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Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells

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The control of damaging inflammation by the mucosal immune system in response to commensal and harmful ingested bacteria is unknown. Here we show epithelial cells conditioned mucosal dendritic cells through the constitutive release of thymic stromal lymphopoietin and other mediators, resulting in the induction of 'noninflammatory' dendritic cells. Epithelial cell–conditioned dendritic cells released interleukins 10 and 6 but not interleukin 12, and they promoted the polarization of T cells toward a 'classical' noninflammatory T helper type 2 response, even after exposure to a T helper type 1–inducing pathogen. This control of immune responses seemed to be lost in patients with Crohn disease. Thus, the intimate interplay between intestinal epithelial cells and dendritic cells may help to maintain gut immune homeostasis.

Epithelial cells (ECs) provide the first line of defense of the organism by preventing the entry of potentially dangerous microorganisms[1]. Nevertheless, bacteria can gain access across mucosal surfaces through specialized ECs called M cells[2]. In addition, dendritic cells (DCs) in the lamina propria are able to open the tight junctions between adjacent ECs to send dendrites out like periscopes to sample bacteria directly from the intestinal lumen[3]. These DCs sample both invasive pathogens and noninvasive commensal bacteria. The extension of the dendrites is regulated by expression of the fractalkine receptor (CX3CR1)[4]. Encounter with bacteria or bacterial products triggers the functional maturation of DCs that leads to the generation of powerful antigen-presenting cells[5,6]. The activation of DCs requires signaling via the class of pattern-recognition receptors called Toll-like receptors (TLRs)[6]. Because pathogens and commensal bacteria share many if not most TLR ligands (such as lypopolysaccharide, bacterial DNA and flagellin)[7], it is unclear how exaggerated inflammatory responses to these bacteria are avoided.

Unique functions have been attributed to mucosal DCs, such as the ability to preferentially promote T helper type 2 (T_H2) differentiation in *in vitro* T cell priming assays[8–12] and to induce B cells to secrete immunoglobulin A (IgA)[13], suggesting that mucosal DCs may be likely to induce a noninflammatory environment. Whether this is an intrinsic property of 'specialized' mucosal DCs or whether the mucosal microenvironment confers this functional phenotype is unknown. Involvement of the local microenvironment in 'dictating' DC function in the spleen has been proposed[14]. It is possible that the mucosal environment 'educates' DCs to mount default noninflammatory responses to preserve gut immune homeostasis. Because DCs are in close contact with ECs[3,15], we hypothesized that the latter might be involved in governing DC function. Here we show that culture of human monocyte–derived DCs (MoDCs) with EC supernatants from polarized Caco-2 cell monolayers or from primary ECs resulted in the induction of 'noninflammatory' DCs. This process was mediated by a combination of thymic stromal lymphopoeitin (TSLP) and other factors that are constitutively expressed by ECs. EC-conditioned DCs lost their ability to release interleukin 12 (IL-12) and to polarize T_H1 responses in response to bacteria. In patients with Crohn disease, expression of TSLP was undetectable in ECs and these cells could not induce the 'noninflammatory' DCs. Thus, homeostasis is maintained by physiological interaction between ECs and DCs, and when such communication is lost, T_H1-mediated intestinal pathologies could arise.

RESULTS

Human colon DCs promote T_H2 cell differentiation

Murine mucosal DCs preferentially promote T_H2 differentiation in *in vitro* T cell priming assays[8–12]. Therefore, we tested whether DCs isolated from human gut also have a bias in the induction of T_H2 differentiation. We isolated DCs from healthy human colon tissue and incubated them first for 24 h with the T_H1-inducing pathogen *Salmonella* enterica serovar *typhimurium* (SL-WT) and then for 5 d with allogeneic naive $CD4^+CD45RA^+$ T cells. Unlike MoDCs, human gut DCs were unable to induce T_H1 responses but instead induced T cell responses that were strongly polarized toward T_H2, even in the

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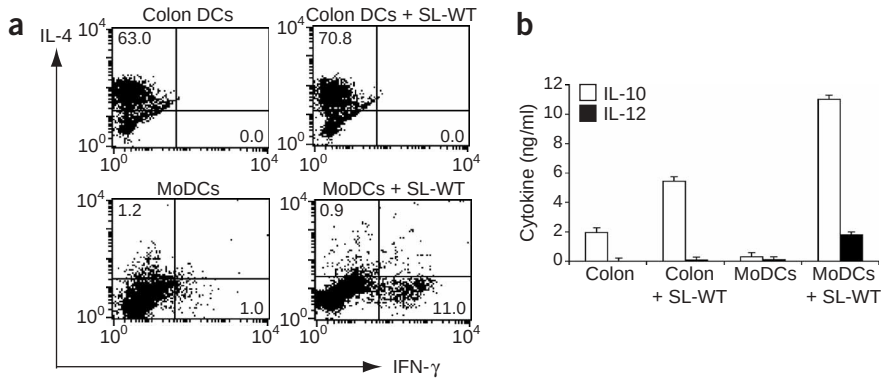

Figure 1 Primary human gut DCs are unable to release IL-12 and promote T_H2 cells in response to *S. typhimurium*. (**a**) Intracellular cytokine staining for IL-4 and IFN-γ in DCs isolated from healthy colon (top) or differentiated from monocytes (bottom), incubated for 24 h with (SL-WT) or without *S. typhimurium* and then incubated for 5 d with purified allogeneic naive $CD4^+$ T cells. Numbers in quadrants indicate the percentage of positive cells. Data are representative of three independent experiments. (**b**) Cytokines (IL-10 and IL-12p70 (IL-12)) in culture supernatants measured by ELISA after 24 h of incubation of MoDCs or colon DCs with *S. typhimurium*. Data are presented as mean ± s.d. of three replicates and are representative of three independent experiments.

presence of bacterial stimulation (**Fig. 1a**). This response was probably due to the inability of bacteria-activated DCs to release IL-12p70, a key cytokine in the induction of T_H1 cell responses[16]. In support of our results, bacteria-activated colon DCs released IL-10 but not IL-12 in response to *Salmonella typhimurium* (**Fig. 1b**). Colon-derived DCs in the absence of bacterial stimulation also promoted T_H2 cell polarization.

EC supernatants 'condition' DCs

We hypothesized that the unique functions of mucosal DCs might reflect an *in vivo* conditioning conferred by the local microenvironment, in particular by ECs that interact closely with the DCs, rather than reflecting intrinsic properties of specialized DC subsets. Thus, we incubated MoDCs with supernatants from the Caco-2 EC line, collected from the basolateral membrane of polarized epithelia. We first tested whether Caco-2 supernatants could induce DC activation. We found only a slight upregulation of the cell surface activation markers CD80 and HLA-DR after 24 h of incubation, versus a much greater increase for MoDCs activated with *S. typhimurium* (**Fig. 2a**). Moreover, the MoDCs were not functionally mature, as there was no induction of IL-10 or IL-12 release (**Fig. 2b**). Nevertheless, when Caco-2-conditioned MoDCs were incubated with allogeneic naive $CD4^+CD45RA^+$ T cells, they promoted greater T_H2 cell polarization (**Fig. 2c**) than that of MoDCs incubated with unconditioned medium. These results suggest that Caco-2 supernatants do not activate MoDCs but instead confer on them the ability to polarize T_H2 responses even in the absence of further stimuli, as with isolated gut DCs. Furthermore, a soluble factor that is constitutively released by ECs was probably responsible for the observed phenotype.

EC derived TSLP favors T_H2 polarization

During allergic inflammation, human ECs release large amounts of TSLP, a potent activator of DCs[17,18]. TSLP-activated DCs can induce the differentiation of allogeneic proallergic T_H2 cells[19] and cytotoxic T cells[20] and induce homeostatic proliferation of autologous $CD4^+$ T cells[21]. We speculated that TSLP could be constitutively released by ECs in physiological conditions and could be involved in 'educating' T_H2-promoting DCs. We tested TSLP expression in the Caco-2 cell line by quantitative RT-PCR and found that it was constitutively expressed (**Fig. 3a**). Because resting MoDCs do not express a functional TSLP receptor (TSLPR)[17], which is made up of the common IL-7 receptor α-chain (IL-7Rα) and TSLPR, we determined if its expression could be induced after exposure to Caco-2 supernatants. Caco-2 supernatants induced the expression of both IL-7Rα and TSLPR in MoDCs (**Fig. 3b**).

We next evaluated the possible involvement of TSLP in the T_H2-promoting ability of 'conditioned' DCs. TSLP expression was suppressed by small interfering RNA (siRNA) in one clone of Caco-2 cells (CL2) but not in another (CL1; **Fig. 3a**). DCs incubated with CL2 but not those incubated with CL1 supernatants lost their ability to drive

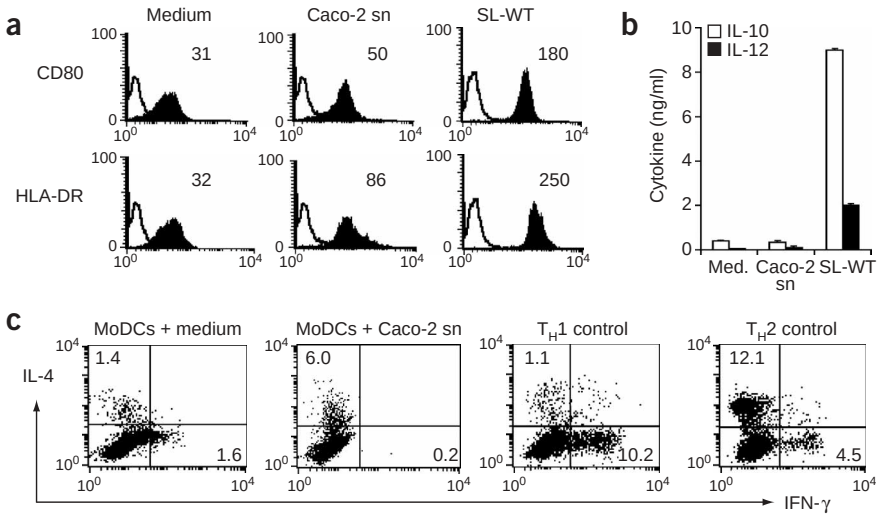

Figure 2 EC supernatants do not induce DC maturation but instead condition T_H2-promoting DCs. (**a**) CD80 and HLA-DR surface expression on MoDCs collected after 24 h of incubation with unconditioned medium (Medium) or with Caco-2 supernatant (Caco-2 sn) or *S. typhimurium* (SL-WT). Filled histograms, stained cells; open histograms, isotype controls. Numbers indicate mean fluorescence intensity. One of three independent experiments. (**b**) Cytokine release (IL-10 and IL-12p70), measured by ELISA, in culture supernatants collected 24 h after MoDCs were incubated as described in **a**. Med., medium only. Data are presented as mean ± s.d. of three replicates and are representative of four independent experiments. (**c**) Intracellular staining for IFN-γ and IL-4 in MoDCs incubated first for 24 h with unconditioned medium or with Caco-2 supernatants and then for 5 d with purified naive $CD4^+$ T cells. Positive controls: for T_H1, T cells activated with unconditioned MoDCs incubated with SL-WT; for T_H2, T cells activated with unconditioned MoDCs incubated with *L. plantarum*. Numbers indicate the percentage of positive cells in the quadrant. Data are representative of four independent experiments.

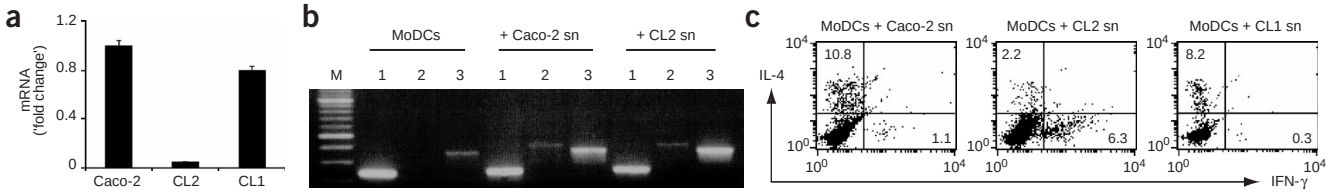

Figure 3 TSLP is constitutively expressed by Caco-2 cells and is able to confer T_H2-inducing ability. (**a**) TSLP mRNA quantified by real-time RT-PCR in the Caco-2 cell line (Caco-2) and in the TSLP siRNA–treated (CL2) or control-treated (CL1) clones. In each sample, mRNA is normalized to that of the 'housekeeping' gene *Gapd*. The 'fold change' in expression is in reference to Caco-2 cell mRNA (ref = 1). Data are presented as mean ± s.d. ($n = 4$) and are representative of five independent experiments. (**b**) Both TSLP receptor chains are induced by Caco-2 or CL2 supernatants. MoDCs were incubated for 24 h in medium (MoDC) or in supernatants of Caco-2 (Caco-2 sn) or CL2 (CL2 sn) cells, and TSLPR and IL-7Rα expression was assessed by RT-PCR. M, molecular weight marker. Lanes: 1, β-actin; 2, TSLPR; 3, IL7-Rα. Data are representative of two independent experiments. (**c**) The capacity of EC-conditioned MoDCs to skew T_H2 cells is lost in CL2-conditioned but not in the CL1-conditioned MoDCs. Intracellular staining for IFN-γ and IL-4 of naive $CD4^+$ T cells incubated for 5 d with Caco-2-, CL2- or CL1-conditioned MoDCs. Positive controls for IFN-γ-producing T cells (activated with unconditioned MoDCs incubated with SL-WT) and IL-4-producing T cells (activated with unconditioned MoDCs incubated with *L. plantarum*) are 13.8% and 11.2% of total T cells, respectively. Numbers indicate the percentage of positive cells in the quadrant. Data are representative of four independent experiments.

T_H2 cell polarization and instead induced T_H1 cells (**Fig. 3c**). Hence, EC-derived factors stimulate the expression of both chains of TSLPR on DCs, which confers the ability to respond to TSLP and to drive T_H2 responses.

EC-conditioned DCs do not drive T_H1 polarization

We assessed whether Caco-2-conditioned DCs that had acquired the ability to skew a T_H2 response could induce a T_H1 response after exposure to *S. typhimurium*. We used two *S. typhimurium* strains: the invasive wild-type strain (SL-WT) and a noninvasive strain (SL-*InvA*) with deletion of *InvA*, which is required to invade host cells. We first conditioned the DCs with Caco-2 supernatants and then incubated them with either invasive or noninvasive *S. typhimurium* derivatives. DCs incubated with medium alone or with Caco-2 supernatants showed similar responses to bacteria in terms of changes in morphology and upregulation of surface markers, such as CD80 and HLA-DR (**Supplementary Fig. 1** online). Caco-2-conditioned DCs released less IL-10 but no IL-12p70 ($P < 0.01$) in response to bacteria compared with DCs not incubated with Caco-2 supernatant (**Fig. 4a**). Whereas the reduction in IL-10 production was independent of TSLP, the suppression of IL-12 production was dependent on TSLP in EC supernatants. When TSLP was either suppressed by siRNA or neutralized by antibody to TSLP (anti-TSLP), IL-12 production was restored, albeit in lower amounts than in unconditioned DCs (**Fig. 4a**). This result suggests that other EC-derived factors also contribute to IL-12 inhibition.

Caco-2 supernatant also reduced IL-1β but not tumor necrosis factor (TNF) release by bacteria activated DCs (**Fig. 4b**). Similar to freshly isolated mouse Peyer's patch DCs[13], Caco-2-conditioned DCs acquired the ability to release IL-6 (**Fig. 4b**). We then tested the ability of Caco-2-conditioned DCs to drive T cell polarization after bacterial activation. Consistent with their inability to release IL-12, Caco-2-conditioned DCs were unable to drive T_H1 differentiation even after exposure to *S. typhimurium* (**Fig. 4c**). In contrast, CL2-conditioned DCs released IL-12 after bacterial encounter and induced T_H1 cell polarization (**Fig. 4c**) without losing their ability to release

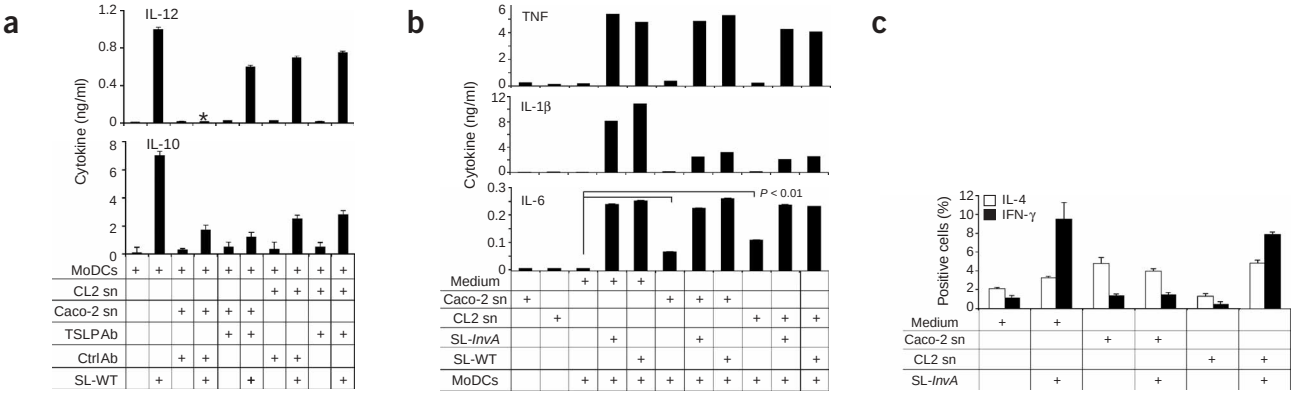

Figure 4 Caco-2-conditioned DCs release IL-6 constitutively but lose the ability to release IL-12 in response to bacteria and to drive T_H1 cell polarization by a TSLP-mediated mechanism. (**a**) Caco-2- or CL2-conditioned medium was pretreated with anti-TSLP (TSLP Ab) or sheep IgG control antibody (Ctrl Ab) or was left untreated, then was incubated with MoDCs. Conditioned MoDCs were subsequently exposed to bacteria for 24 h (SL-WT). Cytokine release was measured by ELISA in culture supernatants. Data are presented as mean ± s.d. of three replicates and are representative of two independent experiments. *, $P < 0.01$, IL-12 release after bacterial exposure by MoDCs incubated in Caco-2 supernatant versus MoDCs incubated in CL2 supernatant or TSLP-neutralized Caco-2 supernatant is highly significant. (**b**) Cytokine production, measured by ELISA, in culture supernatants by MoDCs conditioned by medium, Caco-2 or CL2 supernatants after exposure to bacteria (SL-WT or SL-*InvA*). Data are presented as mean ± s.d. of three replicates and are representative of three independent experiments. The difference in IL-6 release after conditioning MoDCs with Caco-2 or CL2 supernatant versus medium-conditioned MoDCs is highly significant ($P < 0.01$). (**c**) MoDCs were treated with supernatants of Caco-2 or CL2 cultures for 24 h and then incubated overnight with bacteria. The next day, the medium was changed and bacteria-activated conditioned DCs were incubated with allogeneic naive $CD4^+$ T cells for 5 d. Data represent the percentage of cells with positive intracellular cytokine staining for IFN-γ and IL-4 and are presented as mean ± s.d. of four independent experiments.

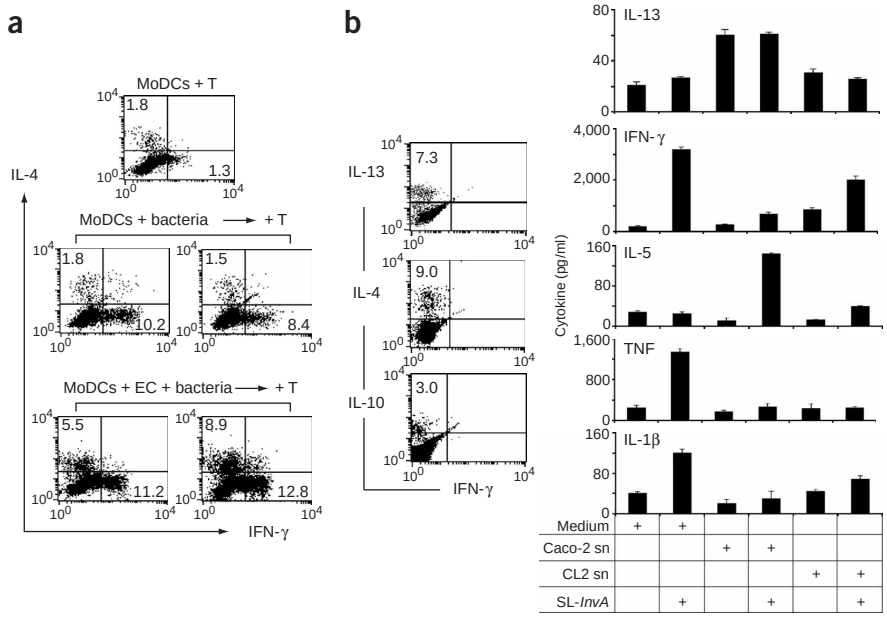

Figure 5 Unconditioned DCs drive T_H1 responses, whereas Caco-2-conditioned DCs induce typical T_H2 cells. (**a**) DCs were treated as follows: left unactivated, activated with bacteria (left, noninvasive SL-*InvA*; right, SL-WT) or seeded from the basolateral side of EC monolayers and activated with bacteria incubated apically. DCs were then incubated for 5 d with naive allogeneic $CD4^+$ T cells. T cells were then stained for intracellular IL-4 and IFN-γ. Data are representative of four independent experiments. Numbers indicate the percentage of positive cells in the quadrant. (**b**) Left, intracellular cytokine staining for IFN-γ and IL-13, IL-4 or IL-10 of naive allogeneic T cells incubated for 5 d with DCs treated for 24 h with Caco-2 supernatants. Positive controls for IFN-γ-producing T cells (activated with unconditioned MoDCs incubated with SL-WT) and IL-4 producing T cells (activated with unconditioned MoDCs incubated with *L. plantarum*) are 12.6% and 10.5% of total T cells, respectively. Right, IL-5, IL-13, IFN-γ, TNF and IL-1β release in culture supernatants, measured by ELISA or flow cytometry, of T cells activated with bacteria-treated, Caco-2-conditioned DCs. Data are presented as mean ± s.d. ($n = 3$) and are one experiment representative of four individual experiments.

IL-6 (**Fig. 4b**). We did not detect any differences in T cell yield either as the number of recovered T cells or as [^{3}H]thymidine-incorporating cells (**Supplementary Fig. 2** online), ruling out the possibility that one of the treatments of DCs was causing the death of a particular T cell type. These results indicate that like isolated gut DCs, Caco-2-conditioned DCs are likely to drive T_H2 responses even after strong T_H1-inducing stimuli. Moreover, EC-derived TSLP blocked IL-12 release by conditioned DCs, but other factors conferred their ability to release IL-6 or to reduce IL-10 production.

Unconditioned DCs can induce T_H1 responses

Next we sought to determine whether DC conditioning by ECs occurred immediately after DCs were seeded on ECs and if the concomitant presence of *S. typhimurium* would promote 'inflammatory' DCs. We attempted to mimic a physiological situation whereby DCs were seeded from the basolateral membrane of Caco-2 monolayers and bacteria were incubated from the apical face. This avoided the immediate binding of bacteria with DCs and allowed us to test whether DCs were conditioned by ECs before they contacted the bacteria. Apical salmonella drives the migration of DCs across the epithelial monolayer[22]. DCs open up the tight junctions between adjacent ECs and capture bacteria directly from the apical face[3]. After 2 h of incubation, we washed bacteria from the apical face and replaced the medium with medium containing antibiotics, to kill any remaining bacteria. We collected DCs 16 h later from the lower chamber and tested them in a T cell polarization assay. Like DCs incubated directly with *S. typhimurium*, DCs in our culture system were able to skew polarization of T cells toward T_H1 (**Fig. 5a**), probably because of the release of IL-12 (data not shown). DCs exposed to the EC monolayer also acquired the ability to induce T cells to produce IL-4, suggesting that some conditioning by ECs occurred in the time frame of the experiment (**Fig. 5a**). This observation probably reflects the proportion of DCs that were unable to directly contact the bacteria. Hence, these results suggest that DCs can activate T_H1 cells after encountering *S. typhimurium* if they can contact bacteria before being exposed to EC conditioning.

Conditioned DCs drive 'classical' T_H2 cells

TSLP-activated DCs induce proallergic inflammatory T_H2 cells that release IL-13, IL-5 and TNF but moderate amounts of IL-4 or IL-10 (ref. 19). However, we found that Caco-2-conditioned DCs induced 'classical' noninflammatory T_H2 cells producing IL-4, IL-5, IL-13 and IL-10 but not TNF or IL-1β (**Fig. 5b**). This result was expected, because in physiological conditions, mucosal tissues should be protected from unwanted inflammatory reactions. Two possibilities could explain this observation. First, in our system, EC-derived factors could block the induction of 'allergic' T cells. Second, TSLP could have a dose-dependent effect. The amount of TSLP expressed by Caco-2 cells or released in the supernatant was much lower than that used before[19], being below detection limit by immunoprecipitation (1 ng; **Fig. 6a**, lane 2). TSLP protein was detected by immunoprecipitation (**Fig. 6a**) only after incubation with *S. typhimurium*, which induced a two- to threefold upregulation of TSLP mRNA in Caco-2 cells (**Fig. 6b**). As expected, even after bacterial activation, TSLP protein was not expressed in the CL2, confirming that the gene was successfully silenced (**Fig. 6a**). Thus, ECs had low expression of TSLP, which was responsible alone or in combination with other EC factors for the induction of typical noninflammatory T_H2 cells.

We added exogenous TSLP to CL2 supernatants or to unconditioned medium before incubating these with the DCs. Consistent with the dose-dependent hypothesis mentioned above, the inhibition of IL-12 release after bacterial activation occurred only within a narrow 'window' of TSLP concentrations (0.07–0.15 ng/ml; **Fig. 6c**). Unconditioned MoDCs incubated with TSLP in the same range of concentrations also showed a reduction in IL-12 secretion (**Fig. 6c**). This observation suggests that TSLP alone is sufficient to reduce IL-12 release by MoDCs in response to bacteria; however, other EC-derived factors allow complete 'shutoff' of IL-12 release. Whether this additional effect of ECs relates to the ability of EC supernatants to upregulate the expression of a functional TSLPR on DCs remains to be established. Our data indicate that physiological amounts of TSLP do not induce DC maturation (**Fig. 2a**) or the release of IL-12 (**Fig. 4a–c**) or the induction of inflammatory T cells (**Fig. 5b**). Therefore, TSLP could act as a 'guardian' of the inflammatory response, as a narrow window of TSLP concentrations allowed the blockade of IL-12 release

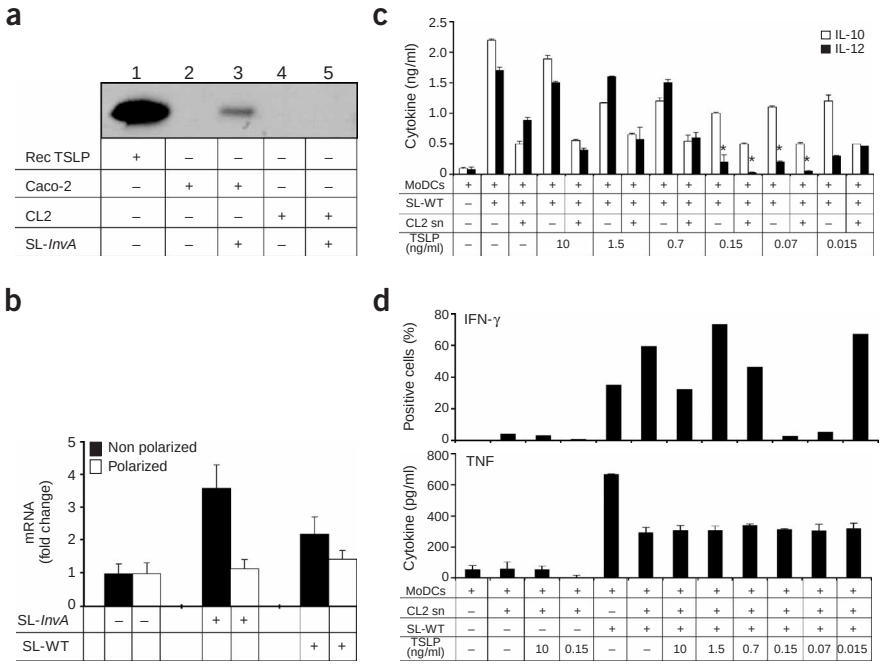

Figure 6 Bacteria upregulate TSLP expression, but 'rescue' of the CL2 phenotype occurs only within a narrow window of TSLP concentration. (**a**) Caco-2 cells (lanes 2,3) and CL2 cells (lanes 4,5) were treated with noninvasive *S. typhimurium* (SL-*InvA*) or were left untreated overnight, then were lysed and were incubated with anti-TSLP for immunoprecipitation, followed by immunoblot. Lane 1: recombinant TSLP (Rec TSLP; 10 ng) not subjected to immunoprecipitation (positive control). (**b**) Bacteria induce upregulation of TSLP only when seeded on nonpolarized Caco-2 cells. TSLP mRNA was quantified by real-time PCR after activation of polarized (open bars) or nonpolarized (filled bars) Caco-2 cells with invasive (SL-WT) or noninvasive (SL-*InvA*) *S. typhimurium*. For each sample, mRNA is normalized to that of *Gapd*. Data are presented as the 'fold change' with reference to unstimulated polarized or nonpolarized Caco-2 cells (ref = 1). (**c**) Sensitivity of DCs to TSLP conditioning. Recombinant TSLP was added in increasing concentrations (0.015–10 ng/ml; bottom row) to CL2 supernatant or to medium before incubation with MoDCs for 24 h. Conditioned MoDCs were treated with *S. typhimurium* (SL-WT). Culture supernatants were collected 24 h later and cytokine release (IL-10 and IL-12p70) was measured by ELISA. *, $P < 0.01$, IL-12 release induced by bacteria in CL2- or medium-conditioned DCs plus 0.07 or 0.15 ng/ml of TSLP versus no added TSLP. Basal IL-12 release by DCs without bacteria plus the various concentrations of TSLP was less than 0.1 ng/ml in all cases. Data are presented as mean ± s.d. of three replicates and are representative of two independent experiments. (**d**) MoDCs conditioned with TSLP concentrations that block IL-12 release are unable to promote T_H1 polarization. MoDCs were conditioned for 24 h with CL2 supernatant with the addition of TSLP (concentration, bottom row). Cells were then activated with SL-WT or were left unactivated, and were used to polarize naive T cells. Data represent the percentage of IFN-γ-positive cells and TNF release as measured by ELISA (mean ± s.d. of three replicates) and are representative of two independent experiments.

and this correlated with the inability of EC-conditioned DCs to skew T_H1 inflammatory responses. When the concentrations of TSLP were outside the small window described above, DCs were able to promote IFN-γ-producing T cells (**Fig. 6d**). T cells activated by EC-conditioned DCs also produced less TNF through a TSLP-independent mechanism (**Fig. 6d**), indicating that other EC factors contribute to limit inflammatory reactions. We did not detect any differences in the number of [^{3}H]thymidine-incorporating cells (**Supplementary Fig. 3** online), ruling out the possibility that one of the treatments of DCs was causing the death of a particular T cell type.

Having shown that TSLP concentration affects the ability of DCs to promote T_H1 cells, we tested whether bacteria upregulated the expression of TSLP when seeded on the apical side of polarized Caco-2 monolayers. TSLP expression increased when bacteria were seeded on nonpolarized but not on polarized Caco-2 cells, suggesting that TSLP was upregulated only after entrance of free *S. typhimurium* into the epithelial layer (**Fig. 6b**). Moreover, the expression of TSLP is likely to be controlled by bacteria-membrane cell contact, as noninvasive *S. typhimurium* also induced TSLP upregulation (**Fig. 6b**). Thus, our data suggest that bacteria crossing the epithelial barrier can bind to basolateral membrane receptors and induce TSLP upregulation. Whether this is a way to induce T_H1 responses after bacterial entrance into the epithelial layer remains to be established.

Colon ECs constitutively express TSLP

To determine whether the ability of ECs to 'educate' DCs was a feature unique to the Caco-2 cell line or if it could be generalized, we tested

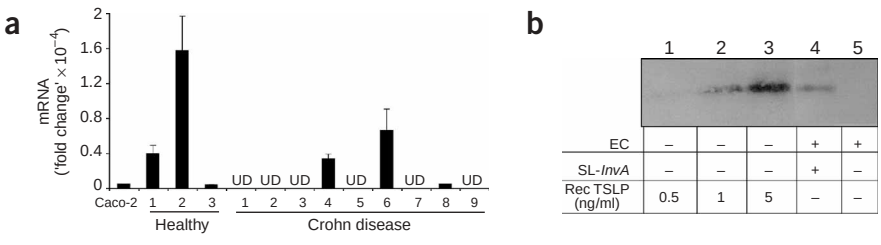

Figure 7 TSLP is constitutively expressed by isolated primary ECs of healthy but not diseased tissue. (**a**) TSLP mRNA, quantified by real-time RT-PCR, in primary ECs isolated from three healthy people (Healthy, 1–3). For each sample, mRNA is normalized to that of *Gapd* and is presented as a 'fold change' relative to *Gapd*. ECs isolated from colon of nine patients with Crohn disease were also analyzed for TSLP mRNA expression (Crohn disease, 1–9). UD, undetectable. Data are presented as mean ± s.d. of three independent quantitative PCR analyses. (**b**) Immunoblot of TSLP expression in primary ECs after immunoprecipitation with anti-TSLP. Isolated primary colon ECs (4×10^6) were treated with *S. typhimurium* (lane 4) or were left untreated (lane 5) overnight, then were lysed and were immunoprecipitated with anti-TSLP. Lanes 1–3, immunoprecipitation of fixed amounts of TSLP (0.5, 1 and 5 ng; Rec TSLP). Data are representative of three experiments using samples from three different donors.

the expression of TSLP by freshly isolated gut cells. Similar to Caco-2 cells, ECs isolated from human healthy colon constitutively expressed TSLP, as shown by quantitative RT-PCR analysis (**Fig. 7a**). Despite the finding that the mRNA expression of TSLP was variable among the various healthy samples analyzed, the protein was always below the detection limit by immunoprecipitation (less than 1 ng) unless cells were incubated with bacteria (**Fig. 7b**). We then tested whether the T_H1-mediated pathology in inflammatory bowel diseases such as Crohn disease[23] is due to deregulation of TSLP expression. Six of nine patients with Crohn disease analyzed had undetectable TSLP mRNA (**Fig. 7a**). These results suggest that TSLP is constitutively expressed by gut ECs and that pathological conditions may result in the deregulated expression of TSLP.

DISCUSSION

We have shown here that similar to the mouse system, human gut DCs are also likely to induce a T_H2 response. This property was not intrinsic to mucosal DCs, as MoDCs were able to acquire the 'mucosal' phenotype when exposed to gut ECs. Thus, EC-derived factors induced the expression of both IL-7Rα and TSLPR, required for the formation of a functional TSLPR, and allowed MoDCs to respond to small amounts of TSLP. This 'tight' crosstalk between ECs and DCs may regulate the generation of inflammatory responses to enteric bacteria. We found that EC-conditioned DCs were phenotypically matured after encountering *S. typhimurium* but were unable to release IL-12 or to drive T_H1 responses. However, it is likely that *in vivo* other EC-derived factors contribute to the skewing of 'classical' T_H2 polarization, as DCs isolated from the gut promoted a much stronger T_H2 response than did EC-conditioned DCs. Moreover, like mouse Peyer's patch DCs, EC-conditioned DCs acquired the ability to produce constitutively IL-6, which drives the development of IgA-producing plasma cells[13]. Our results support the hypothesis that in steady-state conditions, mucosal tissues favor the induction of mucosal immune responses, including the polarization of T cells toward a T_H2 phenotype and the production of IgA[24]. This is consistent with a report showing that oral administration of cholera toxin B subunit induces mainly T_H2 polarization, whereas transcutaneous immunization promotes strong T_H1 responses[25], suggesting that rather than reflecting an intrinsic propensity of cholera toxin B subunit to induce T_H2 responses, the immunological outcome depends mainly on the environment in which the toxin is applied.

Our data suggest that resident mucosal DCs that are in close contact with ECs are conditioned by EC-released TSLP and are unable to induce inflammatory T_H1 responses even to T_H1-inducing pathogens. Thus, the homeostasis of the gut is preserved through the continuous generation of noninflammatory helper T cells and antibody responses that limit bacterial entrance. This is consistent with published data showing that commensal bacteria are not simply 'ignored' or tolerized by the immune system; instead, they promptly induce IgA responses that are mediated by mucosal DCs[26,27]. A study showing the presence of individual intestinal villous M cells[28] further strengthens our results, because it indicates the necessity of keeping inflammatory reactions at bay, as bacteria could enter throughout the intestinal wall.

How then can protective T_H1 responses that are necessary for fighting intracellular pathogens[29–33] be raised against *S. typhimurium*? Here, unconditioned DCs seeded from the basolateral membrane of EC monolayers shortly before apical bacterial addition were able to creep between ECs and to drive T_H1-polarized responses. Hence, we believe that only unconditioned, newly recruited DCs can mount protective T_H1 responses. During salmonella infection, ECs release CCL20 (refs. 34,35), which attracts CCR6-expressing immature DCs at various epithelial sites[15,36]. It is likely that after salmonella infection, DCs are recruited from nearby areas or from the blood and are therefore not subjected to EC conditioning. The immunostimulatory environment specifically generated by infection with invasive salmonella through the release of inflammatory cytokines such as IL-8, IL-1β and TNF[37] allows unconditioned DCs to respond to bacteria and to promote protective T_H1 responses. Moreover, TSLP may function as a 'guardian' for the establishment of inflammatory reactions. A narrow window of TSLP concentrations regulates the capacity of DCs to respond to bacteria and to release IL-12. In fact, at TSLP concentrations outside this window, DCs regain the capacity to release IL-12 and to promote T_H1 cells. Because our data suggest bacteria can induce TSLP upregulation only when attached to the basolateral membrane of ECs, we envisage that the entrance of bacteria across the EC barrier results in large increases in local concentration of TSLP, which favors the generation of T_H1-promoting DCs. Therefore, the initiation of T_H1 responses to invasive salmonella could depend on two concomitant events: the probability that unconditioned DCs will contact and capture salmonella before the anti-inflammatory environment modulates their function, and the ability of salmonella to breach across the gut wall and to upregulate expression of TSLP.

Finally, we have shown that deregulation of TSLP expression could be crucial in the establishment of intestinal inflammatory bowel disease. Nearly 70% of the patients with Crohn disease that we analyzed had undetectable TSLP mRNA, and this correlated with the inability of ECs to control IL-12 release by bacteria-activated DCs (data not shown). Moreover, our preliminary data indicated that DCs isolated from patients with Crohn disease had a natural propensity to drive T_H1 responses without stimulation and that this reflected the higher proportion of T_H1 cells found in patients with Crohn disease than in healthy people. Hence, in pathological conditions, the deregulated control of DC function by ECs could disrupt intestinal homeostasis.

In conclusion, our results suggest the existence of a default mechanism mediated by TSLP released constitutively by ECs that helps maintain the homeostasis of the gut by generating a noninflammatory environment. Defects in this mechanism may induce unwanted T_H1 inflammatory responses, resulting in inflammatory bowel disease. Future studies should determine whether this mechanism is influenced by the presence of probiotic or commensal bacteria, given their regulatory effect on EC function[38–40], and if re-establishment of physiological amounts of TSLP can help restoration of gut immune homeostasis in patients with Crohn disease.

METHODS

DC and EC generation or purification. MoDCs were generated from $CD14^+$ monocytes of healthy people according to a slightly modified protocol[41]. Monocytes were purified by positive selection with anti-CD14 coupled to magnetic beads (Miltenyi). $CD14^+$ cells were incubated for 6 d in complete medium containing 50 ng/ml of granulocyte-monocyte colony-stimulating factor and 20 ng/ml of IL-4 (Peprotech) to obtain immature DCs.

Colon DCs and ECs were isolated from healthy tissue of colon carcinoma (more than 7 cm from the neoplasm) or from colons of patients with Crohn disease (noninflamed areas) according to a protocol established in our laboratory. After surgical excision, mucosa and submucosa were separated from the muscular tunicae. The tissue was dissected with forceps and scissors in 30 ml of medium (Hanks balanced salt solution) without Ca^{2+} and Mg^{2+}, containing 1 mM dithiothreitol, and samples were stirred for 15 min at 37 °C. The suspension was filtered through nylon mesh (70 μm in pore diameter). The filtered solution consisted mainly of enterocytes (95% positive for the EC marker NCL-ESA), which were used for conditioning experiments. The remaining tissue was incubated with 30 ml Hanks balanced salt solution without Ca^{2+} and Mg^{2+}, containing 5 mM EDTA, and was stirred for 15 min at 37 °C.

The suspension was filtered through nylon mesh; the filtered solution was discarded, whereas the tissue was resuspended in 10 ml RPMI medium plus 2% FCS containing 1 g liberase per 100 ml, 20 μg/ml of DNAase, 10 U/ml of hyaluronidase and antibiotics (50 μg/ml of gentamicin and 500× amphotericin B). The tissue was microdissected and was incubated for 2 h at 37 °C. At the end of the reaction, the enzymatic action was blocked with 5 mM EDTA for 10 min at 37 °C. The suspension was filtered through nylon mesh and only the filtered solution was kept. The solution was centrifuged and the cells (containing lamina propria DCs and lymphocytes) were resuspended in Hanks balanced salt solution before separation on a Percoll gradient. The Percoll gradient allowed the separation of lamina propria DCs from lymphocytes. Cells were washed with PBS and their purity was assessed by flow cytometry. All experimental procedures are in compliance with guidelines set by our internal review board (European Institute of Oncology, Milan, Italy).

Bacterial strains. The following *S. typhimurium* strains on SL1344 background were provided by G. Dougan (Imperial College, London, UK): wild-type invasive strain, SL1344 WT; noninvasive strain SL-*InvA*, SPI-I (*InvA*$^-$). SL-*InvA* is defective in *InvA* and is unable to form the productive type three secretion system required for invasion of host cells. Bacteria were grown at 37 °C in Luria broth, supplemented with appropriate antibiotics to preserve carried mutations. *Lactobacillus plantarum* was grown at 37 °C without agitation in MRS broth (Biokar Diagnostic).

Activation of DCs across EC monolayers. Caco-2 cells were cultured for 7–10 d in the upper chambers of Transwell filters (3 μm in pore diameter; Costar) until a transepithelial resistance of 300 Ohm × cm^2 was achieved. Filters were turned upside-down and DCs were cultured for 4 h on the filter facing the basolateral membrane of ECs to allow the cells attach to the filter. Filters were then turned upside-down again into 24-well plates. The Transwells were left untreated or were treated directly with bacteria (10 bacteria to 1 EC) from the apical surface (upper chamber). At 1 h after incubation, bacteria were washed out and the medium was replaced with medium containing antibiotics (100 μg/ml of gentamicin). DCs were collected after 16 h from the lower chamber. DCs were detached from filters by gentle centrifugation and were analyzed by flow cytometry for surface activation markers CD83, CD80 and HLA-DR (all from Pharmingen).

EC and DC conditioning and bacterial activation. Caco-2 or CL2 cells were grown on Transwell filters as described above. Supernatants were collected when the transepithelial resistance was 300 Ohm × cm^2. Alternatively, supernatants were collected after overnight culture of primary ECs. MoDCs were incubated for 24 h with medium alone or with Caco-2, CL2 or EC supernatants. For neutralization experiments, Caco-2 and CL2 supernatants were incubated overnight at 4 °C with sheep anti–human TSLP or with control sheep IgG (both from R&D Systems) before conditioning of MoDCs. Next, conditioned DCs were activated for 1 h by bacteria (10 bacteria to 1 DC) in medium without antibiotics. The medium was replaced with medium containing 100 μg/ml of gentamicin and cells were cultured overnight. Cells were collected and were analyzed for acquisition of activation markers (CD80, CD83 and HLA-DR; Pharmingen) by flow cytometry. Culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA; IL-6, IL-10 and IL-12 p70; R&D systems) or by flow cytometry (IL-1β and TNF Flowcytomix; Bender Medsystems).

Immunohistochemistry. MoDCs conditioned with medium alone or with Caco-2 supernatant were treated with bacteria as described above. Activated cells were processed for immunohistochemistry after seeding for 30 min on gelatin-coated coverslips to allow attachment. Cells were fixed for 20 min at 25 °C, with 3% paraformahaldehyde in PBS and were blocked and permeabilized for 1 h with 3% BSA and 0.01% Triton in PBS. The DAKO Envision Doublestain System (Dako cytomation) was used for staining according to manufacturer's instructions with mouse primary anti–human HLA-DR (Pharmingen).

DC–T cell cocultures. DCs conditioned with Caco-2, CL2 or CL2 supernatant plus TSLP recombinant protein (0.015–10 ng/ml) or incubated with medium alone were incubated with bacteria (10 bacteria per DC) or were left without bacterial incubation as described above and then were incubated with allogeneic CD4$^+$CD45RA$^+$ purified T cells (Miltenyi) in 48-well plates at a ratio of 1:5 (DC/T cell). After 5 d of culture, supernatants were collected and were directly analyzed by ELISA for IFN-γ, TNF and IL-13 (from R&D systems) or by flow cytometry detection (IL-5 and TNF Flowcytomix; Bender Medsystems), according to the manufacturers' instructions. For cytokine intracellular staining, T cell cultures were restimulated for 4 h with phorbol 12-myristate 13-acetate plus ionomycin and then with brefeldin A for additional 2 h. Cells were collected, fixed and permeabilized with Cyto Fix/Perm (Becton Dickinson). Intracellular staining was done with phycoerythrin-conjugated anti-IL-4, anti-IL-10 and anti-IL-13 and with fluorescein isothiocyanate–conjugated anti-IFN-γ (all from Pharmingen). Stained cells were analyzed by flow cytometry.

Analysis of human TSLPR and human IL-7Rα expression. MoDCs (1 × 10^6 cells) were incubated for 24 h in medium alone or in Caco-2 or CL2 supernatants. Total RNA was isolated using TRIZOL reagent (InVitrogen) according to the manufacturer's recommendations. Reverse transcription (SuperScript II; Gibco BRL) used 1 μg of total RNA. The cDNA obtained was used for PCR. Primers for human TSLPR amplification covered the region of nucleotides 158–541; primers for the amplification of human IL-7Rα (hIL7RA) covered the region of nucleotides 68–451 (numbering starts at the start codon). Primer sequences were as follows: TSLPR forward, 5′-GGAC CAACCTGACTTTCCACTACAG-3′, and reverse, 5′-AACACTTCTCGGCATC CAAGC-3′; and hIL7RA, forward, 5′-GCTATGCTCAAAATGGAGACTTGG-3′, and reverse, 5′-AGACGACACTCAGGTCAAAAGGAG-3′. Reactions were heat-denatured for 3 min at 95 °C and then were amplified with 40 PCR cycles each comprising successive incubations at 95 °C for 1 min, at 55 °C 1 min and at 72 °C for 30 s. A further extension step was done at 72 °C for 7 min. PCR reactions were normalized by analysis of expression of the gene encoding β-actin (*Actb*) with the following primers: forward, 5′-GGGTCAGAAG GATTCCTATG-3′, and reverse, 5′-GGTCTCAAACATGATCTGGG-3′. The GeneAmp PCR System 9700 (Applied Biosystems) was used for all PCR. Amplicons were identified by ethidium bromide staining of 1.5% agarose gels.

TSLP RNA interference and quantitative PCR. TRIZOL (InVitrogen) was used for extraction of mRNA from 1 × 10^6 cells (Caco-2 cells or purified human colon ECs purified as above). TSLP expression was measured by quantitative RT-PCR (TaqMan; Beckton Dickinson) with amplicons covering base pairs 529–645 (numbering starts at start codon). Primer sequences were as follows: forward, 5′-CCCAGGCTATTCGGAAACTCAG-3′, and reverse, 5′-CGCCACAATCCTTGTAATTGTG-3′.

Expression of TSLP was 'knocked down' by silencing with siRNA using the pSuper RNAi system (OligoEngine). Caco-2 cells were transformed using pSuper RNAi by the calcium phosphate method according to standard protocols. Two siRNA sequences specific for TSLP were used (starting from nucleotide 120 (5′-GCAGCCTATCTCAGTACTA-3′) or nucleotide 254 (5′-TCCCACCGCCGGCTGCGCG-3′), respectively), but only one was successful in silencing TSLP mRNA. Therefore, two clones (CL1 and CL2) were generated, of which only one (CL2) showed 'silencing' of TSLP. CL1 was used as control, as scrambled sequences are too unrelated to the active probe[42]. Moreover, the functional control of admixing CL-2 supernatant with recombinant protein showed that the effect was due to TSLP.

TSLP immunoprecipitation. Caco-2 cells or primary ECs were grown to subconfluence and were activated for 1 h by bacteria (10 bacteria to 1 EC) in medium without antibiotics. The medium was replaced with medium containing gentamicin (100 μg/ml) and cells were cultured overnight. Cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 2 mM phenylmethylsulfonyl fluoride, 50 μg/ml of aprotinin and leupeptin. For immunoprecipitation procedures, 5 μg antibody/mg total protein was incubated overnight at 4 °C, with gentle rotation, with protein G bound to Sepharose beads (Zymed). After several washes with lysis buffer, this complex was added to the lysates and to the cell supernatants and samples were incubated overnight at 4 °C, with gentle rotation.

After several washes with lysis buffer, sample buffer (30% glycerol, 5% SDS, 0.1 M Tris, pH 6.8, 8% 2-mercaptoethanol and 0.01% bromophenol

blue) was added and samples were boiled for 5 min. Immunoprecipitated proteins were then separated by SDS-PAGE and transferred onto nitrocellulose filters. For immunoblots, filters were incubated for 2 h at 25 °C in TBS (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 5% of milk. Filters were then incubated for 1 h at 25 °C with anti-TSLP (0.3 μg/ml; R&D systems). After three washes with TBS plus 0.2% Tween 20, the filters were incubated with secondary antibody for 30 min at 25 °C. After three washes in TTBS plus 0.2% Tween 20, TSLP was immunodetected by enhanced chemiluminescence (Amersham).

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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