

## RESEARCH ARTICLE

# Secretory IgA in complex with *Lactobacillus rhamnosus* potentiates mucosal dendritic cell-mediated Treg cell differentiation via TLR regulatory proteins, RALDH2 and secretion of IL-10 and TGF- $\beta$

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The importance of secretory IgA in controlling the microbiota is well known, yet how the antibody affects the perception of the commensals by the local immune system is still poorly defined. We have previously shown that the transport of secretory IgA in complex with bacteria across intestinal microfold cells results in an association with dendritic cells in Peyer's patches. However, the consequences of such an interaction on dendritic cell conditioning have not been elucidated. In this study, we analyzed the impact of the commensal *Lactobacillus rhamnosus*, alone or associated with secretory IgA, on the responsiveness of dendritic cells freshly recovered from mouse Peyer's patches, mesenteric lymph nodes, and spleen. *Lactobacillus rhamnosus*-conditioned mucosal dendritic cells are characterized by increased expression of Toll-like receptor regulatory proteins [including single immunoglobulin interleukin-1 receptor-related molecule, suppressor of cytokine signaling 1, and Toll-interacting molecule] and retinaldehyde dehydrogenase 2, low surface expression of co-stimulatory markers, high anti- versus pro-inflammatory cytokine production ratios, and induction of T regulatory cells with suppressive function. Association with secretory IgA enhanced the anti-inflammatory/regulatory *Lactobacillus rhamnosus*-induced conditioning of mucosal dendritic cells, particularly in Peyer's patches. At the systemic level, activation of splenic dendritic cells exposed to *Lactobacillus rhamnosus* was partially dampened upon association with secretory IgA. These data suggest that secretory IgA, through coating of commensal bacteria, contributes to the conditioning of mucosal dendritic cells toward tolerogenic profiles essential for the maintenance of intestinal homeostasis.

*Cellular & Molecular Immunology* (2017) 14, 546–556; doi:10.1038/cmi.2015.110; published online 14 March 2016

**Keywords:** commensal bacteria; dendritic cells; secretory IgA; TLR regulatory proteins; T regulatory cells

## INTRODUCTION

The mucosal surface of the gastrointestinal tract is in constant interaction with the luminal microenvironment, which contains ever-changing commensal microbiota, as well as a variety of bacterial, viral and protozoan pathogens. In this highly antigenic environment, the mucosal immune system must remain hyporesponsive (tolerant) to food antigens, self-antigens and commensal bacteria, while initiating protective responses to pathogens to prevent periodic host infection. In contrast to the systemic compartment, which needs to remain sterile, mucosal surfaces are capable of coping with various challenges while preserving homeostasis. Symbiotic control of the microbiota by the intestinal immune system relies on the modulation

of dendritic cells (DCs) and T cells by intrinsic characteristics associated with microbial-associated molecular patterns.<sup>1–4</sup> Moreover, beyond contributing to host defense against intestinal pathogens, secretory IgA (SIgA) governs the quantitative and qualitative control of the commensal microbiota composition and prevents systemic dissemination.<sup>5–8</sup>

SIgA is the most abundant antibody molecule on the mucosal surfaces of humans and most other mammals.<sup>9,10</sup> While other isotypes are rapidly degraded, intact SIgA molecules have been regularly demonstrated in samples from mucosal surfaces, even in the presence of large numbers of microorganisms. The stability of SIgA depends largely on its secretory component, which is covalently bonded to Fc $\alpha$  and masks potential proteolytic cleavage

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Received: 15 September 2015; Revised: 2 December 2015; Accepted: 2 December 2015

sites in this region.<sup>11</sup> These properties make SIgA particularly well suited to operate with preserved functionality in the enzymatically hostile environment that prevails on gut mucosal surfaces. Furthermore, the presence of SIgA at mucosal surfaces requires that pro-inflammatory processes are kept under control, a feature that is essential to the maintenance of the integrity and functionality of the epithelial barrier.<sup>10,12,13</sup>

We have previously demonstrated that commensal or pathogenic bacteria in complex with SIgA in the lumen are targeted to underlying DCs in the subepithelial dome region of Peyer's patches (PP),<sup>14</sup> but do not interact with lamina propria DCs. This result most likely indicates that this pathway is important to ensure appropriate communication between the luminal contents and the associated immune system. The transport of SIgA-based immune complexes across microfold (M) cells in PPs and capture by local CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>DCs in the subepithelial dome region<sup>15,16</sup> suggests that this process is the primary pathway by which commensal bacteria are exposed to these antigen-presenting cells. DCs in the intestinal lamina propria and MLNs exhibit tightly regulated expression of surface markers that contribute to the induction of Foxp3<sup>+</sup>T regulatory (Treg) cells<sup>17,18</sup> and the production of IL-10 and TGF- $\beta$  under the influence of constitutive environmental signals,<sup>19</sup> including interaction with commensal bacteria.<sup>20</sup> The association of SIgA with bacteria may therefore have an impact on the conditioning of PP and MLN DCs, but this has, to our knowledge, never been investigated.

To examine this hypothesis, we exposed DCs freshly isolated from mouse mucosal PPs and MLNs, or from the spleen, to the commensal-representative bacterium *Lactobacillus rhamnosus* (LPR), alone or in complex with SIgA (SIgA-LPR). Comparison of DC responsiveness with respect to several immunologic markers relevant to the mechanistic dissection of DC conditioning toward a tolerogenic profile was investigated. The results support the concept that the tissue from which DCs are isolated is important in the regulation of immune patterns involved in commensal bacteria sensing, and also reveal the so far unknown contribution of SIgA to the process of DC conditioning at both the mucosal and systemic level.

## MATERIALS AND METHODS

### Mice

Female BALB/c mice (Jackson Laboratory) with conventional microbiota were purchased from Charles River France (L'Arbresle, France). DO11.10 TCR transgenic mice<sup>21</sup> were used as a source of naïve OVA-specific T cells. Female C57BL/6 mice (Jackson Laboratory) were used as a source of splenocytes for a mixed lymphocyte reaction assay. Mice were housed in the animal facility of the Centre Hospitalier Universitaire Vaudois under conventional conditions prior to use at the age of 6–8 weeks. All experiments were performed upon approval of the State of Vaud Veterinary Office.

### Source of SIgA

Culture supernatants of the mouse hybridoma cell clone IgAC5, specific for *Shigella flexneri* serotype 5a LPS, grown at 37 °C in

RPMI-1640 medium complemented with 10% fetal calf serum were used as a source of IgA.<sup>22</sup> To reconstitute SIgA, equimolar amounts of purified polymeric IgAC5 mAb and recombinant mouse secretory component were combined as described.<sup>22</sup>

### Bacteria

The commensal strain *Lactobacillus rhamnosus* (LPR) CGMCC 1.3724 was cultured according to a previously published protocol.<sup>23</sup> Washed bacteria were resuspended at a concentration of  $1 \times 10^8$  CFU/ml. Pure bacterial cultures or bacterial cultures associated with 0.5  $\mu$ g of reconstituted SIgA at room temperature for 1 h<sup>16</sup> were then incubated with DCs isolated from various tissues. For SIgA-LPR complex observations (Figure 1A), SIgA were labeled with indocyanin-3 as previously reported,<sup>15</sup> and the formation of complex between LPR and SIgA-Cy3 was verified using a Zeiss LSM 710 Meta confocal microscope (Carl Zeiss, Jena, Germany). Images were captured with a 63X objective and processed using ZEN 2009 light edition (Zeiss).

### MACS isolation of PP, MLN and splenic DCs and naïve CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells

For preparation of tissue-derived DCs, the procedure of Kadaoui and Corthésy<sup>15</sup> was followed. The resulting purity of CD11c<sup>+</sup> DCs (referred to as totDCs hereafter) was 90–95%.

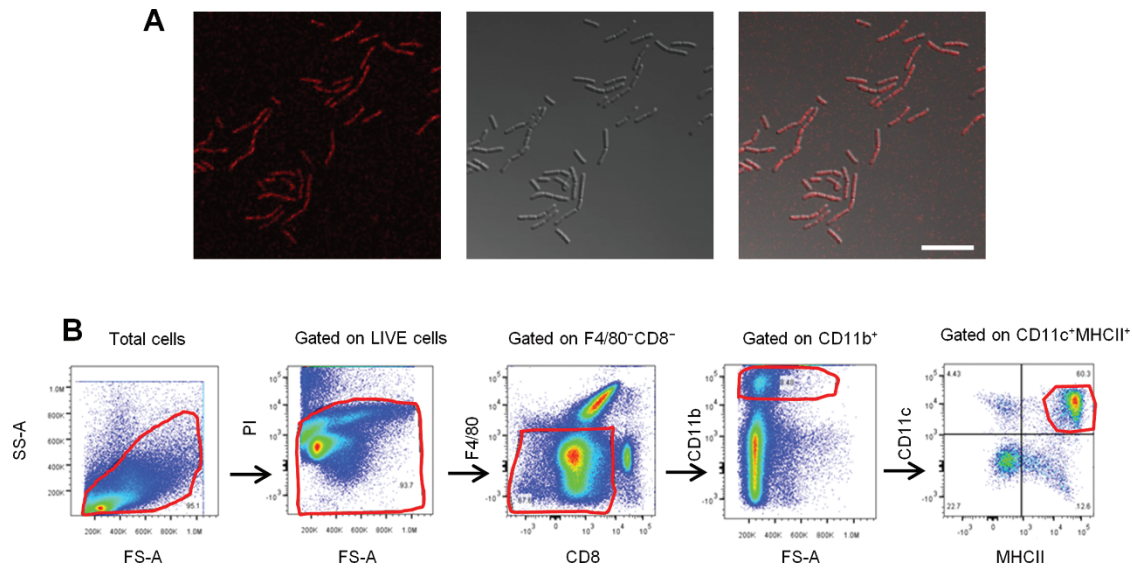
Naïve CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells from DO11.10 mice were isolated from spleen (processed as above) using a CD4<sup>+</sup>CD62L<sup>+</sup> T cell isolation kit II (Miltenyi Biotec) supplemented with biotinylated anti-CD25 mAb; a purity of 90–95% was achieved.<sup>24</sup>

### Conditioning of totDCs from PPs, MLNs and the spleen

DCs isolated by magnetic cell sorting from various tissues were cultured in plain medium serving as a control or conditioned *in vivo* with LPR alone (ratio of 2 bacteria per DC, based on titration experiments<sup>25</sup>), LPR in complex with SIgA, SIgA alone, or challenged with 2  $\mu$ g ml<sup>-1</sup> *E. coli* LPS (*E. coli*, 055: B5; Calbiochem) overnight in 100  $\mu$ l of DMEM complemented with 5% fetal calf serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, 1 mM Na-pyruvate, 10 mM HEPES (pH 7.3) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

### Phenotyping of conditioned DCs

Overnight conditioned DCs were washed twice in PBS containing 1% fetal calf serum and incubated in the same solution with blocking rat anti-mouse CD16/32 mAbs (BD Biosciences) for 20 min at 4 °C. DCs were then labeled with appropriate combinations of mAbs specific for CD11c (APC-conjugated), CD11b (PE-Cy7-conjugated), MHC-II (PE-conjugated), F4/80 (PerCP-Cy5.5-conjugated), CD8 (Pacific Blue-conjugated), CD40 (FITC-conjugated), CD80 (FITC-conjugated), CD86 (FITC-conjugated), or isotype controls (BD Biosciences, Allschwil, Switzerland) for 30 min at 4 °C. Cells were washed and re-suspended in PBS-1% FCS prior to analysis using a LSR II flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA). After initial gating on side and



**Figure 1** Visualization of SlgA-LPR complexes and DC gating strategy. **(A)** Observation of association of LPR with SlgAC5-Cy3 by laser-scanning confocal microscopy (see Materials and Methods); bacteria are visualized by differential interference contrast or by co-localization with SlgAC5-Cy3 as a merged picture. Bars: 5  $\mu$ m. **(B)** DC phenotyping (see Materials and Methods). Gating strategy used for the identification of the CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DC subtype used in the study. DCs were detected following the exclusion of dead cells, selection for F4/80-negative and CD8-negative cells, and then positively selected for CD11b, CD11c and MHCII surface markers. Plots are from one experiment and are representative of at least four independent experiments.

forward scatters, dead cells (propidium iodide staining), F4/80-positive and CD8-positive cells were excluded. Positive selection for CD11c, CD11b and MHCII was then carried, resulting in the isolation of CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs (Figure 1B). An analysis of CD40, CD80 and CD86 surface markers was performed on this particular phenotype with relative changes in expression displayed as the ratio between experimental levels and DCs kept in plain medium (CTRL in figures).

### Real time RT-PCR

PPs, MLNs and splenic (plain or conditioned) CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs were sorted using a FACS Aria II cell sorter (BD Biosciences), following the gating strategy given above. Total RNA was isolated using a Qiagen RNeasy Mini Kit and cDNA was generated with the Superscript First-Strand Synthesis System for RT-PCR and random hexamer primers (Invitrogen) according to the manufacturer's protocols. Two microliters of the reverse transcription reaction containing cDNA was used as a template for quantitative real-time PCR using SYBR Green Master Mix (Roche Applied Science), and gene-specific primers. Sequences of the sense and anti-sense primers were as follows: retinaldehyde dehydrogenase 1 (*Aldh1a1*), 5'-ATGGTTTAGCAGCAGGACTCTTC-3' and 5'-CCAGACATCTTGAATCCACCGAA-3'<sup>26</sup>; retinaldehyde dehydrogenase 2 (*Aldh1a2*), 5'-GACTTGTAGCAGCTGTCTTCACT-3' and 5'-CACCCATTTCTCTCCATTTC-3'<sup>26</sup>; single immunoglobulin interleukin-1 receptor-related molecule (*Sigirr*), 5'-GTGGCTGAAAGATGGTCTGGCAT TG-3' and 5'-CAGGTGAAGTTCCATAGTCTCTGC-3'<sup>27</sup>; suppressor of cytokine signaling 1 (*Socs1*), 5'-CTGCGCTTCTATTGGGGAC-3'

and 5'-AAAAGGCAGTCGAAGGTCTCG-3'<sup>28</sup>; Toll-interacting molecule (*Tollip*), 5'-TTGGCTATGTGCCATTGC-3' and 5'-AGCTTTGAGGTCCTTTCATTACAG-3'<sup>29</sup>; glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5'-CCAGGTTGTCTCC TGGCAG-3' and 5'-CCTGTTGCTGTAGCCGTATTCA-3'. PCRs and analyses were performed using a ABI Prism sequence detection system 7500 (PE Applied Biosystems). PCR conditions were as follows: T<sub>denaturation</sub>: 95 °C, 15 s; T<sub>annealing and extension</sub>: 60 °C, 1 min; 40 cycles. Gene expression was calculated relative to *Gapdh*. The comparative C<sub>T</sub> method ( $\Delta C_T$ <sup>30</sup>), where C<sub>T</sub> represents the cycle number where the SYBR green fluorescence crossed the threshold value set at 0.2, was used to assess relative changes in mRNA levels between samples.

### Cytokine/chemokine secretion by DCs from PPs, MLNs and the spleen

Isolated PP-, MLN- or spleen-derived CD11c<sup>+</sup> DCs ( $1 \times 10^5$  cells) were conditioned or stimulated as indicated above in a final volume of 200  $\mu$ l. Insufficient DCs from the CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> subset could be sorted from PPs for this particular analysis, so we had to compare total MACS-isolated CD11c<sup>+</sup> DCs (referred to as totDCs) from each tissue. Production of IL-12p70, KC, TNF- $\alpha$ , IL-6, and IL-10 by conditioned/activated totDCs was measured in supernatants collected after overnight incubation using Multiplex array technology according to the manufacturer's instructions (Life Technologies, Zug, Switzerland) in a BioPlex 200 instrument (Bio-Rad Laboratories, Reinach, Switzerland). Due to particular sample preparation prior to analysis, TGF- $\beta$  was measured by a sandwich ELISA following the procedure provided by the

supplier (R&D Systems, Abingdon, UK). Cytokine and chemokine levels are expressed as pg/ml (Table 1).

### Stimulation of T lymphocytes

Following overnight conditioning with LPR alone, SigA-LPR or LPS, sorted PP and MLN, and splenic CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs ( $2.5 \times 10^4$ ) were loaded with the OVA 323–339 peptide ( $2 \mu\text{g ml}^{-1}$ ), followed by incubation with freshly isolated naïve CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells ( $2.5 \times 10^5$ ) from transgenic DO11.10 mice for 72 h. No other stimulus was provided to the co-cultures. <sup>3</sup>H-thymidine (0.5  $\mu\text{Ci}$ ) was added to each culture for the last 18 h of the incubation period. The cells were then harvested on glass fiber filters on a PHD cell harvester (Cambridge Technology, Watertown, MA) and proliferation was assessed as the amount of the incorporation of <sup>3</sup>H-thymidine into cell DNA, as measured by  $\beta$ -scintillation counting of the harvested samples. Stimulation indices were measured relative to the CTRL set arbitrarily at 1.0. Conversion of naïve CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells into Treg cells was assessed after 72 h of incubation: cells were stained with anti-CD25 and -CD4 mAbs (BD Biosciences), whereas intracellular Foxp3 expression was evaluated by using an anti-mouse Foxp3 staining kit (eBioscience, San Diego, CA). The percentages of converted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs were determined on an identical number (25 000) of CD4<sup>+</sup> T cells for each experimental condition. In neutralizing experiments, mAbs against mouse IL-10 (JESS-16E3, BD Biosciences) or TGF- $\beta$  (1D11.16.8, BioXcell, West Lebanon, NH) or IgG isotype controls were added to the DC-T cell cultures at a final concentration of  $5 \mu\text{g ml}^{-1}$ .

### Suppression assay

The suppressive capacity of converted Tregs was assessed with a mixed lymphocyte reaction (MLR) assay. Spleens were harvested from BALB/c or C57Bl/6 mice and a single-cell suspension was prepared. After lysis of red blood cells with 155 mMNH<sub>4</sub>Cl-10 mM KHCO<sub>3</sub> lysis buffer (Sigma-Aldrich), cells were washed with PBS, re-suspended in complete medium and tested for viability. Then,  $1 \times 10^5$  responder cells from BALB/c mice were co-cultured with  $1 \times 10^5$  irradiated (3000 rads) stimulator cells from C57BL/6 mice in a 96-well round-bottom plate together with different numbers of sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells from DC cultures previously incubated with SigA-LPR or LPR in a total volume of  $200 \mu\text{l well}^{-1}$ . Expression of Foxp3 by DC-converted CD4<sup>+</sup>CD25<sup>+</sup>Treg cells was confirmed by flow cytometry. After 3 days of culture, cells were pulsed with 1  $\mu\text{Ci}$  of <sup>3</sup>H-thymidine for 18 h. Cells were harvested and <sup>3</sup>H-thymidine incorporation was measured by  $\beta$ -scintillation counting.

### Statistical analysis

The significance of the data were evaluated by nonparametric one-way ANOVA with Bonferroni's multiple comparison test or a paired *t*-test. The results are depicted as the mean  $\pm$  SD of *n* determinations. Differences were considered to be significant when *p* values < 0.05. Graph generation and statistical analyses were performed using GraphPad Prism software version 6.

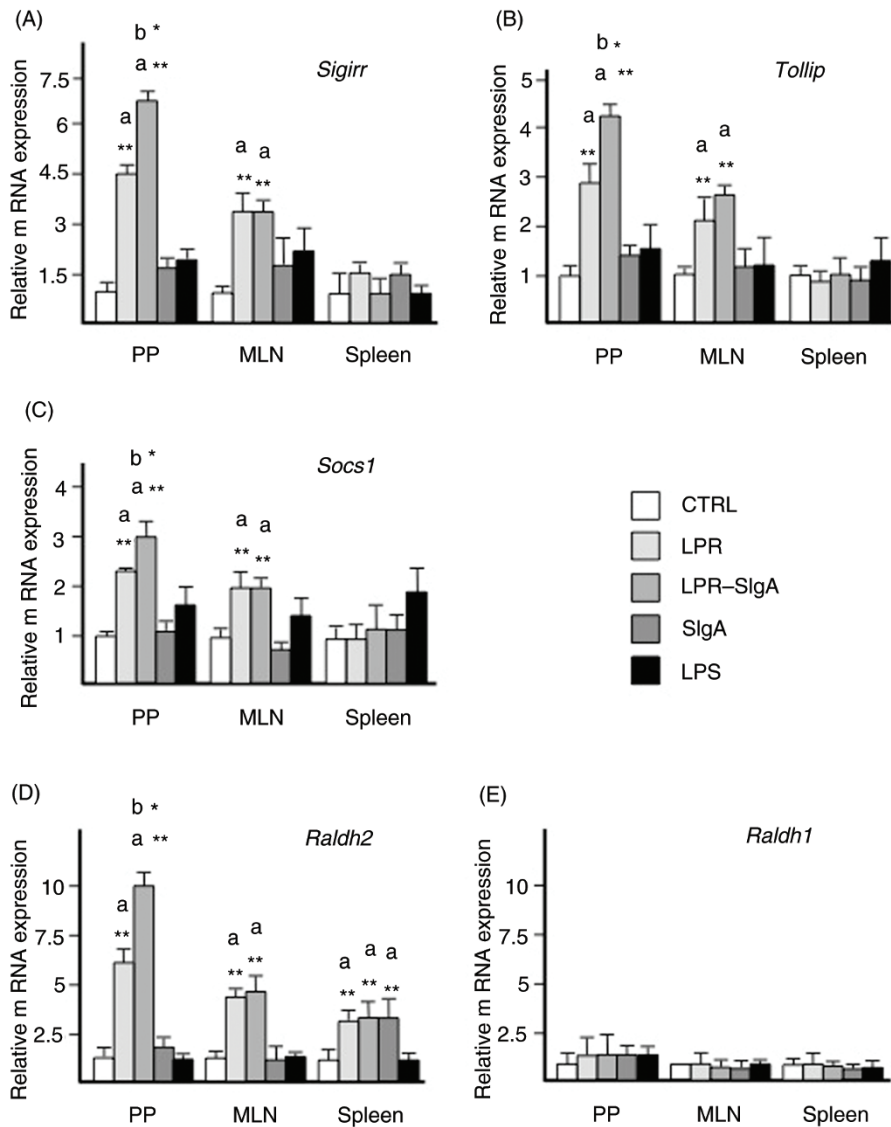
## RESULTS

### Pattern of expression of TLR regulatory proteins in mucosal and splenic conditioned DCs

In steady-state, although intestinal DCs express less TLR2 and TLR4 than their splenic counterparts,<sup>31,32</sup> this expression pattern does not satisfactorily explain how the gut mucosal surface discriminates between symbiotic and pathogenic bacteria.<sup>33</sup> We hypothesized that differential expression of TLR regulatory proteins (SIGIRR, Tollip and SOCS1) in mucosal and splenic CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs (Figure 1B) following conditioning with LPR or SigA-LPR may serve as a mechanism to restrict intestinal DC overreaction. SIGIRR, a protein located at the plasma membrane of immature DCs, sequesters adaptor proteins and prevents signaling via TLR4.<sup>34</sup> Tollip is an adaptor molecule that binds TLR2 and TLR4 to inhibit MyD88-mediated activation and blocks IL-1 receptor associated kinases (IRAKs) function in the TLR pathway.<sup>35</sup> Finally, SOCS1 acts by suppressing cytokine signaling and serves as a negative regulator of TLR4 and 9 signaling upon targeting of IRAK1.<sup>36</sup> As examined by qRT-PCR in sorted CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs, incubation with LPR alone significantly upregulated mRNA expression in DCs isolated from PPs and MLNs, but no effect could be detected on splenic DCs (Figure 2A–C). Because the commensal bacterium LPR in complex with SigA was shown to associate *in vivo* with the CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>DC subtype in PPs,<sup>16</sup> we sought to examine whether such an interaction can further modulate the expression of TLR regulatory proteins in DCs isolated from PPs, MLNs and spleen. The association of SigA with LPR did increase the expression of the three tested genes in a statistically significant manner only in PPs. SigA *per se*, or LPS serving as an indicator of cell fitness and responsiveness post-isolation, had no significant effect. These results indicate that conditioning of mucosal DCs with LPR, and as identified here with SigA-LPR complexes in PPs, upregulates the expression of molecules that dampen TLR signaling.

### Pattern of expression of vitamin A metabolizing enzymes in mucosal and splenic conditioned DCs

The differential expression of RALDH enzymes by mucosal tissues compared with spleen and peripheral lymph nodes suggests that these enzymes are important for the topical production of retinoic acid and the induction of DCs with a tolerogenic phenotype.<sup>37</sup> We therefore examined whether cellular proteins known to intervene in the differentiation of mucosal tolerogenic DCs could be regulated via exposure to LPR or SigA-LPR complexes. Incubation with LPR induced higher expression of *Raldh2* mRNA in PPs than in splenic DCs (Figure 2D). Consistent with the presence of commensal-SigA immune complexes in PPs, SigA bound to LPR further promoted the expression of *Raldh2* mRNA in DCs isolated from this tissue but not in DCs recovered from draining MLNs or spleen. No change in the level of expression of *Raldh1* mRNA was observed in either mucosa-derived or in splenic DCs (Figure 2E), arguing for a specific effect observed in the induction of the *Raldh2* gene. In comparison with the control,



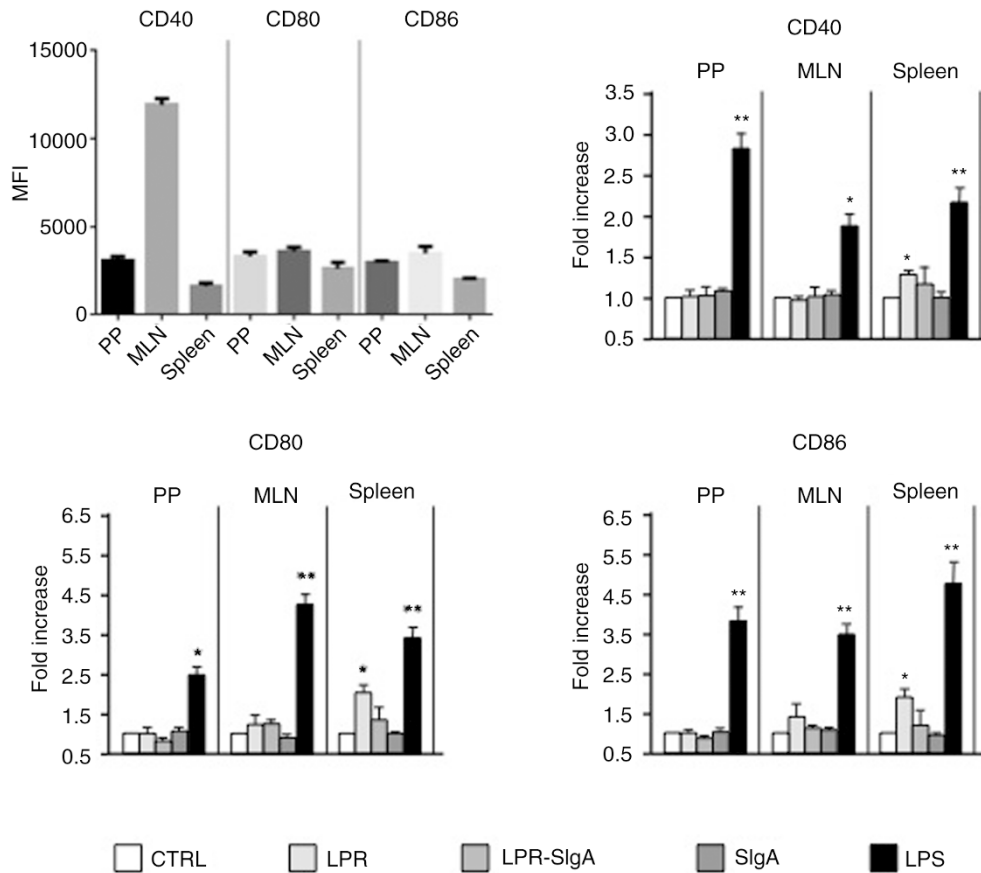
**Figure 2** Analysis by real-time RT-PCR of the expression of TLR regulatory proteins *Sigirr* (A), *Tollip* (B), *Socs1* (C), and of the vitamin A metabolizing enzymes *Raldh2* (D) and *Raldh1* (E) by conditioned, FACS-sorted PP-, MLN- and spleen-derived CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs. All RNA samples were isolated from two independent groups of four animals and analyzed in triplicates. For all gene products tested, the relative expression level was normalized to *Gapdh* RNA with the control (CTRL) set at 1. Data pooled from the two groups are shown as the mean  $\pm$  SD. The significance of the data were evaluated by nonparametric one-way ANOVA with Bonferroni's multiple comparison test (a) or paired *t*-test (b). "a" indicates comparison with CTRL and "b" indicates comparison between LPR-conditioned DCs and SigA-LPR-conditioned DCs \*, *p* values < 0.01; \*\*, *p* values < 0.005.

stimulation with LPS did not change the level of RNA expression for the two forms of RALDH tested (Figure 2D and E). This leads us to conclude that the sensing of the SigA-LPR complex by PP DCs increases the expression of an enzyme instrumental to the production of retinoic acid involved in local immune regulation, particularly at the mucosal level.

**Pattern of surface activation markers on mucosal and splenic conditioned DCs**

Given the observation that TLR regulatory proteins and RALDH2 are upregulated in mucosal D11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs, we next sought to determine whether conditioning with LPR- and SigA-LPR modulates surface expression

of the activation markers CD40, CD80 and CD86, the basal level of expression of which is depicted in Figure 3, upper left panel. In comparison with co-incubation with LPS, which induced strong co-stimulatory marker upregulation, no increase in the surface expression of either marker was detected in PP and MLN DCs exposed to LPR, SigA-LPR or SigA (Figure 3), which is indicative of a tolerogenic phenotype.<sup>38</sup> By contrast, incubation of LPR alone with spleen-derived DCs showed an increased expression of CD40, CD80 and CD86 compared with control cells, although to a lower extent than that induced by LPS. This result indicates that tissue origin leads to a different degree of DC activation with respect to commensal bacteria, with modulation by SigA occurring for splenic DCs only.



**Figure 3** Expression of surface activation markers of conditioned, gated CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs from mouse PPs, MLNs and the spleen. Upper left panel shows background level of expression of CD40, CD80 and CD86. Panels CD40, CD80 and CD86 show changes in expression of the three activation markers post-DC conditioning as a function of the control (CTRL) arbitrarily fixed at 1 and plotted as fold increases. All DC samples were isolated from 3 independent groups of 4 animals and analyzed in triplicate. Data pooled from the three groups are depicted as the mean  $\pm$  SD. The significance of the data were evaluated by nonparametric one-way ANOVA with Bonferroni's multiple comparison test compared with CTRL. \*, *p* values < 0.05; \*\*, *p* values < 0.01.

### Pattern of cytokine/chemokine secretion by conditioned mucosal and splenic DCs

We then sought to examine how the incubation of LPR alone or in complex with SigA, or controls, with DCs, affected their release of pro-inflammatory (IL12p70, KC, TNF- $\alpha$ , IL-6) and anti-inflammatory (IL-10, and TGF- $\beta$ ) cytokines. Because an insufficient quantity of DCs from the CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> subset were sorted from PPs to then allow measurable amounts of cytokines post-incubation with diverse stimuli, we had to rely on cytokine production from the conditioning of total MACS-isolated CD11c<sup>+</sup> DCs (referred to as totDCs in the next two paragraphs) from each tissue. LPR had no significant effect on IL-12p70, KC and TNF- $\alpha$  production by PP and MLN totDCs (Table 1), whereas LPR stimulated secretion by spleen totDCs (*p* = 0.01). Association with SigA significantly decreased LPR-induced production of the three cytokines in splenic totDCs only (*p* = 0.008). Exposure of PP, MLN and splenic totDCs to LPR moderately increased IL-6 production (0.02  $\leq$  *p*  $\leq$  0.006). A down-regulating effect mediated by SigA was observed in splenic totDCs only (*p* = 0.01). Overall, these results show that the pattern of secretion of

the pro-inflammatory cytokines IL-12p70, KC, TNF- $\alpha$ , but not IL-6, is associated with a poor responsive state of mucosal DCs to LPR and SigA-LPR. By contrast, SigA dampens the effect of bacterial exposure in the case of splenic DCs, as was observed for activation markers (Figure 3). Consistent with its activating properties, SigA elevated a response to LPS that could be observed in totDCs recovered from all the tissues (0.008  $\leq$  *p*  $\leq$  0.001).

We observed that IL-10 production was upregulated by LPR in totDCs from PPs (*p* = 0.01), MLNs (*p* = 0.008) and spleen (*p* = 0.03; Table 1). The association of LPR with SigA increased IL-10 production in PP totDCs only (*p* = 0.01). Incubation with LPS triggered IL-10 production in all three tissues (*p* = 0.03). TGF- $\beta$  production in PP totDCs was stimulated in the presence of LPR (*p* = 0.03) and further increased upon association with SigA (*p* = 0.02). No enhancement of TGF- $\beta$  production upon incubation with LPR alone was observed in MLN and splenic totDCs, whereas association with SigA had a positive effect in PPs and MLN (*p* = 0.03; Table 1, sixth column). TGF- $\beta$  was diminished in comparison with the control in all tissues after exposure to LPS (*p*  $\leq$  0.01). Differential fine-tuning of the

**Table 1** Concentrations (pg/ml) of cytokines and chemokines produced by totDCs from mouse PPs, MLNs and the spleen.

	IL-12p70	KC	TNF- $\alpha$	IL-6	IL-10	TGF- $\beta$
<b>PP</b>						
CTRL	63 $\pm$ 9	204 $\pm$ 40	49 $\pm$ 4	142 $\pm$ 29	107 $\pm$ 8	664 $\pm$ 42
LPR	81 $\pm$ 11	243 $\pm$ 11	57 $\pm$ 5	<b>258 <math>\pm</math> 30</b>	<b>218 <math>\pm</math> 13</b>	<b>868 <math>\pm</math> 27</b>
SigA-LPR	85 $\pm$ 5	229 $\pm$ 16	52 $\pm$ 5	<b>230 <math>\pm</math> 17</b>	<b>318 <math>\pm</math> 10</b>	<b>1173 <math>\pm</math> 25</b>
SigA	70 $\pm$ 9	198 $\pm$ 9	78 $\pm$ 6	195 $\pm$ 22	169 $\pm$ 8	619 $\pm$ 52
LPS	<b>208 <math>\pm</math> 36</b>	<b>568 <math>\pm</math> 26</b>	<b>195 <math>\pm</math> 38</b>	<b>969 <math>\pm</math> 91</b>	<b>318 <math>\pm</math> 29</b>	<b>469 <math>\pm</math> 38</b>
<b>MLN</b>						
CTRL	52 $\pm$ 10	225 $\pm$ 27	59 $\pm$ 5	109 $\pm$ 19	63 $\pm$ 15	545 $\pm$ 24
LPR	60 $\pm$ 8	234 $\pm$ 61	72 $\pm$ 7	<b>213 <math>\pm</math> 40</b>	<b>165 <math>\pm</math> 13</b>	602 $\pm$ 49
SigA-LPR	53 $\pm$ 10	281 $\pm$ 56	66 $\pm$ 7	<b>202 <math>\pm</math> 36</b>	<b>164 <math>\pm</math> 8</b>	<b>807 <math>\pm</math> 19</b>
SigA	58 $\pm$ 7	236 $\pm$ 44	63 $\pm$ 6	105 $\pm$ 31	82 $\pm$ 69	608 $\pm$ 65
LPS	<b>225 <math>\pm</math> 11</b>	<b>633 <math>\pm</math> 33</b>	<b>147 <math>\pm</math> 51</b>	<b>712 <math>\pm</math> 95</b>	<b>370 <math>\pm</math> 44</b>	<b>381 <math>\pm</math> 31</b>
<b>Spleen</b>						
CTRL	85 $\pm$ 9	117 $\pm$ 29	325 $\pm$ 84	206 $\pm$ 33	68 $\pm$ 20	549 $\pm$ 72
LPR	<b>194 <math>\pm</math> 9</b>	<b>390 <math>\pm</math> 45</b>	<b>974 <math>\pm</math> 95</b>	<b>493 <math>\pm</math> 90</b>	<b>111 <math>\pm</math> 13</b>	627 $\pm$ 33
SigA-LPR	<b>132 <math>\pm</math> 7</b>	<b>202 <math>\pm</math> 16</b>	<b>547 <math>\pm</math> 37</b>	<b>247 <math>\pm</math> 37</b>	<b>105 <math>\pm</math> 7</b>	555 $\pm$ 43
SigA	92 $\pm$ 11	<b>229 <math>\pm</math> 18</b>	453 $\pm$ 29	199 $\pm$ 25	<b>96 <math>\pm</math> 6</b>	588 $\pm$ 15
LPS	<b>419 <math>\pm</math> 22</b>	<b>874 <math>\pm</math> 32</b>	<b>2231 <math>\pm</math> 60</b>	<b>1130 <math>\pm</math> 89</b>	<b>423 <math>\pm</math> 77</b>	<b>296 <math>\pm</math> 14</b>

Isolated DCs from mouse PPs, MLNs and the spleen were incubated in the presence of various ingredients (left column). IL-12p70, KC, TNF- $\alpha$ , IL-6 and IL-10 were measured by using the Luminex technology, while TGF- $\beta$  was determined by ELISA. DC culture supernatants were analyzed in triplicates. The concentrations (pg/ml) are depicted as means  $\pm$  SD and are representative of at least 2 independent experiments carried out with DCs isolated from 4 animals. Number in bold indicate statistically significant differences by comparison with the CTRL (Two-tailed Student's *t* test). Underlined numbers denote statistically significant differences between LPR and SigA-LPR (Two-tailed Student's *t* test).

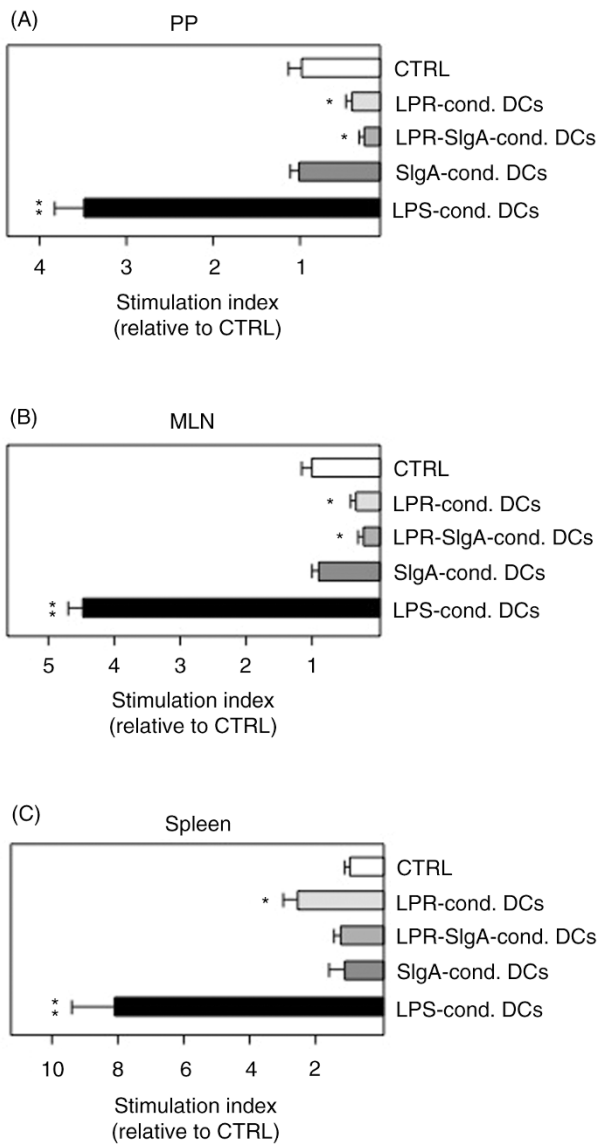
cytokine profile supports the notion that SigA in association with LPR acts as a partner to further modulate DCs toward a more anti-inflammatory pattern.

### Capacity of mucosal and splenic conditioned DCs to induce T cell proliferation and Treg development

Based on the above observation that mucosal and splenic DCs exposed to various stimuli display different properties (Figure 2, Figure 3 and Table 1), we next investigated whether this translated into different functional features. We first analyzed the ability of conditioned and sorted CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs recovered from mucosal PPs and MLN, as well as from the spleen for the systemic environment, to induce the proliferation of isolated naïve antigen-specific CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells using the OVA-specific DO11.10 system. LPS-primed OVA peptide-loaded DCs isolated from mucosal tissues and spleen strongly induced the proliferation of DO11.10 CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells, whereas conditioning of the OVA peptide-loaded PP and MLN DCs with LPR or SigA-LPR not only failed to stimulate such cells but also triggered hypo-responsiveness of T cells compared with the control (Figure 4A and B). Consistent with the pattern of surface markers and secreted cytokines (Figure 3 and Table 1), splenic DCs moderately activated T cells after conditioning with LPR. T cell activation was significantly reduced when in complex with SigA (Figure 4C).

Development of Treg cells post-incubation with conditioned DCs for 72 h was examined next. A marked conversion of naïve DO11.10 CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup>

Foxp3<sup>+</sup>Treg cells was observed upon analysis of an equal amount of CD4<sup>+</sup>T cells following incubation with PP- and MLN-derived LPR-conditioned CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup>DCs compared with control (Figure 5A and B). SigA in association with LPR further increased this differentiation into Treg cells. LPS-treated DCs yielded lower Treg cell conversion compared with control (Figure 5A and B). Incubation of DCs and T cells in the presence of neutralizing mAbs specific for TGF- $\beta$  or IL-10 led to a statistically significant reduction in the expansion of Treg cells, which is in agreement with the role of these cytokines in the generation of Treg cells.<sup>19</sup> At the systemic level, no Treg cells were generated above background when spleen-derived DCs were conditioned by LPR or SigA-LPR (Figure 5C), reflecting the more pro-inflammatory cytokine pattern displayed by such DCs, which is presumably not counteracted by IL-10 and TGF- $\beta$ . Finally, we confirmed the actual regulatory function of Treg lymphocytes by assessing their ability to suppress the proliferation of effector cells using the MLR assay. The addition of mucosal DC-converted Tregs in increasing amounts to a constant amount of responder cells yielded a progressive reduction in proliferation, as reflected by lower <sup>3</sup>H-thymidine incorporation (Figure 5D). The sum of these data shows, at the functional level, that in addition to differences linked to tissue origin, the presence of SigA is essential in driving the homeostatic perception of commensal bacteria by DCs, as exemplified by their differential capacity to affect antigen-specific T cell proliferation and to generate antigen-specific Treg cells with suppressive function.



**Figure 4** Proliferative responses of purified naïve DO11.10 CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells incubated in the presence of conditioned, FACS-sorted CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup> DCs pulsed with the OVA 323–339 peptide. DCs were isolated from PPs (A), from MLNs (B) and from the spleen (C). The results are presented as stimulation indices, with control (CTRL) serving as the reference and show means ± SD of one representative experiment performed 2–3 times with cells isolated from 4 animals. The significance of the data were evaluated by nonparametric one-way ANOVA with Bonferroni’s multiple comparison test compared with CTRL. \**p* values < 0.05; \*\**p* values < 0.01.

**DISCUSSION**

The novelty of our data resides in the observation that SlgA plays an important role in the sensing of the microbiota by further promoting the tolerogenic conditioning of mucosal DCs. At the mechanistic level, our results show SlgA-mediated potentiation of the expression of the TLR regulatory proteins SIGIRR, Tollip and SOCS1, and the vitamin A metabolizing enzyme RALDH2 in PP DCs conditioned by LPR. At the functional level, this translates in basically unchanged DC activation

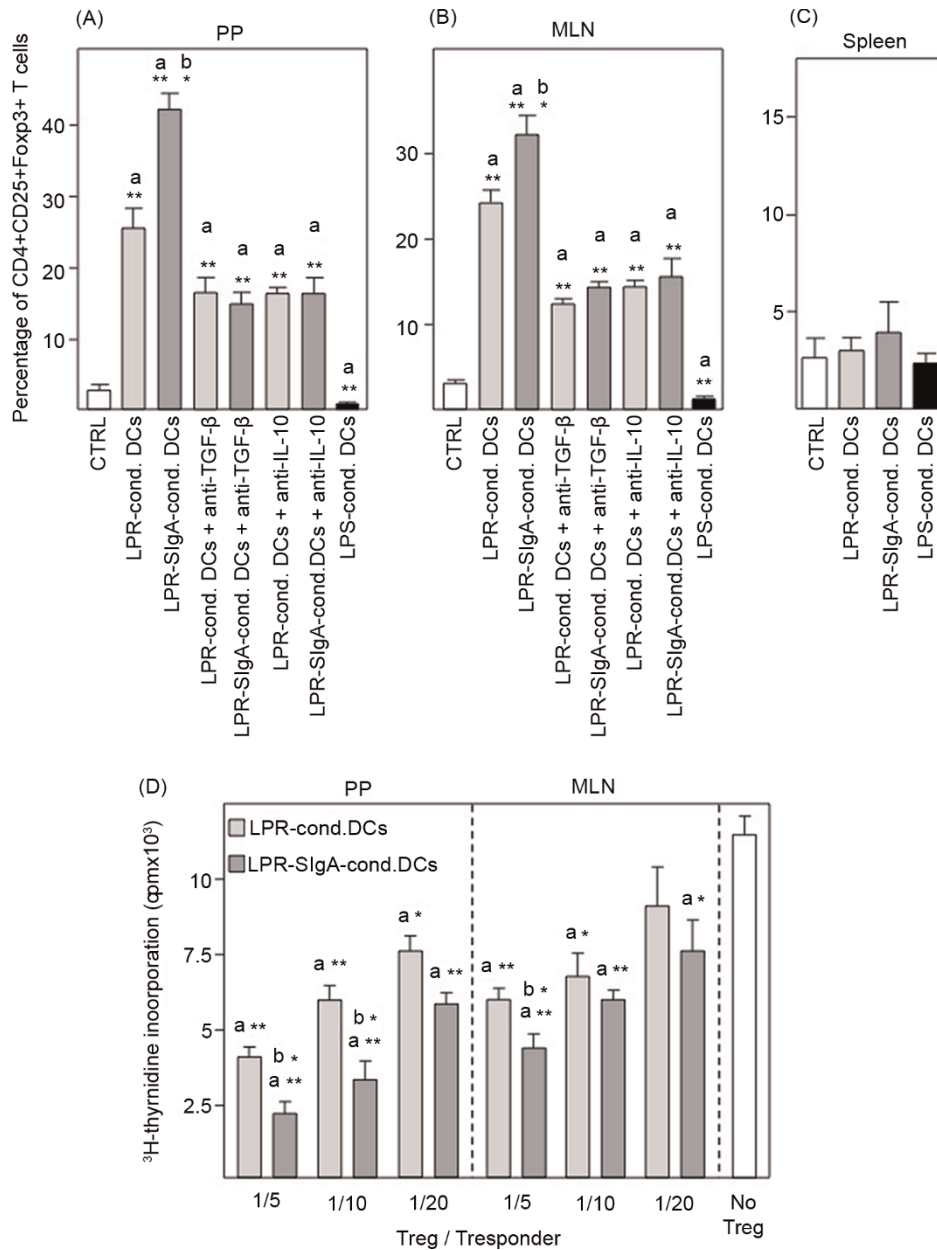
markers, the production of IL-10 and TGF-β by mucosal DCs, into a reduction of antigen-specific T cell proliferation and differentiation into functional Tregs. These findings provide a link between the need for SlgA to control the expansion<sup>39</sup> and character<sup>40</sup> of the intestinal microbiota, the bacterial sensing by PPs,<sup>16</sup> and the development of a tolerogenic phenotype of local DCs that is important for intestinal homeostasis.<sup>41</sup>

The default response of the intestinal immune system to most antigens is the induction of immunological tolerance, which is difficult to reconcile with the constant exposure to ligands for TLR and other pattern recognition receptors.<sup>42</sup> Our work provides insights into this paradox by showing that contrary to splenic DCs, PP and MLN DCs have increased expression of proteins involved in the negative regulation of TLR-mediated responses (SIGIRR, Tollip, SOCS1) following conditioning with LPR. The presence of SlgA further supported the expression of such proteins in PP DCs only, i.e., cells undergoing massive exposure to environmental antigens. Such differences may thus represent one of the mechanisms by which DCs residing in different tissues are likely to have a different reaction to their surrounding environment.<sup>43</sup> This result adds to the complexity of research examining how the regulation of TLR signaling by commensals may contribute to the quenching of immune responses induced by pathogenic bacteria that express TLR ligands. Noteworthy, mice lacking either SIGIRR or SOCS1 have increased susceptibility to inflammatory intestinal diseases.<sup>44</sup>

In comparison with the control, enhanced production of IL-10 and TGF-β and to a lesser extent IL-6 by DCs isolated from the PPs and MLNs is worth noting, as these three cytokines have crucial homeostatic functions in the intestinal environment<sup>41</sup> and participate in the isotypic switch leading to production of local IgA.<sup>1,45</sup> The unchanged pattern of pro-inflammatory cytokines resulting from incubation with LPR and SlgA-LPR undergoes drastic modifications after incubation with LPS, strongly suggesting that PP and MLN DCs have the intrinsic capacity to discriminate between harmless and harmful signals. In comparison to the mucosal environment, DCs isolated from the spleen secrete more pro-inflammatory and less anti-inflammatory cytokines when exposed to LPR, yet this is no longer true when associated with SlgA, showing similarity to the *in vivo* situation.<sup>12</sup> The highest reactivity of spleen-derived DCs exposed to LPR is most likely required to rapidly detect and eliminate commensal bacteria that have gained access to this normally sterile compartment.<sup>46</sup>

The positive effect of SlgA is marked as a function of the parameters examined in DC responsiveness. Such differences may be explained by the presence of endogenous SlgA bound to DCs isolated from the MLNs and PPs (J. Mikulic, unpublished data). Our approach of using tissue-derived DCs has, however, allowed us to unravel the contribution of SlgA in this process. In the absence of the prototype IgA receptor CD89 in mice, an anticipated development will be to identify the receptor(s) for SlgA on mucosal DCs and to determine how crosslinking alters DC signaling and responsiveness. A clue could come from the





**Figure 5** Generation of functional CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Treg cells after co-culture of purified naïve DO11.10 CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells with conditioned, FACS-sorted, CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD8<sup>-</sup>DCs pulsed with the OVA 323–339 peptide. Conversion of naïve DO11.10 CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup> T cells into Treg cells after incubation in the presence of PP-derived DCs (A) as such and in the presence of TGF-β or IL-10 neutralizing mAbs. (B) Same experiment as in (A), with co-cultures performed in the presence of conditioned, FACS-sorted DCs from MLN. (C) No conversion into Treg is observed with conditioned and sorted DCs from splenic origin. (D) Treg obtained as in (A) and (B) were sorted by FACS and added at different ratios (Treg: spleen cells) to a mixed lymphocyte reaction (MLR) assay (1 × 10<sup>5</sup> responder cells: 1 × 10<sup>5</sup> stimulator cells). Proliferation was evaluated by <sup>3</sup>H-thymidine incorporation. The results show mean ± SD and are representative of 2–3 independent experiments (DCs from 4 animals for each) performed in triplicate. c.p.m., counts per minute. The significance of the data were evaluated by nonparametric one-way ANOVA with Bonferroni’s multiple comparison test (a) or paired *t*-test (b). In (A) and (B), “a” indicates comparison with control (CTRL) and “b” indicates comparison between LPR-conditioned DCs and SigA-LPR-conditioned DCs. In panel D, “a” indicates comparison with No Treg and “b” indicates comparison between LPR-conditioned DCs and SigA-LPR-conditioned DCs. \*, *p* values < 0.05; \*\*, *p* values < 0.005.

observation that the specific ICAM-3 grabbing nonintegrin receptor 1 (SIGNR1) has been shown to bind SigA on the surface of bone marrow-derived DCs and can induce signaling favoring non-inflammatory responses.<sup>38</sup> Collectively, these data extend, at the functional level, our previous observations

made in mice that show that immune complexes consisting of commensal bacteria and SigA specifically target PPs CD11c<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup> DCs.<sup>16</sup>

Although a convenient source of cells, the use of *in vitro* generated monocyte-derived DCs does not truly reflect the

intrinsic nature of DCs and their *in situ* activities. This is particularly true when testing interactions with antibody ligands<sup>15</sup> or the effects of bacteria on gut-associated DCs.<sup>47</sup> The different responsiveness of DCs incubated with LPR or LPS used as a surrogate of pathogen-like signal suggests that DCs from mucosal and systemic tissues have intrinsic properties making them capable of discriminating between stimuli of different natures. It is noteworthy that incubation with dead commensal bacteria led to different results,<sup>47,48</sup> an observation that can be explained by the recent finding that the signature of microbial viability plays a role in alerting the immune system.<sup>49</sup>

We found that in contrast to splenic DCs, PP- and MLN-derived DCs conditioned by LPR or SigA-LPR develop characteristics of tolerogenic/homeostatic cells unable to stimulate naïve T cells, yet promote differentiation into Treg cells. The suppressive function of Treg cells converted by conditioned DCs from either the PPs or MLNs was confirmed by the MLR assay. In the physiological context of newborns, it is plausible that, right after birth, the combination of newly colonizing bacteria and maternal SigA from breast milk contributes to actively establish a state of tolerance toward gut microbiota important for the proper development of the immunomodulatory function of the gut.<sup>6</sup> Such a hypothesis may provide an explanation for the finding that an exquisite specificity exists in the TCRs of Treg cells from the mouse colon compared with Treg cells from other organs.<sup>50</sup>

In conclusion, SigA appears to play an important role in the facilitation of communication in the mucosal environment between DCs and commensal bacteria as illustrated with LPR in this study. Although the anti-inflammatory properties of SigA toward mucosal pathogens have been documented in many instances, these data underscore its role in contributing to the tolerogenic outcome of the interaction between mucosal DCs and commensal bacteria. Our work brings the first pieces of evidence toward deciphering the pathways through which SigA, in complex with commensal bacteria, may contribute to educate mucosal DCs, especially in PPs, to promote local homeostasis.

## ACKNOWLEDGMENTS

We acknowledge support from the Swiss Science Research Foundation (grants Nos. 3100-138422 and 3100-156806 to B.C.). The commensal bacterial strain LPR was kindly provided by Nestlé Research Center, Vers-chez-les-Blanc, Switzerland. We thank Dr. Gilles Bioley for critical reading of the manuscript.

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