TLR5 Signaling Stimulates the Innate Production of IL-17 and IL-22 by CD3 neg CD127+ Immune Cells in Spleen and Mucosa

Laurye Van Maele, Christophe Carnoy, Delphine Cayet, Pascal Songhet, Laure Dumoutier, Isabel Ferrero, Laure Janot, François Erard, Julie Bertout, Hélène Leger, Florent Sebbane, Arndt Benecke, Jean-Christophe Renauld, Wolf-Dietrich Hardt, Bernhard Ryffel and Jean-Claude Sirard

J Immunol 2010; 185:1177-1185; Prepublished online 21 June 2010;
doi: 10.4049/jimmunol.1000115
http://www.jimmunol.org/content/185/2/1177

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/06/21/jimmunol.1000115.DC1

References
This article cites 51 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/185/2/1177.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
TLR5 Signaling Stimulates the Innate Production of IL-17 and IL-22 by CD3\(^\text{neg}\)CD127\(^+\) Immune Cells in Spleen and Mucosa

Laurye Van Maele,*†‡§ˌ,1 Christophe Carnoy,*†‡§ˌ,1 Delphine Cayet,*†‡§ˌ,1
Pascal Songhet,‖ Laure Dumoutier,# Isabel Ferrero,** Laure Janot,††,‡‡
François Erard,††,‡‡ Julie Bertout,¶ Hélène Leger,§§,§ˌ††† Florent Sebbane,*†‡§ˌ,
Arndt Benecke,§§,§ˌ††† Jean-Christophe Renauld,# Wolf-Dietrich Hardt,‖
Bernhard Ryffel,††,‡‡ and Jean-Claude Sirard*†,‡§ˌ.

In adaptive immunity, Th17 lymphocytes produce the IL-17 and IL-22 cytokines that stimulate mucosal antimicrobial defenses and tissue repair. In this study, we observed that the TLR5 agonist flagellin induced swift and transient transcription of genes encoding IL-17 and IL-22 in lymphoid, gut, and lung tissues. This innate response also temporarily enhanced the expression of genes associated with the antimicrobial Th17 signature. The source of the Th17-related cytokines was identified as novel populations of CD3\(^\text{neg}\)CD127\(^+\) immune cells among which CD4\(^+\)expressing cells resembling lymphoid tissue inducer cells. We also demonstrated that dendritic cells are essential for expression of Th17-related cytokines and so for stimulation of innate cells. These data define that TLR5-induced activation of CD3\(^{\text{neg}}\)CD127\(^{+}\) cells and production of Th17-related cytokines may be crucial for the early defenses against pathogen invasion of host tissues.


Toll-like receptors are key players in innate immunity and are essential for sensing microbial components and triggering the host defense (1). At the luminal interface, the TLR response is mediated by the epithelium and mainly consists of neutrophil recruitment and activation (2). After microbes cross the epithelium, sensing occurs within the lamina propria. However, the nature of the TLR-mediated innate cells and defense factors that are triggered by microbial desequstration has yet to be defined.

Recent studies highlighted the contribution of IL-17A, IL-17F, and IL-22 to defensive reactions within the mucosa (3–6). These cytokines help orchestrate innate immunity by stimulating epithelial cells to produce defense molecules, matrix proteases, and tissue repair molecules (7, 8). The source of IL-17A, IL-17F, and IL-22 varies. During an adaptive response, the lymphocytes that differentiate into Th17 cells are the main producers of cytokines (9). IL-17A can rapidly be produced during innate responses to bacteria or microbial molecular patterns by γδ T lymphocytes in a TLR4-dependent manner, NKT cells activated with α-galactosylceramide, or lymphoid tissue inducer (LTI)-like cells following stimulation with the TLR2/Dectin-1 agonist zymosan (10–12). NK-like and LTI-like innate lymphocytes expressing IL-7Rα, Nkp46, the transcription factor RORγt, and eventually CCR6 are sources of IL-22 and/or IL-17 in mucosa under steady-state conditions (13–18). Interestingly, microbial flora-colonizing mucosa are required to switch on lasting IL-17 and IL-22 production (15, 17). In absence of these innate lymphocytes, infectious colitis is exacerbated, suggesting an operational role of IL-22 and IL-17 in the gut’s innate immunity (15, 17). However, the link between TLR-mediated signaling, Th17-related cytokine production by innate immune cells, and mucosal defenses has not been defined.

The ability of TLR5 signaling to induce mucosal production of IL-17 and IL-22 and thereby promote antimicrobial defense has never been investigated. TLR5 detects flagellins—the main protein of bacterial flagella (19). Flagellins are expressed by bacteria, particularly pathogenic bacteria, in the gut and the lung and activate epithelial TLR5 signaling (19–21). Flagellin expression is switched off as soon as bacteria translocate into the lamina propria (22). Detection of flagellin molecules represents therefore an alarm signal for subepithelial invasion and/or disruption of the epithelial barrier function. TLR5 signaling is rapidly induced in the lamina propria dendritic cells (DCs) of the small intestine (23). In the current study, we show that flagellin activates (via DCs) the splenic and mucosal production of IL-17 and IL-22 and the subsequent expression of target genes. This TLR5-mediated response was associated with a unique population of immune cells express-
ing CD127 but not CD3 that resembles LTi cells, LTi-like, or NK-like innate lymphocytes. Our findings suggest that CD3<sup>−</sup>CD127<sup>+</sup> innate immune cells may be instrumental to the host’s mucosal defense through the early production of Th17-related cytokines.

**Materials and Methods**

**Mice**

Specific pathogen-free mouse strains C57BL/6J, C57BL/6J-Ly5.1, BALB/c, and Tcrb<sup>−/−</sup>, Tert<sup>−/−</sup>, Tcrb<sup>−/−</sup>/Tert<sup>−/−</sup>, Th5<sup>−/−</sup> (24); Mydb88<sup>−/−</sup> (25); transgenic animals for pre-TCRs, Cd11c-<sup>−/−</sup>-EGFP (Itons-<sup>−/−</sup>-EGFP) (26); Rag2<sup>−/−</sup>Il2g<sup>−/−</sup> backcrossed on C57BL/6J mice; Cd1d<sup>−/−</sup> backcrossed on BALB/c background; and C.B-17 scid (SCID) mice were purchased from Charles River Laboratories (Wilmington, MA), The Jackson Laboratory (Bar Harbor, ME), or Janvier Laboratories (St. Berthevin, France) or bred in an accredited establishment (number A59107; Institut Pasteur de Lille, Lille, France; Transgeno Institute Centre National de la Recherche Scientifique, Orleans, France; RCHCI at Eidgenössische Technische Hochschule Zurich, Zurich, Switzerland; Ludwig Institute for Cancer Research, Brussels, Belgium; and Ludwig Institute for Cancer Research, Lausanne, Switzerland). Animals (6–16 wk old) were used according to national regulations and ethical guidelines.

For bone marrow (BM) chimera, recipient mice were irradiated (1000–1500 rad) and reconstituted 2–24 h later with BM cells (4–20 × 10<sup>6</sup> cells i.v.). These mice were used at 10–16 wk posttransplantation, and the degree of chimerism was assessed by measuring CD45.1 and CD45.2 surface expression by leukocytes. The current protocol yielded 96.7% reconstitution for Cd11c-<sup>−/−</sup>/EGFP→C57BL/6 (wild-type [WT]) in spleen and 98.2% for Th5<sup>−/−</sup>→WT and 97.2% for WT→Th5<sup>−/−</sup> in lung. Depletion of Cd11c<sup>−/−</sup> cells was achieved by injecting i.p. diphtheria toxin (DTX) as described previously (26). Depletion of Gr1<sup>−/−</sup> T cells (~90% depletion) and NK cells (~72% depletion) was performed by injecting i.p., 24 h prior to flagellin treatment, 100 μg mAb specific for TCRβ-chain (GL3 clone) or NK1.1 (PK136) or irrelevant mAb HB152 as control.

**Flagellin administration**

LPS-depleted flagellin Flic from Salmonella typhimurium (5 μg) produced as previously described (21), ultrapure LPS from Escherichia coli (serotype 0111:B4, 5 μg; InvivoGen, Toulouse, France), or phosphorothioate CpG oligonucleotide (5′-TCCATGACCTGGTCGTGCT-3′, 5 μg; Eurogentec, Angers, France) diluted in PBS were injected i.v. or i.p to mice.

**Flow cytometry and sorting**

Spleens were digested with collagenase D (0.5 mg/ml; Roche, Basel, Switzerland) and DNase I (40 μg/ml; Sigma-Aldrich, St. Louis, MO) during 10 min at 37°C. Cells were stained for CD127-FITC, CD45.1-FITC, CD45.2-PE, PE-conjugated lineage-specific Ab (CD3, B220, Gr1, CD11b, and CD11c), MHCIi-PE, NK1.1-PerC-Perc5.5, CD4-APC, CD11c-APC, and CD45.1-Pacific Blue (BD Biosciences, San Jose, CA; BioLegend, San Diego, CA; and eBioscience, San Diego, CA) and sorted on a BD FACS Aria.

**DC culture**

DCs were differentiated from BM as described previously (25). On day 7 or 11, BMDCs were stimulated for 2 h and analyzed.

**Determination of cytokine production**

CCL20 and IL-22 (R&D Systems, Minneapolis, MN) and IL-17A (Bio-Science) levels were measured by ELISA in serum, and tissue homogenates were prepared with T-PER Reagent (Pierce, Rockford, IL) supplemented with protease inhibitors (Roche).

**Gene expression**

Total RNA was extracted with the Nucleospin RNA II kit (Macherey-Nagel, Hoerd, France) and reverse transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). A preamplification of cDNA from sorted cells was performed prior to real-time PCR using the PreAmp kit (Applied Biosystems). cDNA was amplified using SYBR Green-based real-time PCR (Supplemental Table I) or commercial TaqMan assays (Applied Biosystems). For high-throughput analysis, TaqMan Low Density Arrays (Applied Biosystems) were used. Analysis was carried out using Real-Time-SoftWin (inventory tool) (Granada, Spain), Real-time PCR (ΔΔCt) or tive mRNA levels (2<sup>−ΔΔCt</sup>) were determined by comparing 1) the cycle thresholds (Ct) for the gene of interest and Actb (ΔCt) and 2) ΔCt values for treated and control groups (ΔΔCt). Ct upper limit was fixed to 33 cycles.

**Microarrays**

Total RNA (2 μg) was processed on the Mouse Whole Genome Arrays version 2.0 (Applied Biosystems) (27). Data were analyzed using the NEONORM method, and heat maps were created as described previously (27, 28). Gene Ontology was analyzed using the Panther Protein Classification System (www.pantherdb.org).

**Statistical analysis**

The Mann-Whitney U test and the Graphpad Prism software 5.0 were used in analyses. The Limma test with Benjamini-Hochberg false discovery rate (FDR) correction was used for high-throughput PCR with TaqMan Low Density Arrays. Results were considered significant for p < 0.05 indicated by an asterisk. Results are expressed as arithmetic means ± SD.

**Results**

**Systemic TLR5 signaling enhances Il17a, Il17f, and Il22 gene expression in lymphoid tissues**

To establish whether TLR stimulation promotes the rapid expression of the Th17-related cytokines, mice were treated i.p. or i.v. with a TLR4 agonist (LPS), a TLR5 agonist (flagellin), or a TLR9 agonist (CpG). Gene expression in spleen and lymph nodes was then monitored (Fig. 1A, 1B). Flagellin administration triggered within 2 h~1000-fold increase of Il22 mRNA levels. Similarly, Il17a and Il17f gene expression was upregulated. A TLR5-mediated, Th17-related innate response was also observed in the mediastinal and inguinal lymph nodes and, to a lesser extent, in the liver (Fig. 1, Supplemental Fig. 1). LPS was initially shown to promote Il22 expression in many tissues (29). We found that LPS also enhanced the Th17-related innate response but to a lesser extent than flagellin did (Fig. 1A, 1B, Supplemental Fig. 1). Although TLR9-mediated signaling activated the response in lymph nodes, it was devoid of any effect in the spleen.

The flagellin-dependent response was transient and peaked at 2 h; mRNA levels returned to baseline levels after 24 h (Fig. 1C, 1D). Gene profiling showed that the expression of genes specific for TLR-, IL-17R-, and IL-22R–mediated signaling was significantly enhanced in spleen (Fig. 1E). These genes encode pleiotropic and Th17-promoting cytokines (TNF-α, IL-1β, and IL-6), chemokines that are specific for neutrophils, monocytes, and lymphocytes (CCL1−1, −2, −5, −9, and −10), antimicrobial molecules like CAMP and HAMP, lipocalin 2, S100A9, and tissue remodeling proteases matrix metalloproteinases 3 and 13. Strikingly, the expression of chemokine CCL20, which is specific for the recruitment of DC precursors, Th17 lymphocytes, LTi-like or NK-like cells (12, 14, 30, 31), was significantly upregulated. The transcription of the IFN-γ encoding gene was also upregulated by flagellin treatment. However, we did not observe any change in the expression of genes coding for IL-21, RORγt, or TGF-β (i.e., other factors involved in Th17 differentiation). As shown in Fig. 1F, IL-22 levels rose significantly in serum and spleen from flagellin-treated animals, whereas IL-17A was hardly detectable in serum but rose 3-fold in the spleen. In conclusion, TLR5 signaling in lymphoid tissues promotes the rapid production of the innate cytokines IL-17A, IL-17F, and IL-22—a pattern that resembles a Th17-related innate response.

**TLR5-mediated innate immune responses require common γ-chain–dependent immune cells**

TLR5 is expressed by monocyte/macrophage/DC lineages, NK cells, CD4<sup>+</sup> lymphocytes, and radioreistant stromal cells but not B lymphocytes (25, 32–35). To define the cells involved in the early production of IL-17 and IL-22, we used BM chimera expressing or not TLR5 and tested their ability to respond to flagellin. As shown in Fig. 2A and 2E, TLR5-competent hematopoietic cells were required to trigger flagellin-mediated Il17 and Il22 gene expression. By using SCID, pre-TCRa animals harboring enhanced number of γδ
lymphocytes, Tcrb<sup>-/-</sup> and Tcdn<sup>-/-</sup> mice, we demonstrated that neither B cells nor TCR<sub>ab</sub>-/TCR<sub>gd</sub>-expressing T lymphocytes were required for flagellin-mediated Il17 and Il22 expression (Fig. 2B, Supplemental Fig. 2A, 2B, 2D). In contrast, the response was impaired in Rag2<sup>-/-</sup>Il2rg<sup>-/-</sup> mice that have almost normal DCs but lack B and T lymphocytes as well as NK, NKT, LTi, NK-like, and LTi-like cells, all of which depend on the IL receptor common \( \gamma \)c-encoded by Il2rg gene (Fig. 2C, 2E) (12, 15, 17, 36). The impairment was not a collapse of TLR5 signaling, because the liver was still responsive to flagellin (Fig. 2D). Our experiments using genetically deficient animals and depleting Abs suggested that CD1d-restricted NKT cells or NK cells were not drivers of the TLR5-mediated response (Supplemental Fig. 2C, 2D). Therefore, our data showed that the cells expressing Th17-related cytokines after flagellin stimulation require the IL receptor \( \gamma \)c for differentiation or activation but are not NK, NKT, or TCR<sub>gd</sub>-expressing innate lymphocytes.

We next sought to determine which \( \gamma \)c-dependent innate immune cells are involved in TLR5-mediated response. Recent work has suggested that innate lymphocytes expressing IL-7R (i.e., the CD127 or IL-7R\( \alpha \)–chain and the CD132 or \( \gamma \)c) and LTi/NK cell markers are sources of IL-22 and IL-17 (12–17). To determine whether innate immune cells produce Th17-related cytokines, splenic cells from mock- and flagellin-treated animals were sorted on the basis of lineage markers (CD11b, CD11c, Gr1, CD3, and B220), NK.1, CD4, and CD127 (Fig. 3A). After TLR5 stimulation, Lin<sup>−/−</sup>NK.1.1<sup>−/−</sup>CD127<sup>−/−</sup>CD4<sup>−/−</sup> and Lin<sup>−/−</sup>NK.1.1<sup>−/−</sup>CD127<sup>−/−</sup>CD4<sup>-/−</sup> were sorted.
express Ccr6, but we were unable to detect any mRNA for Rorc or Ncr1 encoding RORγt and NKP46. In addition, we observed that, upon flagellin stimulation, Ifng expression was mainly upregulated in NK cells rather than LinnegCD127+ cells (data not shown). Our data provide evidence that LinnegCD127+ innate immune cells, and especially the CD4+ fraction, which resembles LTI cells, can rapidly produce IL-17 and IL-22 cytokines following TLR activation.

**TLR5-mediated activation of Th17-related innate responses requires DCs**

To determine whether the TLR5-mediated upregulation of Th17-related innate response is a direct innate lymphocyte activation process or requires DC stimulation, DTX-mediated ablation of CD11c+ cells was performed in a Cd11c-DTR-EGFP BM chimera. The DTX treatment depleted 93.8 ± 2.2% of CD11c+MHCIIhigh DCs (Fig. 4A). In addition, we found that DTX treatment did not eliminate the CD3negCD4+CD127+ cells in the spleen of a Cd11c-DTR-EGFP chimera and did not alter the TLR5-mediated production of IL-22 (Supplemental Fig. 3). As shown in Fig. 4B, systemic administration of flagellin to DC-depleted animals resulted in impaired Il17f or Il22 transcription, compared with controls. Thus, our experiments demonstrated that DCs are necessary for TLR5-mediated expression of Th17-related cytokines.
Th17 differentiation depends on tissue-derived TGF-β and IL-1β, and IL-6 produced by DCs and the maintenance of Th17 phenotype have been associated to DC-derived IL-23 (9). In vivo, DC cell ablation was found to attenuate the upregulation of Il6 transcription in response to flagellin (Fig. 4C). We did not observe any alteration in Il1b, Il23b coding for the p40 chain of IL-12 or IL-23, and Tgfb gene expression (data not shown). Intriguingly, Il22a (coding for the p19 chain of IL-23) transcription was enhanced by DTx treatment; it is possible that CD11c<sup>hi</sup>MHCII<sup>hi</sup> cells having infiltrated the spleen in DTx-treated mice support Il22a upregulation (Fig. 4A, 4C). In this study, we found that flagellin promoted the expression of both Il12b and Il23a in BM-derived DCs (Fig. 4D). In response to flagellin, DCs can therefore produce both IL-6 and IL-23, with the subsequent expression of an innate, Th17-related signature. However, in vivo, IL-23 alone appears unable to induce gene activation. In response to flagellin, DC-mediated IL-6 production could therefore promote the Th17-related signature by Lin<sup>−</sup>CD127<sup>+</sup> cells.

Previous studies suggested that DCs produce IL-22 (4, 38). In this study, we found that flagellin promoted the expression of Il12b in BM-derived DCs but did not have any effect on Il17a, Il17f, and Il22 transcription (Fig. 4D). LPS, which strongly increased Il12b and Il23a transcription, did not upregulate expression of Il17a and Il17f in DCs but did enhance Il22 mRNA levels ~8-fold, compared with untreated cells. Analysis of transcription in splenic DCs sorted from mice treated with flagellin also showed that DCs were not potent source of IL-22 (Fig. 3D, 3E). Taken as a whole, our data suggested that DCs are not a major source of TLR5-mediated, Th17-related cytokine production but contribute to induction of the latter.

**TLR5 signaling triggers an intestinal, Th17-related, innate response**

Because Th17-related cytokines are important in the control of inflammation and infection in the mucosa (3–6, 38, 39), we next assessed the impact of flagellin administration on intestinal tissues. Flagellin strongly enhanced the production of IL-22 within 2 h of administration from the duodenum to the proximal colon; the level then returned to the baseline at 8 h (Fig. 5). CCL20 synthesis was assessed the impact of flagellin administration on intestinal tissues. Flagellin strongly enhanced the production of IL-22 within 2 h of administration from the duodenum to the proximal colon; the level then returned to the baseline at 8 h (Fig. 5). CCL20 synthesis was successfully modulated by LinnegCD127+ cells in a DC-mediated manner. The second class was involved in the positive or negative transcriptional control of IL-17R (i.e., C/EBPβ and C/EBPδ), IL-22R (i.e., SOCS-1, SOCS-3, and STAT5A) and TLR (i.e., activating transcription factor 3 and I-kBα) signaling. The third group includes TLR, IL-17R, and IL-22R signaling target genes coding for antimicrobial molecules or factors regulating epithelium barrier function. Interestingly, our analysis also identified antiviral genes. In contrast to previous studies of Th17 cytokine-mediated signaling, we did not find any transcriptional modulation of the mucin and β-defensin genes. The fourth group corresponds to genes encoding factors involved in recruitment, development, or function of various immune cells. Relative to controls, chemokines specific for innate immune cells like neutrophils, NK, NKT, or LTI cells, T and B lymphocytes, or monocytes were significantly enriched—suggesting that several cell types may enter the tissues and participate in the immune response. The last group includes genes required for TLR4 or TLR2 signaling (i.e., LBP, CD14, and MAL). In conclusion, intestinal gene expression profiling showed that flagellin promotes a transient immune response involving regulators and effectors of both TLR- and Th17-mediated immunity.

**Discussion**

The way in which TLR signaling activates the host’s innate defenses during mucosal invasion by pathogens is subject to debate. In the current study, we showed that TLR5 signaling induces systemic and mucosal innate expression of the Th17-related IL-17 and IL-22 cytokines by stimulating Lin<sup>−</sup>CD127<sup>+</sup> cells in a DC-dependent manner. Overall, our data suggest that Lin<sup>−</sup>CD127<sup>+</sup> cells may play a major role as innate lymphocytes in the early orchestration of a TLR-dependent, protective response to mucosal invasion by pathogens.

Th17-related cytokines contribute to adaptive immunity in response to various inflammatory and infectious diseases (5, 6, 30, 39); however, their impact on the early phase of infection is poorly understood. The effect of TLR signaling on IL-17 and IL-22 was previously suggested because their synthesis was enhanced after administration of TLR2 and TLR4 agonists (12, 29). Very recently, TLR5 signaling was also associated to such response (40). In this study, we analyzed the immune response to the TLR5 activator
flagellin, with a focus on mucosa. Our rationale was that because flagellin expression is specifically restricted to luminal compartment, its desequestration is likely to be an alarm signal for mucosal invasion (22). We found that systemic flagellin administration promotes the swift, intense, transient production of IL-17A, IL-17F, and IL-22 and factors controlling Th17 differentiation. For example, flagellin modulates expression of the genes coding for the aryl hydrocarbon receptor repressor, the aryl hydrocarbon receptor nuclear translocator-like factor ARNTL and the activating transcription factor-like factor BATF—all of which are involved in Th17 differentiation (41, 42). Moreover, the flagellin-induced innate response and the Th17 adaptive response share many effectors, such as chemokines, antimicrobial peptides, antiapoptotic factors, and tissue remodeling factors. Therefore, we hypothesized that flagellin-mediated response enables the rapid and transient recruitment of systemic and mucosal defenses.

Our results suggest that the Lin<sup>neg</sup>CD127<sup>+</sup> cells, especially the CD4<sup>+</sup> fraction, have a pivotal role in the TLR-mediated response via the production of IL-17 and IL-22. These cells are similar to LTi (CD3<sup>neg</sup>CD127<sup>+</sup>CD4<sup>+</sup>), LTi-like (ROR<sub>γt</sub><sup>neg</sup>Lin<sup>neg</sup>CD127<sup>+</sup>CD4<sup>+</sup>), and NK-like (ROR<sub>γt</sub>NKp46<sup>Lin<sup>neg</sup></sup>CD127<sup>+</sup>NK1.1<sup>neg</sup>) cells (12, 14–17). LTi-like/NK-like cells constitutively produce Th17-related cytokines in a process that depends on gut flora, γ<sub>c</sub>, and ROR<sub>γt</sub> (15, 17). Moreover, the LTi-like cells were shown to upregulate the production of IL-17 and IL-22 in response to microbial products. The development of Lin<sup>neg</sup>CD127<sup>+</sup>CD4<sup>+</sup> and Lin<sup>neg</sup>CD127<sup>+</sup>CD4<sup>neg</sup> cells identified in this study requires the γ<sub>c</sub>. These subsets also express the CCR6 but not the NKp46 encoding gene, suggesting a common ontogeny with LTi and LTi-like cells (12, 13). We were unable to detect any expression of ROR<sub>γt</sub> in the Lin<sup>neg</sup>CD127<sup>+</sup> cells; expression below our assay’s detection threshold is one possible explanation for this failure. Furthermore, Rorc expression was downregulated in gut after flagellin injection. Interestingly, we noted enhanced intestinal expression of the gene encoding NFIL3—a factor that is essential for NK cell development (43). Recent studies demonstrated that increased expression of IL-7 enhances the number of LTi cells (37) and that deficiency in IL-7 affects the number of NK-like IL-22 expressing population (44). Additional work will be needed to define the ontogeny and transcriptional factors involved in the differentiation of Lin<sup>neg</sup>CD127<sup>+</sup> cells.

DCs have an important role in integrating microbial signals and activating immune cells like Th17 lymphocytes (9). When DCs were depleted, the Th17-related innate response to flagellin was impaired, indicating that DCs are necessary for the activation of Lin<sup>neg</sup>CD127<sup>+</sup> cells. Similarly, transcription of Il6 was attenuated, which suggests that, in Th17 differentiation, DC-derived IL-6 like may contribute to Lin<sup>neg</sup>CD127<sup>+</sup> cell activation (9). Our findings suggested that IL-1β, IL-23, or TGF-β (or at least the amounts produced by DCs) are not required for Lin<sup>neg</sup>CD127<sup>+</sup> cell activation. However, IL-23 might be important for an alternative activating pathway for production of IL-17 or IL-22 by LTi-like and NK-like cells because 1) cells are activated in vitro by supplementing the culture medium with IL-23 and 2) CD3<sup>neg</sup>CD4<sup>+</sup> WT distal ileum. D, Contribution of TLR5 signaling in the hematopoietic compartment is essential for the Th17-related signature. mRNA levels 2 h after flagellin treatment are expressed as WT→Thr5<sup>−/−</sup>→Thr5<sup>+</sup><sup>−/−</sup> or WT mice. E, Contribution of the γ<sub>c</sub> to intestinal response. mRNA levels are expressed relative to PBS-treated WT mice. In A, B, and D, all data displayed statistically significant changes, except where denoted as “ns” for “nonsignificant.” Results are given as means ± SD. Mann-Whitney U test relative to PBS group was used in A and B. Limma test was used in C–E.
The contribution of IL-22 was recently suggested as instrumental against inflammatory colitis and gut infections (40, 48). The relevance of TLR5 signaling in defense has recently been assessed. Flagellin-mediated protection of rodents and nonhuman primates against lethal irradiation was associated with CSF3-mediated granulopoiesis and the antiapoptotic effect of superoxide dismutase 2 (47). Flagellin treatment has been linked to resistance mediated granulopoiesis and the antiapoptotic effect of superoxide dismutase 2 (47). Flagellin treatment has been linked to resistance mediated granulopoiesis and the antiapoptotic effect of superoxide dismutase 2 (47). Flagellin-mediated protection of rodents and nonhuman primates against lethal irradiation was associated with CSF3-mediated granulopoiesis and the antiapoptotic effect of superoxide dismutase 2 (47). Flagellin treatment has been linked to resistance mediated granulopoiesis and the antiapoptotic effect of superoxide dismutase 2 (47). Flagellin treatment has been linked to resistance mediated granulopoiesis and the antiapoptotic effect of superoxide dismutase 2 (47). Flagellin treatment has been linked to resistance mediated granulopoiesis and the antiapoptotic effect of superoxide dismutase 2 (47). Flagellin treatment has been linked to resistance mediated granulopoiesis and the antiapoptotic effect of superoxide dismutase 2 (47).

The Journal of Immunology 1183

<table>
<thead>
<tr>
<th>Group</th>
<th>Genes</th>
<th>Expression</th>
<th>Contribution to</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Ahrr</td>
<td>–</td>
<td>Transcriptional regulation of IL-22 gene</td>
</tr>
<tr>
<td></td>
<td>Arntl</td>
<td>o</td>
<td>Transcriptional regulation of IL-22 gene</td>
</tr>
<tr>
<td></td>
<td>Batf</td>
<td>+</td>
<td>Master regulation of Th17 differentiation</td>
</tr>
<tr>
<td></td>
<td>Il1b, Il6, Il1r1, Traf5f15</td>
<td>+</td>
<td>Activation of Th17 differentiation</td>
</tr>
<tr>
<td></td>
<td>Il17f, Il22</td>
<td>+</td>
<td>Th17 cell phenotype</td>
</tr>
<tr>
<td></td>
<td>Nfj3</td>
<td>+</td>
<td>Regulation of NK cell differentiation</td>
</tr>
<tr>
<td></td>
<td>Pfng</td>
<td>o</td>
<td>Inhibition of Th17 differentiation</td>
</tr>
<tr>
<td></td>
<td>Rorc</td>
<td>–</td>
<td>Master regulation of Th17, Treg, LTi, LTi-, and NK-like cell differentiation</td>
</tr>
<tr>
<td>II</td>
<td>Atf3, If5, Map3k8, Njkb1</td>
<td>+</td>
<td>Activation of IL-17R and TLR signaling</td>
</tr>
<tr>
<td></td>
<td>Cebp, Cebpδ</td>
<td>+</td>
<td>Activation of IL-17R signaling</td>
</tr>
<tr>
<td></td>
<td>Dusp1, Njkbia, Njkie, Zip36</td>
<td>+</td>
<td>Repression of IL-17R and TLR signaling</td>
</tr>
<tr>
<td></td>
<td>If54</td>
<td>+</td>
<td>Activation of IL-17R and TLR signaling</td>
</tr>
<tr>
<td></td>
<td>Socs1, Socs3</td>
<td>+</td>
<td>Activation of IL-22R signaling</td>
</tr>
<tr>
<td></td>
<td>Stat5a</td>
<td>+</td>
<td>Activation of IL-17R and IL-22R signaling</td>
</tr>
<tr>
<td>III</td>
<td>Adm, Areg, Ereg, Nrg1, Sprt1a, Spr22, Tj12</td>
<td>+</td>
<td>Epithelial barrier function</td>
</tr>
<tr>
<td></td>
<td>Bcl3, Birc3</td>
<td>+</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td></td>
<td>Hamp, lipocalin 2, S100a8, S100a9</td>
<td>+</td>
<td>Antimicrobial activity</td>
</tr>
<tr>
<td></td>
<td>Igf15, Igf20, Oxa2, Oxa3</td>
<td>+</td>
<td>Antimicrobial activity via superoxides</td>
</tr>
<tr>
<td></td>
<td>Ncf1, Nox1, Sod2</td>
<td>+</td>
<td>Inflammation control</td>
</tr>
<tr>
<td></td>
<td>Retnla, Retnlg</td>
<td>+</td>
<td>Antimicrobial activity?</td>
</tr>
<tr>
<td></td>
<td>Saa2</td>
<td>o</td>
<td>Acute phase protein</td>
</tr>
<tr>
<td></td>
<td>Saa3</td>
<td>+</td>
<td>Acute phase protein</td>
</tr>
<tr>
<td></td>
<td>Serpine1, Serpina3g, Timp1</td>
<td>+</td>
<td>Inflammation control</td>
</tr>
<tr>
<td>IV</td>
<td>Adamt4, Adamt3, Adamt8</td>
<td>+</td>
<td>Tissue remodeling</td>
</tr>
<tr>
<td></td>
<td>Cecl2, Cecl3, Cecl4</td>
<td>+</td>
<td>Monocyte recruitment</td>
</tr>
<tr>
<td></td>
<td>Cecl17, Cecl22</td>
<td>+</td>
<td>T cell recruitment</td>
</tr>
<tr>
<td></td>
<td>Cecl11, Cecl2, Cecl15</td>
<td>+</td>
<td>Granulocyte recruitment</td>
</tr>
<tr>
<td></td>
<td>Cecl7, Cecl9, Cecl10, Cecl11</td>
<td>+</td>
<td>Immune cells recruitment</td>
</tr>
<tr>
<td></td>
<td>Cxcl13</td>
<td>o</td>
<td>B and LTi cell recruitment</td>
</tr>
<tr>
<td></td>
<td>Ifig, Ifn-regulated genes</td>
<td>+</td>
<td>Activation of immunity</td>
</tr>
<tr>
<td></td>
<td>Jlb</td>
<td>+</td>
<td>Lymphotixin β receptor signaling in stromal cells</td>
</tr>
<tr>
<td></td>
<td>Mmp3, Mmp13</td>
<td>+</td>
<td>Tissue remodeling</td>
</tr>
<tr>
<td></td>
<td>Tnfrsf1a, Tnfrsf1b</td>
<td>+</td>
<td>TNFRI and TNFRII signaling</td>
</tr>
<tr>
<td></td>
<td>Vcam1</td>
<td>+</td>
<td>LTi-mediated organization of lymphoid tissues</td>
</tr>
<tr>
<td>V</td>
<td>Cd14</td>
<td>+</td>
<td>LPS binding and TLR4 signaling</td>
</tr>
<tr>
<td></td>
<td>Mal</td>
<td>o</td>
<td>LPS binding and TLR4 signaling</td>
</tr>
<tr>
<td></td>
<td>Th2, Pglypp1</td>
<td>+</td>
<td>Activation of TLR2 and TLR4 signaling</td>
</tr>
</tbody>
</table>

Microarray gene expression data from the distal ileal segments of mice (n = 3) treated i.p. with flagellin for 2 and 8 h were compared with data from mock animals in a time course analysis. All genes differentially expressed and the corresponding method and annotation are included as Supplemental Fig. 5 and Supplemental Table II.

<table>
<thead>
<tr>
<th>Microarray genes expression data from the distal ileal segments of mice (n = 3) treated i.p. with flagellin for 2 and 8 h were compared with data from mock animals in a time course analysis. All genes differentially expressed and the corresponding method and annotation are included as Supplemental Fig. 5 and Supplemental Table II.</th>
<th>Microarray genes expression data from the distal ileal segments of mice (n = 3) treated i.p. with flagellin for 2 and 8 h were compared with data from mock animals in a time course analysis. All genes differentially expressed and the corresponding method and annotation are included as Supplemental Fig. 5 and Supplemental Table II.</th>
</tr>
</thead>
<tbody>
<tr>
<td>o, similar expression relative to mock animals; +, upregulation relative to mock animals; –, downregulation relative to mock animals; ††, data from a comparison between mock and 2-h flagellin-treated animals.</td>
<td>o, similar expression relative to mock animals; +, upregulation relative to mock animals; –, downregulation relative to mock animals; ††, data from a comparison between mock and 2-h flagellin-treated animals.</td>
</tr>
</tbody>
</table>

In the intestine, TLR5 signaling activates lamina propria DCs, which then promote Th17 differentiation (23). In contrast, intestinal DCs do not respond to TLR4 stimulation (23, 46). Flagellin treatment enhanced the transcription of the genes coding for CD14, LPB, MAL, and TLR2 (Supplemental Fig. 2, Table I). These findings suggest that responsiveness to TLR2 and TLR4 agonists may be reactivated or amplified after TLR5 stimulation, allowing the production of a second wave of effectors.

The relevance of TLR5 signaling in defense has recently been assessed. Flagellin-mediated protection of rodents and nonhuman primates against lethal irradiation was associated with CSF3-mediated granulopoiesis and the antiapoptotic effect of superoxide dismutase 2 (47). Flagellin treatment has been linked to resistance against inflammatory colitis and gut infections (40, 48). The TLR5-induced circulating and local production of IL-17/IL-22 may be the main driving force behind these protective effects. The contribution of IL-22 was recently suggested as instrumental in the control of infection with enterococci (40). In response to IL-17R and IL-22R signaling, epithelial and stromal cells produce antimicrobial peptides (RegIII), CXC chemokines, and growth factors (CSF3) for neutrophils, all of which are involved in mucosal protection (3–7, 38). Flagellin treatment prompted the expression of similar factors. In addition, our study identified other potential effectors of the TLR/IL-17R/IL-22R axis, such as antiviral molecules (ISG15, ISG20, OAS2, and OAS3), acute-phase proteins (SAA2, SAA3, and PTX3), and superoxide-mediated killing (NCF1, NOX1, and superoxide dismutase 2).
lymphoid follicles (13). Our work revealed that flagellin upregulated gut expression of genes coding for lymphotixin β, CXC13, and CCL20. We previously showed that flagellin triggers CCL20 production in intestinal epithelial cells (31). Hence, TLR5 signaling in both epithelial and hematopoietic cells may increase the development of secondary/tertiary lymphoid tissues. Recent studies showed that TLR2, TLR4, and Nod1 are involved in the development of lymphoid follicles (50, 51). Pattern recognition receptors in general and TLRs in particular may have a pivotal role in simultaneously conditioning the antimicrobial environment and new ectopic sites for the development of mucosal adaptive immunity.

In conclusion, the current study found that Lin<sup>−</sup>CD127<sup>+</sup> cells constitute a rapidly reacting, innate source of IL-17A, IL-17F, and IL-22 in response to TLR5 signaling. We hypothesize that this immune reaction occurs during microbial penetration into the lamina propria and stimulates innate effectors to locally clear the infection. Similar cell populations have been identified in humans (14, 16), and so, it remains to be seen whether TLR stimulation can promote activation of these innate immune cells.

Acknowledgments

We thank Michel Simonet and François Trottein for critical reading of the manuscript, Shizuo Akira for generous gift of Il2rg<sup>−/−</sup> mice, and Christelle Faveeuw and Luxmi Koodun for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


