit IL-10 production through impairing ERK activation by other TLR ligands. Nonetheless, further experiments need to be done in order to confirm the role of the ERK signaling pathway and the potential contribution of other pathways. Furthermore, LPS-induced phosphorylation of IκB was not altered in flagellin-treated macrophages suggesting that the NFκB pathway is not involved in the negative regulation of IL-10 observed in flagellin-treated macrophages. Since NFkB is a master regulator of TLR mediated cytokine induction, our data indicating that activation of this pathway by LPS is not affected in flagellin-treated macrophages supports that TLR5 engagement is not affecting the global immune response elicited by other TLRs but targeting specific pathways.

We observed that flagellin enhances the production of IL-12p70 by macrophages in response to other TLR ligands such as LPS or CpG. Given the previous demonstration (107-108) (and also in our data) that IL-10 is a powerful inhibitor of IL-12p70 production**,** one possible explanation for our findings is that flagellin enhances IL-12p70 by inhibiting IL-10. However, this assumption might not be the only explanation. Treatment of macrophages with flagellin and CpG resulted in increased IL-12p70 production, but it was not accompanied by inhibition of IL-10. Therefore, the inhibitory effect of flagellin on IL-10 and its ability to enhance IL-12p70 production might be independent from each other and mediated by different mechanisms.

Overall our findings suggest a potential scenario in which the ability of flagellin to inhibit IL-10 could facilitate the induction of inflammatory responses by flagellated bacteria. This effect would be particularly important in organs such as colon and lungs. In these organs immune responses are tightly regulated mainly by the production of IL-10 to avoid inflammation caused by commensal microorganisms (170-171) (88). Furthermore, flagellin has been shown to play a central role in inducing inflammatory responses in lungs and gut (80) (83) (172). Our data support that in the absence of flagellin, IL-10 would be produced in response to other TLR ligands such as LPS that is also expressed by flagellated bacteria. In this scenario, it is likely that the inhibitory effects of IL-10 would prevail and lead to minimum or none inflammatory response. Conversely, in the presence of flagellin and its IL-10 inhibitory effects, proinflammatory pathways (i.e. IL-12) triggered by flagellin itself or other TLR-ligands would unleash stronger inflammatory responses. Interestingly, enhanced inflammation might favor systemic dissemination of flagellated bacteria (173). This concept is supported by recent studies in mice with genetic disruption of TLR5 (79). In these mice, it was predicted that if the main effect of flagellin's recognition by TLR5 is to induce host's protective inflammatory responses then the absence of TLR5 would lead to increased susceptibility to infection by flagellated microorganisms. At odds with this prediction, TLR5 -/- mice were found to be less susceptible to systemic infection following oral challenge with S*almonella typhimurium* (79). Given our results, it is possible that in the absence of flagellin-TLR5 interaction, IL-10 would continue to be produced in response to other TLR ligands. In this case, resistance rather than susceptibility will be the outcome due to

the inability of the bacteria to induce inflammation, not as a protective mechanism but as a mechanism to facilitate its systemic dissemination.

Therefore, for the first time our studies show the effect of a TLR ligand inhibiting the production of a specific cytokine. Furthermore, the observation that flagellin specifically suppresses IL-10 production in response to selected TLR ligands provides a potential explanation to how TLR5 might shape the inflammatory responses elicited by flagellated pathogens.

Studies coming from Groux's group (144) among others have shown that differentiation of bone marrow derived DCs in the presence of IL-10 results in cells with increased ability to produce IL-10. These cells (tolerogenic-DCs) in our hands responded in a similar way producing much higher levels of IL-10 than regular BM-DCs upon LPS stimulation. As observed before *in vivo* and also in macrophages, flagellin treatment of BM-DCs or tolerogenic-DCs did not result in IL-10 protein production. Surprisingly, flagellin-treatment of tolerogenic-DCs resulted in much higher levels of IL-12 p40-70 production than treatment of BM-DCs. One possible explanation for this result could come from the differences in TLR5 expression among these DC subsets, as TLR5 mRNA was much more abundant in tolerogenic-DCs than in BM-DCs. Interestingly, TLR5 expression might be specifically enhanced in tolerogenic-DCs since a similar increase in expression was not observed in other TLRs such as TLR4 or TLR9. Based on the fact that the major difference between both subsets has been the exposure or not to IL-10, we explored the possibility that IL-10 treatment of DCs would result in increase expression of TLR5 mRNA. In our experiments treatment of differentiated BM-DCs with IL-10 did not induce any changes in TLR5 expression. This result indicate that IL-10 might

enhance TLR5 expression in non-differentiated cells but not in fully differentiated DCs or perhaps a longer exposure to IL-10 might be needed.

IL-10 production by APCs has been shown to induce differentiation of regulatory T cells that produce high levels of IL-10 and limit inflammation (38). Based on this evidence we could hypothesize that antigen presentation by flagellin-matured tolerogenic-DCs would not result in regulatory T cell differentiation. In order to verify this hypothesis we decided to differentiate naïve CD4+ T cells *in vitro* using tolerogenic-DCs treated with flagellin or LPS and analyze their responses upon restimulation. Naïve CD4+ T cells primed with flagellin or LPS-activated BM-DCs produced IFN-γ, IL-4 and IL-10. IFN-γ is a hallmark of Th1 differentiation while IL-4 in combination with IL-10 is produced by Th2 cells. The presence of these cytokines might indicate that Th1 and Th2 cells are induced by activated BM-DCs. T cells differentiated by tolerogenic-DCs showed a different phenotype when the DCs were treated with LPS or flagellin. LPS-treated tolerogenic-DCs induced T cells that produced IFN-γ, IL-10 and very low levels of IL-4. Differentiation of T cells with this same phenotype has already been described by Roncarolo's group (161) when antigen presentation occurred in the presence of IL-10. In their study in order to assess if T cells differentiated in the presence of IL-10 had a regulatory/suppressor phenotype they explored the ability of these cells to block inflammatory responses using an inflammatory bowel disease model (IBD). In this model transfer of CD45RBhigh (naïve) CD4+ T cells into SCID mice, mice that lack T and B cells, induces the development of IBD that results in colitis and weight lost. Cotransfer of IL-10 producing T cells together with CD45RB high CD4+T cells prevented colitis development and weight lost indicating that T cells differentiated in the presence of IL-10

display regulatory properties. Due to their regulatory properties IL-10 producing T cells are commonly denominated IL-10 secreting type 1 regulatory T cells (Tr1) (38). Tr1 differentiation might be abrogated when flagellin-treated tolerogenic-DCs act as APCs since T cells differentiated in these conditions did not produce IL-10 upon restimulation. T cells differentiated with flagellin-treated tolerogenic-DCs produced IFN-γ but very low levels of IL-4 and IL-10 suggesting that the dominant response elicited has a Th1 character.

In summary, our results might indicate that antigen presentation by tolerogenic-DCs activated with LPS results in naïve T cell differentiation into Tr1 cells meanwhile flagellin-treated tolerogenic-DCs induce Th1 differentiation. Tr1 differentiation has been shown to be dependent on IL-10 (174) that seems to act on the APC and not directly over the T cell (119-121) (175). This evidence suggests that the differences in T cell differentiation between flagellin-treated and LPS-treated tolerogenic-DCs might be mainly due to the production or not of IL-10 by the APC. Our data supports that the use of flagellin or other TLR5 agonists as adjuvant in situations associated with the presence of high levels of IL-10 would be specially indicated. In contrast the use of other TLR ligands could result in enhanced suppression of immune responses through the expansion of T cells with a regulatory phenotype.

IL-10 production can be detected at steady conditions in gut and lungs (170) (176) where it plays a fundamental role in the prevention of anti-microbial responses (135). Interestingly, analysis of the TLR5 expression levels in different murine tissues have found the highest expression in the gut (79) an evidence that supports our data suggesting that exposure to IL-10 increases TLR5 expression.

IL-10 signaling in the mucosa is likely to affect DCs in a similar way as seen in our *in vitro* experiments. In fact DCs from the gut but not from the spleen produce high levels of IL-10 upon stimulation with CD40 and microbial products (88). A similar phenomenon might also be occurring in the lungs, since pulmonary DCs produce IL-10 and have been shown to induce Tr1 development and antigen specific T cell tolerance (171).

Furthermore, IL-10 has been also detected in many tumors produced by tumor cells or immune cells recruited to the tumor site (121). The presence of IL-10 in the tumor environment has been linked with the generation of regulatory T cells (38). Furthermore, in a recent study exploring the phenotype of tumor infiltrating DCs (TIDC) using different carcinoma models (mammary, liver lung and colon) these cells were characterized as having low expression levels of costimulatory molecules (CD86 and CD40) and hyporesponsiveness to maturating stimuli. Interestingly, in order to induce maturation of TIDC using CpG (TLR9 ligand) *in vivo,* CpG should be administered along with IL-10 blocking antibodies bringing even more evidence of the suppressive role of IL-10 on TIDCs (177). This evidence points towards a similar phenotype between TIDCs and tolerogenic-DCs as IL-10 might be acting as the shaping agent in both situations.

A question that remains to be answered in order to use TLR5 agonists to activate TIDCs would be if the degree of differentiation at which TIDCs are exposed to IL-10 could make a difference in their ability to express TLR5. Our data suggest that IL-10 might enhance TLR5 expression in DCs going through the differentiation proccess but not in those that are fully differentiated. Nonetheless, DC precursors circulate in the

blood and they have been shown to be recruited to the tumor environment (178) where exposure to IL-10 might result in increase of TLR5 expression .

In summary, our study supports that stimulation of TLR5 might play an important role in counteracting the anti-inflammatory effects of IL-10. TLR5 engagement limits the production of IL-10 by innate immune cells. Furthermore it abrogates the differentiation of IL-10 producing T cells that otherwise dominate the immune response in IL-10 rich environments. Further studies will explore the flagellin-based vaccine we have created in our laboratory. The finding that flagellin can prime T cell responses in tolerogenic situations supports the use of this novel strategy to overcome tolerance to tumor antigens, a barrier that we need to remove if we want to effectively harness antitumor immune responses

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