

1 **Toll-like Receptor 5 signaling stimulates the innate production of IL-17 and IL-22**
2 **by CD3^{neg}CD127⁺ immune cells in spleen and mucosa¹**

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18

1 **Abstract**

2 In adaptive immunity, T helper-17 lymphocytes produce the IL-17 and IL-22 cytokines
3 that stimulate mucosal antimicrobial defenses and tissue repair. Here, we observed that
4 the TLR5 agonist flagellin induced swift and transient transcription genes encoding IL-
5 17 and IL-22 in lymphoid, gut and lung tissues. This innate response also temporarily
6 enhanced the expression of genes associated with the antimicrobial Th17 signature.
7 The source of the Th17-related cytokines was identified as novel populations of
8 CD3^{neg}CD127⁺ immune cells among which CD4-expressing cells resembling lymphoid
9 tissue inducer cells. We also demonstrated that dendritic cells are essential for
10 expression of Th17-related cytokines and so for stimulation of innate cells. These data
11 define that TLR-induced activation of CD3^{neg}CD127⁺ cells and production of Th17-
12 related cytokines may be crucial for the early defenses against pathogen invasion of
13 host tissues.

14

1 **Introduction**

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

9 Recent studies highlighted the contribution of IL-17A, IL-17F and IL-22 to defensive
10 reactions within the mucosa (3-6). These cytokines help orchestrate innate immunity by
11 stimulating epithelial cells to produce defense molecules, matrix proteases and tissue
12 repair molecules (7, 8). The source of IL-17A, IL-17F and IL-22 varies. During an
13 adaptive response, the lymphocytes that differentiate into T helper 17 (Th17) cells are
14 the main producers of cytokines (9). IL-17A can rapidly be produced during innate
15 responses to bacteria or microbial molecular patterns by $\gamma\delta$ T lymphocytes in a TLR4-
16 dependent manner, natural killer T (NKT) cells activated with α -galactosylceramide or
17 lymphoid tissue inducer (LTi)-like cells following stimulation with the TLR2/Dectin-1
18 agonist zymosan (10-12). NK-like and LTi-like innate lymphocytes expressing IL-7R α ,
19 Nkp46, the transcription factor ROR γ t and eventually CCR6 are sources of IL-22 and/or
20 IL-17 in mucosa under steady-state conditions (13-18). Interestingly, microbial flora
21 colonizing mucosa are required to switch on lasting IL-17 and IL-22 production (15, 17).
22 In absence of these innate lymphocytes, infectious colitis is exacerbated, suggesting an
23 operational role of IL-22 and IL-17 in the gut's innate immunity (15, 17). However, the

1 link between TLR-mediated signaling, Th17-related cytokine production by innate
2 immune cells, and mucosal defenses has not been defined.

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

18

1 **Materials and methods**

2 **Mice.** Specific-pathogen free (SPF) mouse strains C57BL/6J, C57BL/6J-Ly5.1, BALB/c,
3 and *Tcrb*^{-/-}, *Tcrd*^{-/-}, *Tcrb*^{-/-}*Tcrd*^{-/-}, *Tlr5*^{-/-} (24), *Myd88*^{-/-} (25), transgenic animals for pre-
4 TCR α , *Cd11c-DTR-EGFP* (*Itagx-DTR/EGFP*) (26), *Rag2*^{-/-}*Il2rg*^{-/-} backcrossed on
5 C57BL/6J mice, *Cd1d*^{-/-} backcrossed on BALB/c background, and C.B-17 *scid* (SCID)
6 mice were purchased from Charles River Laboratories, the Jackson laboratory, or
7 Janvier (France) or bred in an accredited establishment (#A59107, Institut Pasteur de
8 Lille ; Transgenose Institute CNRS, Orleans; RCHCI at ETHZ; LICR Brussels and
9 Lausanne branches). Animals (6-16 weeks old) were used according to national
10 regulations and ethical guidelines.

11 For bone marrow (BM) chimera, recipient mice were irradiated (1000-1500 rads) and
12 reconstituted 2-to-24h later with BM cells ($4-20 \times 10^6$ cells i.v.). These mice were used
13 at 10-16 weeks post-transplantation and the degree of chimerism was assessed by
14 measuring CD45.1 and CD45.2 surface expression by leukocytes. The current protocol
15 yielded 96.7% reconstitution for *Cd11c-DTR/EGFP*→C57BL/6 (WT) in spleen, 98.2% for
16 *Tlr5*^{-/-}→ WT and 97.2% for WT→*Tlr5*^{-/-} in lung. Depletion of CD11c⁺ cells was achieved
17 by injecting i.p. diphtheria toxin (DTX) as described (26). Depletion of $\gamma\delta$ T cells (about
18 90% depletion) and NK cells cells (about 72% depletion) was performed by injecting i.p.,
19 24h prior to flagellin treatment, 100 μ g mAb specific for TCR δ chain (GL3 clone) or
20 NK1.1 (PK136) or irrelevant mAb HB152 as control.

21

22 **Flagellin administration.** LPS-depleted flagellin FliC from *Salmonella typhimurium*
23 (5 μ g) produced as described (21), ultrapure LPS from *Escherichia coli* (serotype

1 0111:B4, 5µg, Invivogen), or phosphorothioate CpG oligonucleotide
2 (TCCATGACGTTTCCTGATGCT, 5µg, Eurogentec) diluted in PBS were injected i.v. or
3 i.p to mice.

4
5 **Flow cytometry and sorting.** Spleens were digested with collagenase D (Roche, 0.5
6 mg/ml), DNase I (Sigma 40µg/ml) during 10 min at 37°C. Cells were stained for CD127-
7 FITC, CD45.1-FITC, CD45.2-PE, PE-conjugated lineage-specific antibodies (CD3,
8 B220, Gr1, CD11b, CD11c), MHCII-PE, NK1.1-PercP-Cy5.5, CD4-APC, CD11c-APC,
9 and CD45.1-Pacific Blue (Becton Dickinson, BioLegend and eBioscience) and sorted on
10 a BD FACSAria™.

11
12 **Dendritic cell culture.** DC were differentiated from bone marrow as described (25). On
13 day 7 or 11, BMDCs were stimulated for 2h and analyzed.

14
15 **Determination of cytokine production.** CCL20 and IL-22 (R&D Systems) and IL-17A
16 (eBioscience) levels were measured by ELISA in serum and tissue homogenates
17 prepared with T-PER Reagent (Pierce) supplemented with protease inhibitors (Roche).

18
19 **Gene expression.** Total RNA was extracted with the Nucleospin RNA II kit (Macherey
20 Nagel) and reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied
21 Biosystems). A pre-amplification of cDNA from sorted cells was performed prior to real
22 time PCR using the PreAmp kit (Applied Biosystems). cDNA was amplified using SYBR
23 Green-based real time PCR (Table S1) or commercial TaqMan assays (Applied

1 Biosystems). For high throughput analysis, Taqman Low Density Arrays (Applied
2 Biosystems) were used. Analysis was carried out using Real Time StatMiner software
3 (Integromics). Relative mRNA levels ($2^{-\Delta\Delta Ct}$) were determined by comparing (i) the cycle
4 thresholds (Ct) for the gene of interest and *Actb* (ΔCt) and (ii) ΔCt values for treated and
5 control groups ($\Delta\Delta Ct$). Ct upper limit was fixed to 33 cycles.

6

7 **Microarrays.** Total RNA (2 μ g) was processed on the Mouse Whole Genome Arrays
8 v2.0 (Applied Biosystems) (27). Data were analyzed using the NeONORM method and
9 heatmaps were created as described (27, 28). Gene Ontology was analyzed using the
10 Panther Protein Classification System (<http://www.pantherdb.org>). Microarray data were
11 deposited in the publicly available database: <http://mace.ihes.fr> with accession number:
12 [2844328654](http://mace.ihes.fr).

13

14 **Statistical analysis.** The Mann-Whitney test and the Graphpad Prism software 5.0
15 were used in analyses. The Limma test with Benjamini-Hochberg FDR correction was
16 used for high throughput PCR with Taqman Low Density Arrays. Results were
17 considered significant for $P < 0.05$ indicated by “*”. Results are expressed as arithmetic
18 means \pm SD.

19

1 **Results**

2 Systemic TLR5 signaling enhances *Il17a*, *Il17f* and *Il22* gene expression in lymphoid 3 tissues

4 To establish whether or not TLR stimulation promotes the rapid expression of the Th17-
5 related cytokines, mice were treated i.p. or i.v. with a TLR4 agonist (lipopolysaccharide
6 or LPS), a TLR5 agonist (flagellin) or a TLR9 agonist (CpG). Gene expression in spleen
7 and lymph nodes was then monitored (Fig. 1A-B). Flagellin administration triggered
8 within 2h about 1,000-fold increase of *Il22* mRNA levels. Similarly, *Il17a* and *Il17f* gene
9 expression was upregulated. A TLR5-mediated, Th17-related innate response was also
10 observed in the mediastinal and inguinal lymph nodes and, to a lesser extent in the liver
11 (Fig. 1 and Fig. S1). LPS was initially shown to promote *Il22* expression in many tissues
12 (29). We found that LPS also enhanced the Th17-related innate response but to a
13 lesser extent than flagellin did (Fig. 1A-B and Fig. S1). Although TLR9-mediated
14 signaling activated the response in lymph nodes, it was devoid of any effect in the
15 spleen.

16 The flagellin-dependent response was transient and peaked at 2h; mRNA levels
17 returned to baseline levels after 24h (Fig. 1C-D). Gene profiling showed that the
18 expression of genes specific for TLR-, IL-17R- and IL-22R-mediated signaling was
19 significantly enhanced in spleen (Fig. 1E). These genes encode pleiotropic and Th17-
20 promoting cytokines (TNF- α , IL-1 β , and IL-6), chemokines that are specific for
21 neutrophils, monocytes and lymphocytes (CXCL-1, -2, -5, -9, and -10), antimicrobial
22 molecules like CAMP and HAMP, lipocalin 2 (*Lcn2*), S100A9 and tissue remodeling
23 proteases MMP-3 and -13. Strikingly, the expression of chemokine CCL20 which is

1 specific for the recruitment of DC precursors, Th17 lymphocytes, LTi-like or NK-like cells
2 (12, 14, 30, 31) was significantly upregulated. The transcription of IFN- γ encoding gene
3 was also upregulated by flagellin treatment. However, we did not observe any change in
4 the expression of genes coding for IL-21, ROR γ t or TGF- β (i.e. other factors involved in
5 Th17 differentiation). As shown in Fig. 1F, IL-22 levels rose significantly in serum and
6 spleen from flagellin-treated animals whereas IL-17A was hardly detectable in serum
7 but rose 3-fold in the spleen. In conclusion, TLR5 signaling in lymphoid tissues
8 promotes the rapid production of the innate cytokines IL-17A, IL-17F and IL-22 - a
9 pattern which resembles a Th17-related innate response.

10

11 TLR5-mediated innate responses require γ_c chain-dependent immune cells

12 TLR5 is expressed by monocyte/macrophage/DC lineages, NK cells, CD4⁺ lymphocytes
13 and radioresistant stromal cells but not B lymphocytes (25, 32-35). To define the cells
14 involved in the early production of IL-17 and IL-22, we used bone marrow (BM) chimera
15 expressing or not *Tlr5* and tested their ability to respond to flagellin. As shown in Fig. 2A
16 and 2E, TLR5-competent hematopoietic cells were required to trigger flagellin-mediated
17 *Il17* and *Il22* gene expression. By using SCID, *preTCR α* animals harboring enhanced
18 number of $\gamma\delta$ lymphocytes, *Tcrb*^{-/-} and *Tcrd*^{-/-} mice, we demonstrated that neither B cells
19 nor TCR $\alpha\beta$ -/TCR $\gamma\delta$ -expressing T lymphocytes were required for flagellin-mediated *Il17*
20 and *Il22* expression (Fig. 2B and Fig. S2A-B and S2D). In contrast, the response was
21 impaired in *Rag2*^{-/-}*Il2rg*^{-/-} mice that have almost normal DCs but lack B and T
22 lymphocytes as well as NK, NKT, LTi, NK-like and LTi-like cells, all of which depend on
23 the interleukin receptor γ_c chain encoded by *Il2rg* gene (Fig. 2C and 2E) (12, 15, 17, 36).

1 The impairment was not a collapse of TLR5 signaling, since the liver was still
2 responsive to flagellin (Fig. 2D). Our experiments using genetically deficient animals
3 and depleting antibodies suggested that CD1d-restricted NKT cells or NK cells were not
4 drivers of the TLR5-mediated response (Fig. S2C-D). Therefore, our data showed that
5 the cells expressing Th17-related cytokines after flagellin stimulation require the
6 interleukin receptor γ_c chain for differentiation or activation but are not NK, NKT or
7 TCR $\gamma\delta$ -expressing innate lymphocytes.

8

9 CD3^{neg}CD127⁺ LTi-like cells produce IL-17 and IL-22 in response to TLR5 signaling

10 We next sought to determine which γ_c chain-dependent innate immune cells are
11 involved in TLR5-mediated response. Recent work has suggested that innate
12 lymphocytes expressing IL-7R (i.e. the CD127 or IL-7R α chain and the CD132 or γ_c
13 chain) and LTi/NK cell markers are sources of IL-22 and IL-17 (12-17). To determine
14 whether or not innate immune cells produce Th17-related cytokines, splenic cells from
15 mock- and flagellin-treated animals were sorted on the basis of lineage (Lin) markers
16 (CD11b, CD11c, Gr1, CD3, and B220), NK1.1, CD4 and CD127 (Fig. 3A). After TLR5
17 stimulation, Lin^{neg}NK1.1^{neg}CD127⁺CD4^{neg} and Lin^{neg}NK1.1^{neg}CD127⁺CD4⁺ cells were
18 found to strongly upregulate *Il17f* and *Il22* expression about 80- and 500-fold,
19 respectively (Fig. 3B). These subsets account for about 0.5-1% of Lin^{neg}NK1.1^{neg} cells
20 and 0.02-0.1% of splenocytes in a C57BL/6 mouse. Similar observations were done
21 using CD3^{neg}CD127⁺CD4⁺ cells from SCID mice (Fig. 3D). Both subsets were found to
22 express CD45, indicating an hematopoietic origin and their classification as immune
23 cells (Fig. S3A). We also found that the CD3^{neg}CD127⁺CD4⁺ and CD3^{neg}CD127⁺CD4^{neg}

1 cells are mostly absent in the *Rag2^{-/-}Il2rg^{-/-}* mouse (Fig. S3D); This is consistent with
2 previous observations (12, 17). In spleen, the frequency of $\text{Lin}^{\text{neg}}\text{NK1.1}^{\text{neg}}\text{CD127}^+\text{CD4}^+$
3 matched the number of LTi cells (12, 37). We also detected upregulation of *Il22*
4 transcript levels in CD4 lymphocytes ($\text{Lin}^+\text{CD4}^+$) and NK cells ($\text{Lin}^+\text{NK1.1}^+$) after flagellin
5 administration. However, the $\text{Lin}^{\text{neg}}\text{NK1.1}^{\text{neg}}\text{CD127}^+\text{CD4}^+$ cells were the most potent
6 producers, since *Il17f* and *Il22* mRNA levels were 100- to 1,000-fold higher in this
7 population than in $\text{Lin}^{\text{neg}}\text{NK1.1}^{\text{neg}}\text{CD127}^+\text{CD4}^{\text{neg}}$ cells, CD4 or NK cells (Fig. 3C). Like
8 the NK-like or LTi-like cells, $\text{Lin}^{\text{neg}}\text{NK1.1}^{\text{neg}}\text{CD127}^+\text{CD4}^+$ innate lymphocytes express
9 *Ccr6* but we were unable to detect any mRNA for *Rorc* or *Ncr1* encoding ROR γ t and
10 NKp46. In addition, we observed that, upon flagellin stimulation, *Ifng* expression was
11 mainly upregulated in NK cells rather than $\text{Lin}^{\text{neg}}\text{CD127}^+$ cells (data not shown). Finally,
12 we observed that the $\text{Lin}^{\text{neg}}\text{CD127}^+$ cells were absent in *Rag2^{-/-}Il2rg^{-/-}* animals (Fig.
13 S3D). Our data provide evidence that $\text{Lin}^{\text{neg}}\text{CD127}^+$ innate immune cells, and especially
14 the CD4^+ fraction which resembles LTi cells can rapidly produce IL-17 and IL-22
15 cytokines following TLR activation.

16

17 TLR5-mediated activation of Th17-related innate responses requires dendritic cells

18 To determine whether the TLR5-mediated upregulation of Th17-related innate response
19 is a direct innate lymphocyte activation process or requires DC stimulation, diphtheria
20 toxin (DTX)-mediated ablation of CD11c^+ cells was performed in a *Cd11c-DTR-EGFP*
21 bone marrow chimera. The DTX treatment depleted $93.8 \pm 2.2\%$ of $\text{CD11c}^+\text{MHCII}^{\text{high}}$
22 DCs (Fig. 4A). In addition, we found that DTX treatment did not eliminate the
23 $\text{CD3}^{\text{neg}}\text{CD4}^+\text{CD127}^+$ cells in the spleen of a *Cd11c-DTR-EGFP* chimera and did not

1 alter the the TLR5-mediated production of IL-22 (Fig. S3). As shown in Fig. 4B,
2 systemic administration of flagellin to DC-depleted animals resulted in impaired *Il17f* or
3 *Il22* transcription, compared with controls. Thus, our experiments demonstrated that
4 DCs are necessary for TLR5-mediated expression of Th17-related cytokines.
5 Th17 differentiation depends on tissue-derived TGF β and IL-1 β , and IL-6 produced by
6 DCs and the maintenance of Th17 phenotype has been associated to DC-derived IL-23
7 (9). *In vivo*, DC cell ablation was found to attenuate the upregulation of *Il6* transcription
8 in response to flagellin (Fig. 4C). We did not observe any alteration in *Il1b*, *Il12b* coding
9 for the p40 chain of IL-12 or IL-23, and *Tgfb* gene expression (data not shown).
10 Intriguingly, *Il23a* (coding for the p19 chain of IL-23) transcription was enhanced by DTX
11 treatment; it is possible that CD11c^{low}MHCII^{neg} cells having infiltrated the spleen in DTX-
12 treated mice support *Il23a* upregulation (Fig. 4A and 4C). Here, we found that flagellin
13 promoted the expression of both *Il12b* and *Il23a* in BM-derived DCs (Fig. 4D). In
14 response to flagellin, DCs can therefore produce both IL-6 and IL-23, with the
15 subsequent expression of an innate, Th17-related signature. However, *in vivo*, IL-23
16 alone appears unable to induce gene activation. In response to flagellin, DC-mediated
17 IL-6 production could therefore promote the Th17-related signature by Lin^{neg}CD127⁺
18 cells.
19 Previous studies suggested that DCs produce IL-22 (4, 38). Here, we found that flagellin
20 promoted the expression of *Il12b* in BM-derived DCs but did not have any effect on
21 *Il17a*, *Il17f* and *Il22* transcription (Fig. 4D). Lipopolysaccharide which strongly increased
22 *Il12b* and *Il23a* transcription did not upregulate expression of *Il17a* and *Il17f* in DCs but
23 did enhance *Il22* mRNA levels about 8-fold, compared with untreated cells. Analysis of

1 transcription in splenic DC sorted from mice treated with flagellin also showed that DC
2 were not potent source of IL-22 (Fig. 3D-E). Taken as a whole, our data suggested that
3 DCs are not a major source of TLR5-mediated, Th17-related cytokine production but
4 contribute to induction of the latter.

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

7 Since Th17-related cytokines are important in the control of inflammation and infection
8 in the mucosa (3-6, 38, 39), we next assessed the impact of flagellin administration on
9 intestinal tissues. Flagellin strongly enhanced the production of IL-22 within 2h of
10 administration from the duodenum to the proximal colon; the level then returned to the
11 baseline at 8h (Fig. 5). CCL20 synthesis was also strongly induced in the small intestine
12 (Fig. S4A). In contrast, gut IL-17A production changed moderately following flagellin
13 treatment. In any intestine segments, TLR5 signaling strongly enhanced transcription of
14 the *Il17* and *Il22* genes and those encoding antimicrobial peptides, neutrophil-specific
15 chemokines and growth factors and tissue remodeling/repair molecules (Fig. 5C and
16 Fig. S4B-E). A similar pattern was also observed in lung tissue, but regression to
17 baseline levels was not observed at 8h, suggesting that the kinetic may be different in
18 the respiratory tract (Fig. S5). In conclusion, systemic flagellin administration promotes
19 mucosal Th17-related innate responses.

20 We used the same type of analysis than for the response in spleen and lymph nodes to
21 investigate the effects of flagellin on mucosa. The gut and pulmonary flagellin-mediated
22 Th17-like innate response was stronger in $WT \rightarrow Tlr5^{-/-}$ than in $Tlr5^{-/-} \rightarrow WT$ animals (Fig.
23 5D and Fig. S5). These findings indicated that TLR5-competent hematopoietic cells are

1 also instrumental in the mucosal response. DC depletion impaired the upregulation of
2 the intestinal Th17-related signature (Fig. S4F). Lastly, we compared Th17-related gene
3 expression in *Rag2^{-/-}Il2rg^{-/-}* and WT animals (Fig. 5E). As previously reported, steady-
4 state levels of *Il22*, *Il17* and *Ifng* transcripts in the ileum were significantly lower in *Rag2^{-/-}*
5 *Il2rg^{-/-}* than in WT animals (17). After TLR5 stimulation, transcription of these genes
6 was not enhanced in the *Rag2^{-/-}Il2rg^{-/-}* intestine. Taken as a whole, our data suggest
7 that TLR5 signaling elicits a mucosal Th17-related response by innate immune cells that
8 require the γ_c chain and help from DCs.
9 Finally, the expression of many genes was upregulated in the gut of *Rag2^{-/-}Il2rg^{-/-}*
10 animals, suggesting that TLR5 signaling promotes the activation of non-lymphoid cells
11 (Fig. 5E). Experiments with the *Tlr5^{-/-}* chimera reinforced these conclusions since
12 radioresistant cells, likely structural/stromal cells, contributed to TLR5-mediated
13 responses, especially in lung tissue (Fig. 5D and Fig. S5).

14

15 The flagellin-induced transcriptional signature has features of Th17- and TLR-mediated
16 responses.

17 To further characterize the response to flagellin, we performed a microarray time course
18 analysis of gene expression in the distal ileum of treated mice. Biological processes
19 including signaling and defense pathways, cytokine- and chemokine-mediated
20 immunity, NF- κ B signaling, granulocyte- and macrophage-mediated immunity, cell
21 proliferation and differentiation and apoptosis were significantly modulated 2h-8h after
22 flagellin administration (Fig. S6 and Table S2). On the basis of these data, we arbitrarily
23 defined 5 groups of genes according to their potential role in the gut immune response

1 (Table 1). The first group of genes encodes modulators of IL-17 and IL-22 production as
2 well as differentiation of Th17, LT α i, LT α i-like, NK and NK-like cells. The second class was
3 involved in the positive or negative transcriptional control of IL-17R (i.e. C/EBP β and
4 C/EBP δ), IL-22R (i.e. SOCS-1, SOCS-3 and STAT5A) and TLR (i.e. ATF3 and I- κ B α)
5 signaling. The third group includes TLR-, IL-17R- and IL-22R-signaling target genes
6 coding for antimicrobial molecules or factors regulating epithelium barrier function.
7 Interestingly, our analysis also identified antiviral genes. In contrast to previous studies
8 of Th17 cytokine-mediated signaling, we did not find any transcriptional modulation of
9 the mucin and β defensin genes. The fourth group corresponds to genes encoding
10 factors involved in recruitment, development or function of various immune cells.
11 Relative to controls, chemokines specific for innate immune cells like neutrophils, NK,
12 NKT or LT α i cells, T and B lymphocytes or monocytes were significantly enriched -
13 suggesting that several cell types may enter the tissues and participate in the immune
14 response. The last group includes genes required for TLR4 or TLR2 signaling (i.e. LBP,
15 CD14 and MAL). In conclusion, intestinal gene expression profiling showed that flagellin
16 promotes a transient immune response involving regulators and effectors of both TLR-
17 and Th17-mediated immunity.

18

1 **Discussion**

2 The way in which TLR signaling activates the host's innate defenses during mucosal
3 invasion by pathogens is subject to debate. In the present study, we showed that TLR5
4 signaling induces systemic and mucosal innate expression of the Th17-related IL-17
5 and IL-22 cytokines by stimulating Lin^{neg}CD127⁺ cells in a DC-dependent manner.
6 Overall, our data suggest that Lin^{neg}CD127⁺ cells may play a major role as innate
7 lymphocytes in the early orchestration of a TLR-dependent, protective response to
8 mucosal invasion by pathogens.

9 Th17-related cytokines contribute to adaptive immunity in response to various
10 inflammatory and infectious diseases (5, 6, 30, 39); however, their impact on the early
11 phase of infection is poorly understood. The effect of TLR signaling on IL-17 and IL-22
12 was previously suggested since their synthesis was enhanced after administration of
13 TLR2 and TLR4 agonists (12, 29). Very recently, TLR5 signaling was also associated to
14 such response (40). Here, we analyzed the immune response to the TLR5 activator
15 flagellin, with a focus on mucosa. Our rationale was that since flagellin expression is
16 specifically restricted to luminal compartment, its desequestration is likely to be an
17 alarm signal for mucosal invasion (22). We found that systemic flagellin administration
18 promotes the swift, intense, transient production of IL-17A, IL-17F and IL-22 and factors
19 controlling Th17 differentiation. For example, flagellin modulates expression of the
20 genes coding for the aryl hydrogen receptor (AHR) repressor AHRR, the AHR nuclear
21 translocator-like factor ARNTL and the ATF-like factor BATF - all of which are involved
22 in Th17 differentiation (41, 42). Moreover, the flagellin-induced innate response and the
23 Th17 adaptive response share many effectors, such as chemokines, antimicrobial

1 peptides, anti-apoptotic factors and tissue remodeling factors. Therefore, we
2 hypothesized that flagellin-mediated response enables the rapid and transient
3 recruitment of systemic and mucosal defenses.

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

1 Dendritic cells have an important role in integrating microbial signals and activating
2 immune cells like Th17 lymphocytes (9). When DCs were depleted, the Th17-related
3 innate response to flagellin was impaired, indicating that DCs are necessary for the
4 activation of Lin^{neg}CD127⁺ cells. Similarly, transcription of *Il6* was attenuated, which
5 suggests that, in Th17 differentiation, DC-derived IL-6 like may contribute to
6 Lin^{neg}CD127⁺ cell activation (9). Our findings suggested that IL-1 β , IL-23 or TGF- β (or at
7 least the amounts produced by DCs) are not required for Lin^{neg}CD127⁺ cell activation.
8 However, IL-23 might be important for alternative activating pathway for production of
9 IL-17 or IL-22 by LTI-like and NK-like cells because (i) cells are activated *in vitro* by
10 supplementing the culture medium with IL-23 and (ii) CD3^{neg}CD4⁺ cells express IL-23R
11 (12, 13, 15, 45). In the intestine, TLR5 signaling activates lamina propria DCs, which
12 then promote Th17 differentiation (23). In contrast, intestinal DCs do not respond to
13 TLR4 stimulation (23, 46). Flagellin treatment enhanced the transcription of the genes
14 coding for CD14, LPB, MAL and TLR2 (Table 1 and S2). These findings suggest that
15 responsiveness to TLR2 and TLR4 agonists may be re-activated or amplified after TLR5
16 stimulation, allowing the production of a second wave of effectors.

17 The relevance of TLR5 signaling in defense has recently been assessed. Flagellin-
18 mediated protection of rodents and non-human primates against lethal irradiation was
19 associated with CSF3-mediated granulopoiesis and the anti-apoptotic effect of SOD2
20 (47). Flagellin treatment has been linked to resistance against inflammatory colitis and
21 gut infections (48) (40). The TLR5-induced circulating and local production of IL-17/IL-
22 22 may be the main driving force behind these protective effects. The contribution of IL-
23 22 was recently suggested as instrumental in the control of infection with enterococci

1 (40). In response to IL-17R and IL-22R signaling, epithelial and stromal cells produce
2 antimicrobial peptides (RegIII), CXC chemokines and growth factors (CSF3) for
3 neutrophils all of which are involved in mucosal protection (3-7, 38). Flagellin treatment
4 prompted the expression of similar factors. In addition, our study identified other
5 potential effectors of the TLR/IL-17R/IL-22R axis, such as antiviral molecules (ISG15,
6 ISG20, OAS2 and OAS3), acute phase proteins (SAA2, SAA3 and PTX3) and
7 superoxide-mediated killing (NCF1, NOX1 and SOD2).

8 The Lin^{neg}CD127⁺CD4⁺ cells herein described resemble the LT_i cells that are
9 instrumental in the development of secondary/tertiary lymphoid tissues (such as Peyer's
10 patches or isolated lymphoid follicles) (13, 49). Intestinal LT_i cells express lymphotoxin
11 β (LTβ), and CCR6 and CXCR5, the receptors for CCL20 and CXCL13, respectively
12 (13, 49). CXCL13 and CCL20 are produced by epithelial/stromal cells and are involved
13 in the clustering of lymphocytes, DCs and LT_i to form lymphoid follicles (50, 51). Ectopic
14 epithelial expression of CXCL13 increases the number of LT_i cells (which produce IL-22
15 constitutively) and isolated lymphoid follicles (13). Our work revealed that flagellin
16 upregulated gut expression of genes coding for LTβ, CXCL13, and CCL20. We
17 previously showed that flagellin triggers CCL20 production in intestinal epithelial cells
18 (31). Hence, TLR5 signaling in both epithelial and hematopoietic cells may increase the
19 development of secondary/tertiary lymphoid tissues. Recent studies showed that TLR2,
20 TLR4 and Nod1 are involved in the development of lymphoid follicles (50, 51). Pattern
21 recognition receptors in general and TLRs in particular may have a pivotal role in
22 simultaneously conditioning the antimicrobial environment and new ectopic sites for the
23 development of mucosal adaptive immunity.

1 In conclusion, the present study found that Lin^{neg}CD127⁺ cells constitute a rapidly
2 reacting, innate source of IL-17A, IL-17F and IL-22 in response to TLR signaling. We
3 hypothesize that this immune reaction occurs during microbial penetration into the
4 lamina propria and stimulates innate effectors to locally clear the infection. Similar cell
5 populations have been identified in humans (14, 16) and so it remains to be seen
6 whether TLR stimulation can promote activation of these innate immune cells.

7

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5

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14

1 **Figure Legends**

2

3 **Fig. 1: TLR5 signaling promotes a swift, transient, Th17-related response.**

4 C57BL/6 mice (n=3-4) were treated i.p. or i.v. with PBS, flagellin, LPS or CpG. Tissues
5 were assayed 2h later (or as indicated) for cytokine production or mRNA levels. (A)
6 Spleen and (B) mesenteric lymph node gene expression in response to i.v. injection of
7 TLR agonists. Kinetic analysis of transcription in mesenteric (C) and mediastinal (D)
8 lymph nodes after i.v. flagellin injection. (E) Spleen transcriptional signature after i.p.
9 flagellin injection. mRNA levels are expressed relative to the PBS group. (F) Cytokine
10 production in spleen extract (per whole spleen) and serum (per ml) after i.p. flagellin
11 injection. The results are representative of 2 to 4 experiments. Results are given as
12 means \pm SD. Asterisks indicate $P < 0.05$ in Mann-Whitney test relative to PBS group. In
13 panel E, all genes display $P < 0.05$ in Limma test.

14

15 **Fig. 2: The interleukin receptor γ_c chain is required for the TLR5-mediated, Th17-**

16 **related innate response.** Mice (n=3-4) were treated i.p. with PBS or flagellin. Lymphoid
17 tissues and serum were sampled 2h after injection for the quantification of mRNA levels
18 and ELISA. (A) Response in mediastinal lymph nodes from C57BL/6 (WT) \rightarrow *Tlr5*^{-/-} and
19 *Tlr5*^{-/-} \rightarrow WT bone marrow chimera. The spleen response in SCID (B) and *Rag2*^{-/-}*Il2rg*^{-/-}
20 (C) mice. (D) The flagellin-mediated liver response. mRNA levels are expressed relative
21 to the PBS group. (E) IL-22 production in serum. Results are given as means \pm SD.
22 Asterisks indicate $P < 0.05$ in Mann-Whitney test relative to PBS group.

23

1 **Fig. 3: Lin^{neg}CD127⁺ innate cells upregulate *I17* and *I22* expression following**
2 **TLR5 stimulation.** C57BL/6 (A-C) or SCID mice (D-E) mice (n=3-4) were treated i.p.
3 with PBS or flagellin. Spleens were sampled after 2h for cell sorting and quantification of
4 mRNA levels. (A) Sorting scheme. Cells were sorted as Lin⁺CD4⁺, Lin⁺NK1.1⁺,
5 Lin^{neg}NK1.1^{neg}CD127⁺CD4⁺ cells and Lin^{neg}NK1.1^{neg}CD127⁺CD4^{neg} cells. (B) Regulation
6 of *I17f* and *I22* expression following flagellin stimulation. mRNA levels and statistical
7 significance are expressed relative to PBS. (C) Levels of transcripts in sorted cells
8 following flagellin stimulation. mRNA levels levels and statistical significance are
9 expressed relative to Lin⁺CD4⁺ cells. The data correspond to the mean of 3 independent
10 sorting. Results are given as means \pm SD. Asterisks indicate $P < 0.05$ in Mann-Whitney
11 test. (D-E) Spleens cells were used such as (input) or sorted as CD3^{neg}CD4⁺CD127⁺,
12 DC (CD11c⁺MHCII⁺) or NK (NKp46⁺). In D, mRNA levels in flagellin-treated animals are
13 expressed relative to PBS. In E, mRNA levels levels are expressed relative to input
14 cells.

15
16 **Fig. 4: Dendritic cells regulate TLR5-dependent, Th17-related innate responses.**
17 *Cd11c-DTR-EGFP*→C57BL/6 chimera mice (n=4) were injected i.p. with 100ng
18 diphtheria toxin (DTX) or untreated (no DTX). (A) Depletion of splenic dendritic cells
19 was assessed 24h later by flow cytometry. (B, C) The flagellin-specific transcriptional
20 response. 24h after DTX injection, mice were treated i.p. with PBS or flagellin. Spleen
21 was sampled after 2h for the quantification of transcripts specific for Th17-related
22 cytokine genes (B) and DC-specific cytokine genes (C). mRNA levels are expressed
23 relative to the no-DTX→PBS group. (D) Bone marrow-derived DCs from C57BL/6 mice

1 were treated or not with LPS or flagellin (1 μ g/ml) for 2 h and mRNA levels were
2 determined relative to mock DCs. Statistical significance was expressed relative to no-
3 DTX (B, C) or relative to the mock DCs (D). Results are given as means \pm SD. Asterisks
4 indicate $P < 0.05$ in Mann-Whitney test.

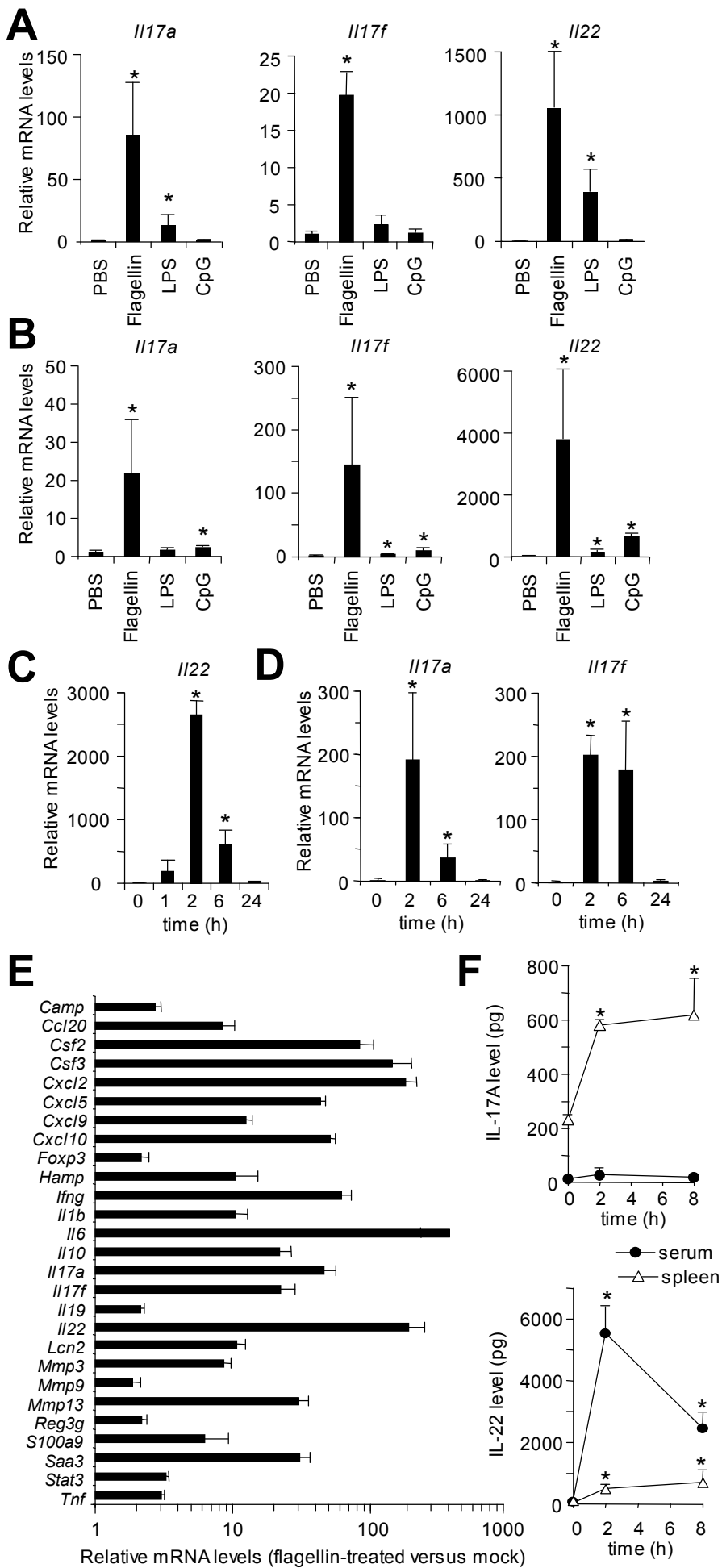
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6 **Fig. 5: TLR5 signaling promotes γ_c chain-dependent, Th17-related innate**
7 **responses in the gut.** Mice (n=3-4) were treated i.p. with PBS or flagellin and gut
8 segments (5 cm for proximal duodenum, jejunum, distal ileum , 3 cm for proximal colon
9 and the whole cecum) were sampled at the indicated times for quantification of cytokine
10 production and mRNA levels. (A, B) Cytokine production in C57BL/6 (WT) animals.
11 Tissue segments were homogenized within 1 mL of lysis buffer. The results are
12 representative of 2 experiments. (C) Time course analysis of the transcriptional
13 response in WT distal ileum. (D) Contribution of TLR5 signaling in the hematopoietic
14 compartment is essential for the Th17-related signature. mRNA levels 2h after flagellin
15 treatment are expressed as WT \rightarrow *Tlr5*^{-/-} relative to *Tlr5*^{-/-} \rightarrow WT mice. (E) Contribution of
16 the γ_c chain to intestinal response. mRNA levels are expressed relative to PBS-treated
17 WT mice. In panels A, B, and D, all data displayed statistically significant changes,
18 except where denoted as “ns” for “non-significant”. Results are given as means \pm SD.
19 Mann-Whitney test relative to PBS group was used in A and B. Limma test was used in
20 C to E panels.

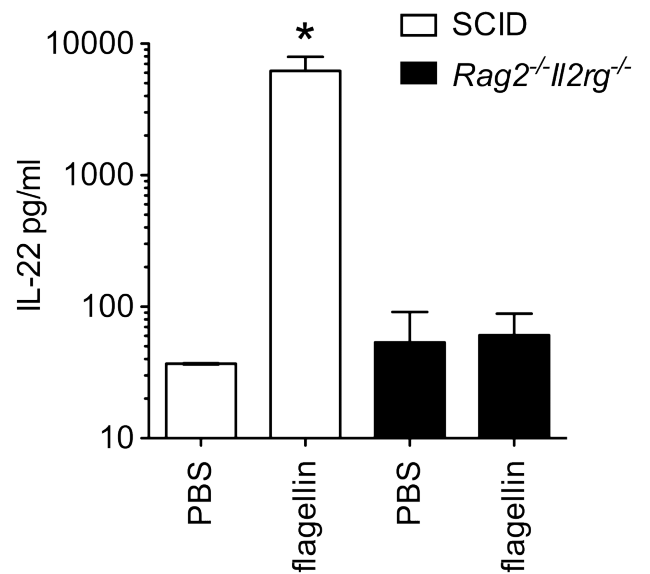
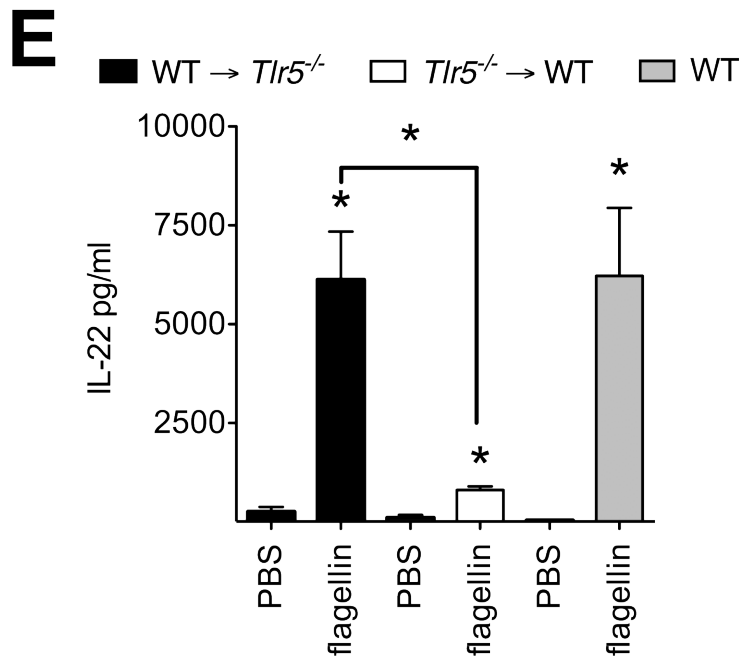
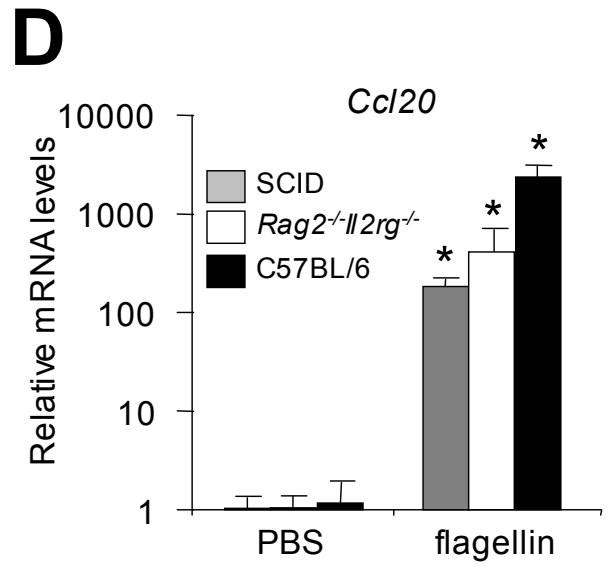
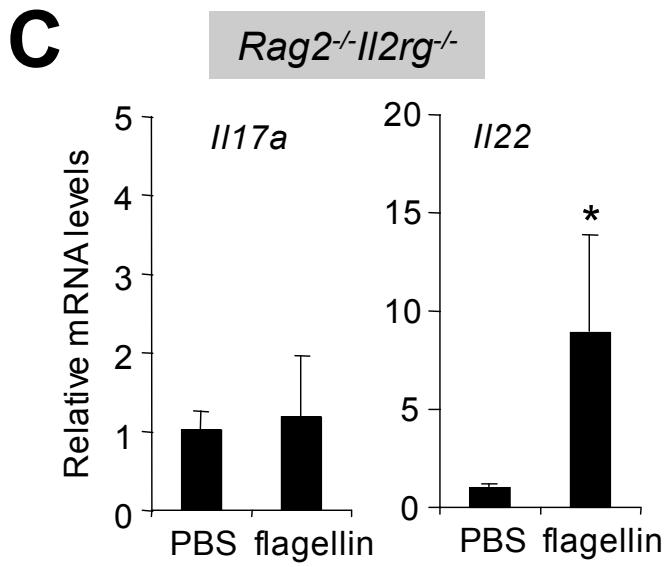
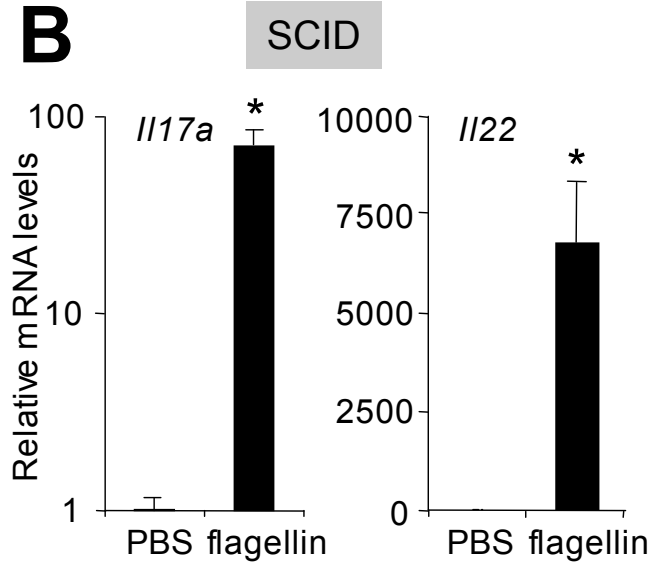
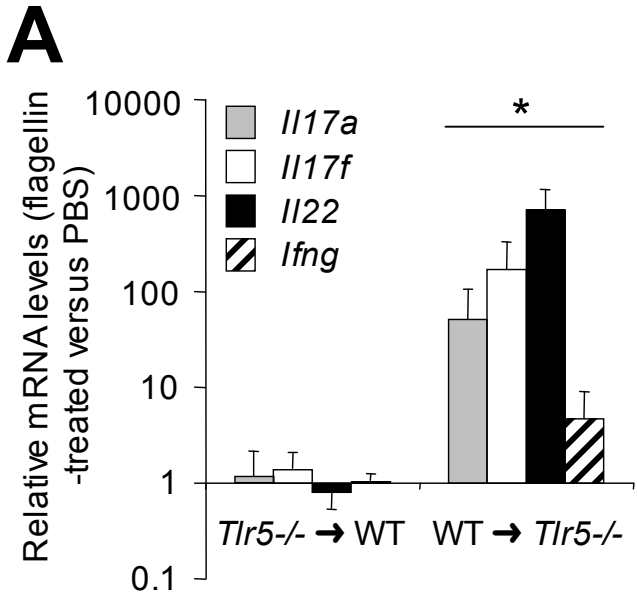
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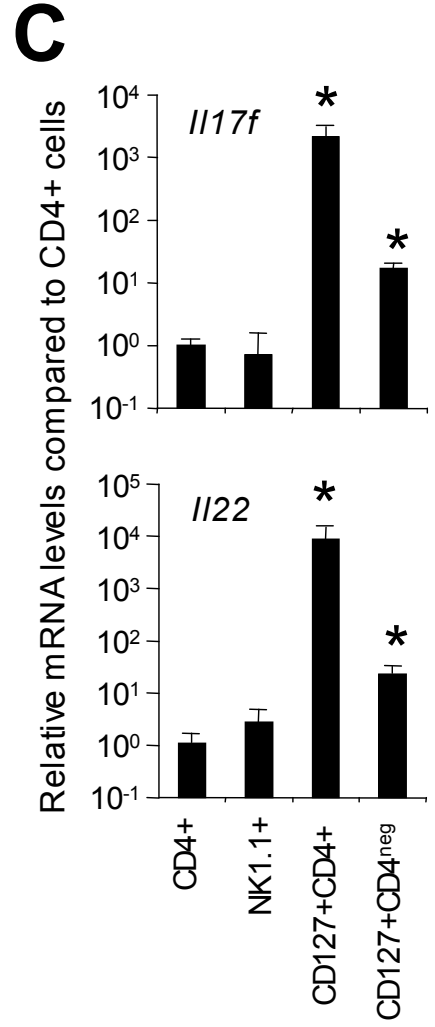
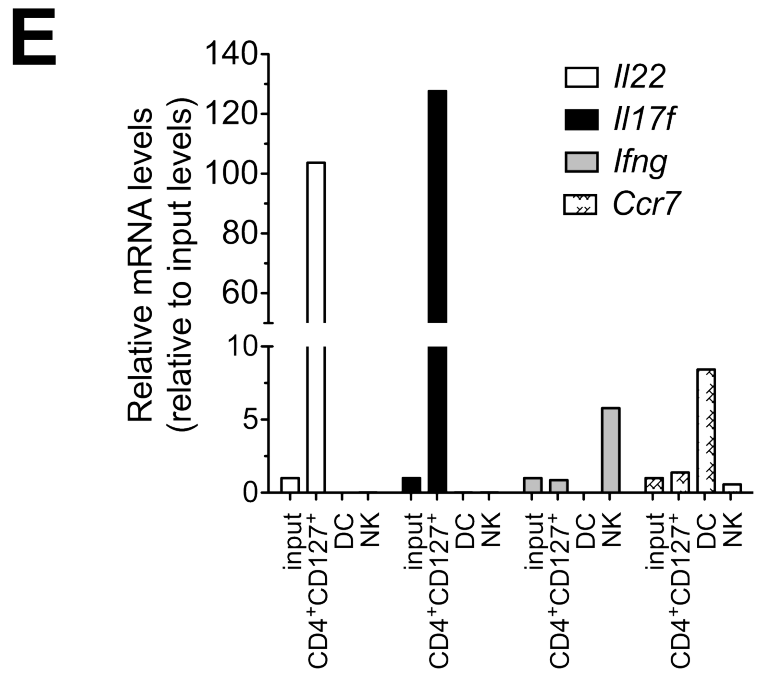
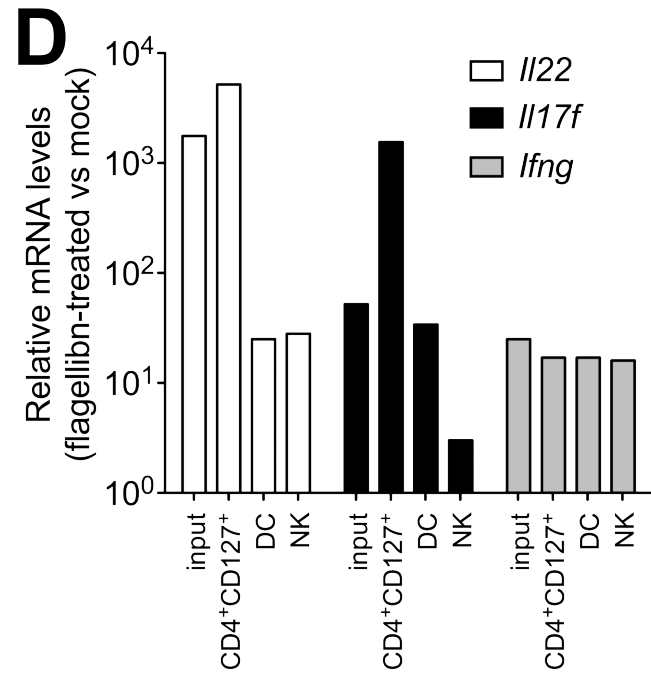
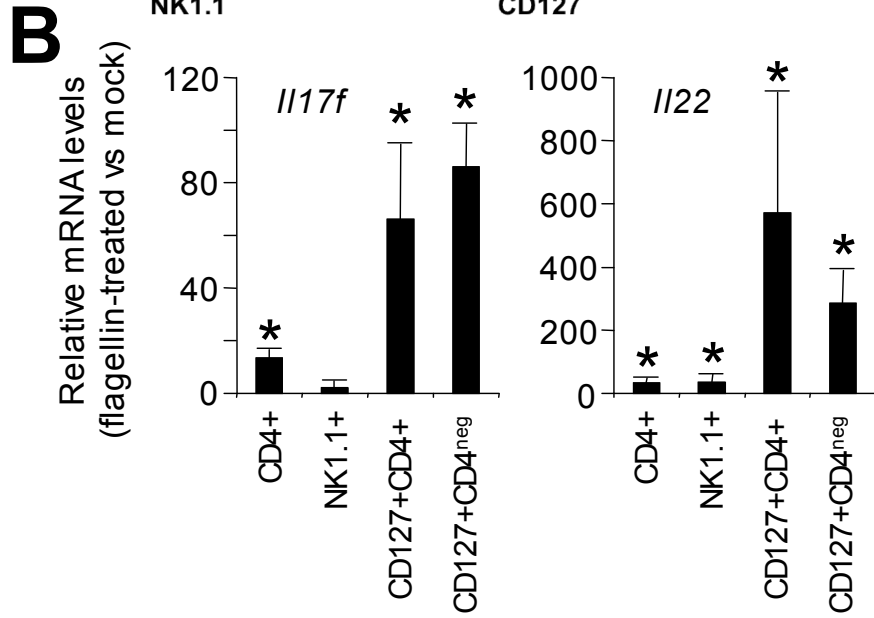
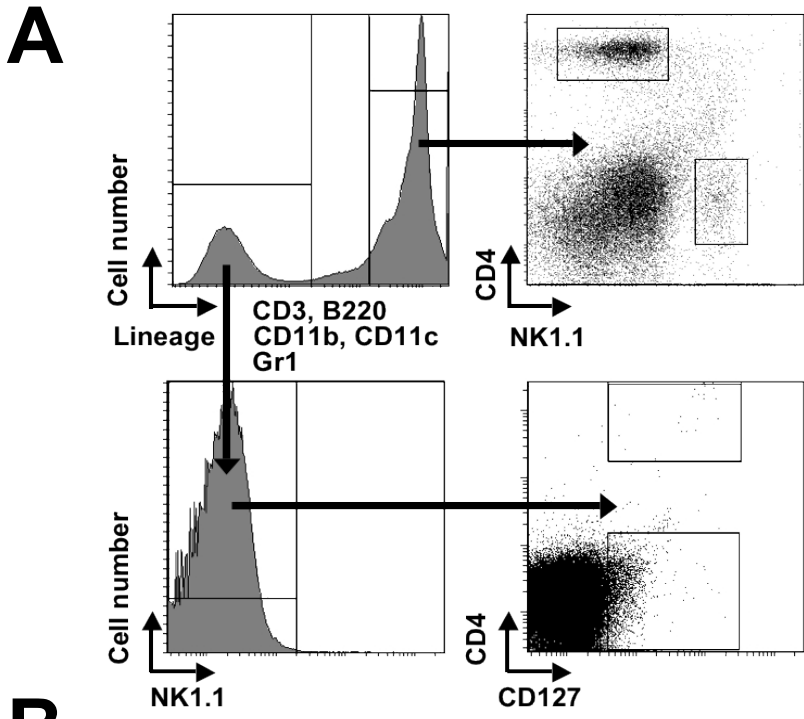
1 **Table Legends**

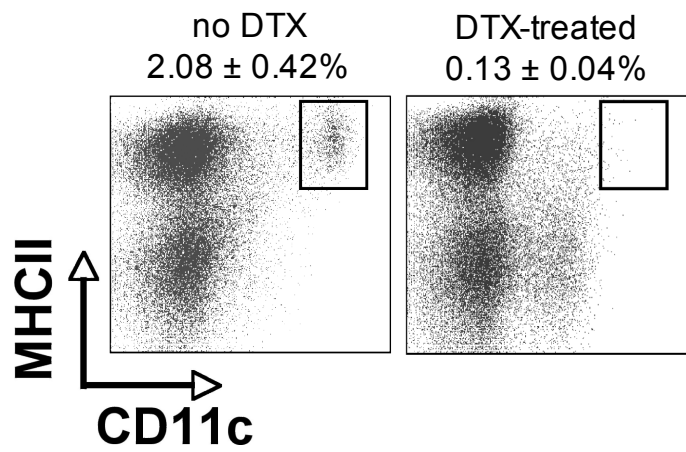
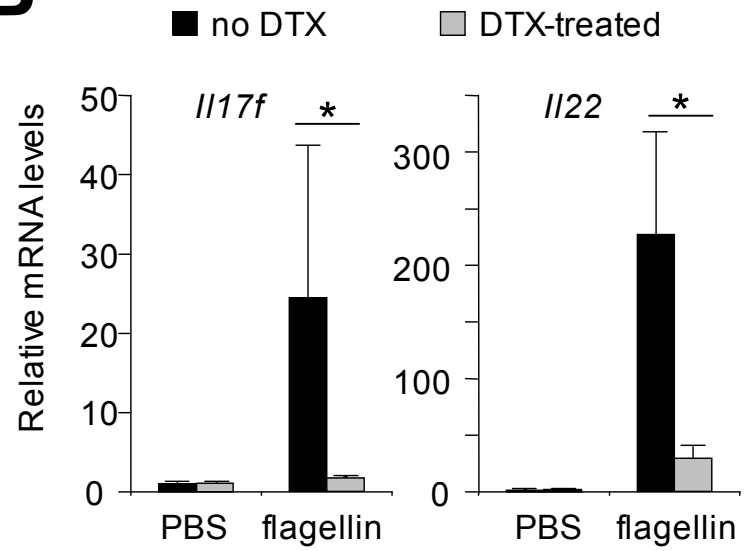
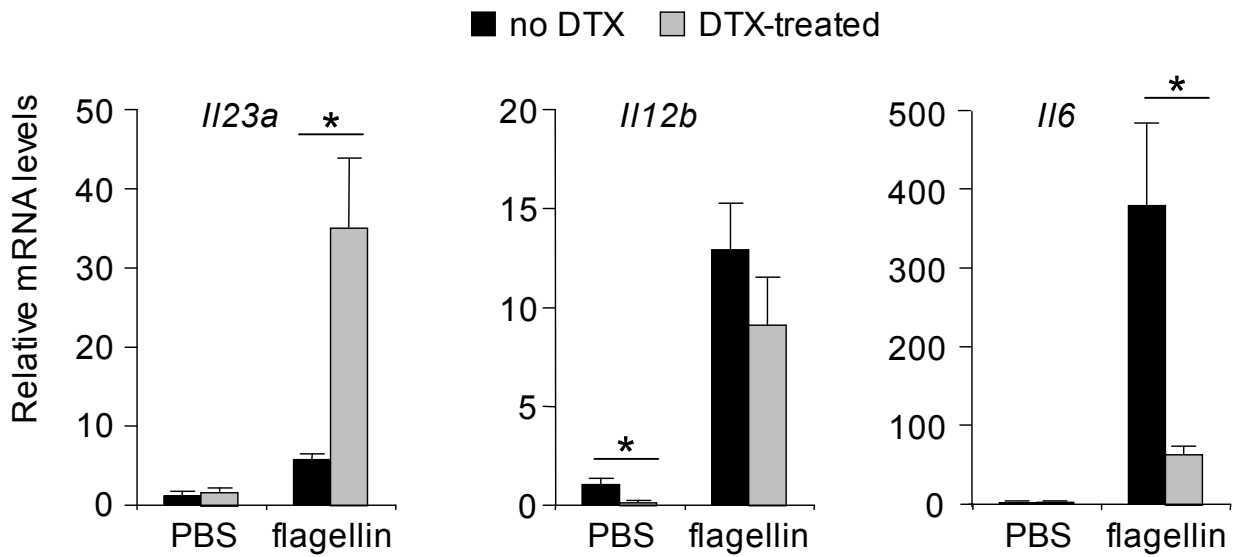
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3 **Table 1: Flagellin-induced gene expression in the ileum displays signatures that**
4 **are reminiscent of Th17- and TLR-mediated responses.** Microarray gene expression
5 data from the distal ileal segments of mice (n=3) treated i.p. with flagellin for 2h and 8h
6 was compared with data from mock animals in a time course analysis. All genes
7 differentially expressed and the corresponding method and annotation are included as
8 Fig. S5 and Table S2. The signs "o", "+", and "-" indicate similar expression,
9 upregulation and downregulation relative to mock animals. "§" indicates data from a
10 comparison between mock and 2h flagellin-treated animals.







A**B****C****D**