The regulatory role of the oral commensal *Streptococcus mitis* on human monocytes

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Abstract

Streptococcus mitis colonizes all niches of the human oral cavity from early infancy and throughout life. Monocytes patrol blood vessels, lymphoid and non-lymphoid tissues, and migrate into infected tissue where they participate in the inflammatory cascade and immune regulation. Here, we studied the effect of *S. mitis* on monocytes. Transcriptome analysis of monocytes exposed to *S. mitis* (SmMo) revealed increased transcription of chemotactic factors (*CCL2, CCL3, CCL20, CXCL1, CXCL2*) and cytokines (*IL1A, IL1B, IL6, IL23, IL36G, TNF*), indicating that *S. mitis* may trigger recruitment of leukocytes and initiate inflammation. Increased transcription in SmMo of *IL1B, IL6* and *IL23* indicated that *S. mitis* may participate in the induction of Th17 responses, and agreed with our earlier findings of *S. mitis*-mediated memory Th17 reactivity. Furthermore, *S. mitis* inhibited tetanus toxoid-specific CD4 T cell proliferation. This can be due to the increased secretion of IL-10 and expression of PD-L1 that was observed in SmMo. PGE2 can modulate IL-10 and PD-L1 expression, concomitant with that of CCR7, IL-12 and IL-23 that also were changed. This, along with increased SmMo transcription of *PTGS2* (COX2) and *PTGER4* (EP4), pointed to a role of PGE2. Measurement of PGE2 secretion by SmMo showed indeed a marked increase and chemical inhibition of PGE2 production lowered the PD-L1 expression on SmMo. In conclusion, our findings show that *S. mitis* may trigger immune modulation by recruiting immune cells to the site of infection, while at the same time dampening the severity of the response through expression of IL-10, PGE2 and PD-L1.

Introduction

Streptococcus mitis is one of the earliest commensal colonizers of the human oral cavity, and resides in the oral mucosa from early infancy and throughout life. In immunocompetent persons, *S. mitis* is rarely associated with disease since it survives poorly in blood and is immediately cleared [1]. In immunocompromised patients, however, it is the oral commensal streptococcal species that is most frequently associated with endocarditis or septicemia [2]. *S. mitis* is closely related to the opportunistic *Streptococcus pneumoniae* [3]. Despite their close relationship and significant difference in pathogenic potential, *S. mitis* shows a superior intrinsic ability to inhibit memory T helper cell proliferation compared with *S. pneumoniae* [4]. How *S. mitis* mediates its inhibitory properties is unknown.

The oral cavity is continuously exposed to heavy loads of both colonizing and foreign microbes and a wound in the mouth can therefore be potentially quite ominous. The lining of the oral cavity consists of a thick, robust, multilayered epithelium. In addition, the oral epithelia host an array of resident immune cells, including antigen-presenting cells (APCs), which can react to any threats by inducing inflammation and immune responses. In inflamed tissue, the most abundant APCs are monocytes [5].

Monocytes are members of the mononuclear phagocyte system with high phagocytic capacity [6]. They arise from common monocyte progenitors (cMoPs) in the bone marrow [7], egress from the bone marrow into the blood as mature monocytes in a CCR2 dependent manner [5], and constitute about 10 % of blood leukocytes. Monocytes have long been thought to have their main role in replenishing tissue macrophages and dendritic cells. Now, it is recognized that monocytes can contribute to fighting infection in tissues, as well as patrolling non-lymphoid and lymphoid tissues in steady state without necessarily acquiring dendritic cell or macrophage phenotype [8]. Monocytes show great plasticity, and when they migrate into the tissues, they can differentiate into other types of effector cells depending on their microenvironment [5].

In the case of oral wounding, the underlying tissues will become exposed to the ubiquitously occurring *S. mitis* and the bacterial cells will be detected by different cell types, including monocytes, for adequate initiation of inflammatory and immune responses. As the effect of *S. mitis* on monocytes is undescribed, the aim of this study was to investigate how exposure to *S. mitis* affects monocyte phenotype (Supp. Fig. 1).

Materials and methods

Bacterial strains and inactivation

A selection of nine oral and nasopharyngeal streptococcal species were used in this study to investigate differences in response to distinct strains within the same species and between different species. Glycerol stocks were prepared for each strain from 10 mL of overnight cultures grown in tryptic soy broth (TSB; ThermoFisher, Waltham, MA, USA) at 37 °C with 5 % CO2 saturation. The bacteria were pelleted, re-suspended in 700 µL TSB and mixed with 300 µL of 100 % glycerol (final concentration of 30 %) before they were frozen at – 80°C.

For UV-inactivation, overnight cultures were diluted in fresh TSB and propagated to OD600 of 0.5-0.8. The bacterial cells were pelleted by centrifugation at 10000 g for 10 min and re-suspended in 16 mL PBS. 4 mL of bacterial solution were added to each of four petri dishes (100 x 15 mm; VWR International, Radnor, PA), swirled to ensure even distribution and UV-inactivated in a Hoefer UVC 500 Ultraviolet Crosslinker (Hoefer, Hollister, MA, USA) without lids for 30 min. Inactivated bacterial cells were aliquoted and frozen at -80 °C until further use. Inactivation was confirmed by negative viable counts and the quantification of bacterial cells was done by visual count at 60 x magnification in a microscope (Nikon, Melville, NY, USA) using a Neubauer counting chamber (Marienfeld-Superior, Germany) to determine the number of cocci regardless of chain length.

Escherichia coli (*E. coli*) was prepared in the same manner as the streptococcal species but were grown on Luria-Bertani agar and broth (BD diagnostics, Franklin Lakes, NJ, USA) at 37 °C in aerobic conditions and shaking at 150 RPM.

Isolation of PBMCs and monocytes

Buffy coats from healthy human donors were obtained from the Blood Bank at the Oslo University Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep (Alere, Oslo, Norway) gradient centrifugation according to the manufacturer's protocol. In brief, 60 mL blood was diluted in equal amounts of PBS, carefully added on top of 13 mL Lymphoprep in 50 mL tubes, and centrifuged at 800 g for 30 min. The PBMCs were collected and washed twice in PBS. After the second wash, the cells were re-suspended in 50 mL complete RPMI (cRPMI) (RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2mM L-glutamine, 1x amphotericin, penicillin, streptomycin (APS) solution and 10 % v/v fetal bovine serum (FBS) (all from Sigma-Aldrich)). The cells were quantified by MOXI Z mini automated cell counter (ORFLO Technologies, Ketchum, ID; 6 - 15 µm) and further processed or frozen in cryomedium comprising 50 % v/v FBS, 40 % v/v cRPMI and 10 % v/v DMSO (Sigma).

Monocytes were isolated from PBMCs by positive selection of CD14+ cells using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. In brief, PBMCs were suspended in buffer and incubated with microbeads at 4 °C for 20 min. The cells were washed once and re-suspended in ice-cold buffer before they were applied to a MACS LS column (Miltenyi). The column was thoroughly washed with buffer before it was removed from the magnet and 6 mL of buffer was added to the reservoir to elute the CD14+ cells by use of the plunger. The elution process was repeated a second time. The cells were pelleted and re-suspended in cRPMI for quantification and further stimulations.

Stimulations

For the microarray analysis, $5x10^6$ monocytes were co-cultured with UV-inactivated *S. mitis* (multiplicity of infection (MOI) 1:1) in cRPMI in 96 well U-bottom plates (Corning Incorporated, Corning, NY, USA). After 1 and 16 h, the cells were collected in Eppendorf tubes, pelleted at 800 g in room temperature (RT) for 5 min and lysed in 700 µL RLT buffer (Qiagen, Valencia, CA, USA) supplemented with 1 % v/v 2-mercaptoethanol (Sigma) for RNA extraction using RNease mini kit (Qiagen).

For the T cell proliferation experiments, PBMCs were labeled with 2.5 µM carboxyfluorescein succinimidyl ester (CFSE; ThermoFisher) at RT and in the dark for 4 min before they were washed three times in 10 mL PBS with 5 % v/v FBS. After the final wash, the cells were re-suspended in cRPMI and quantified before $1x10^6$ monocytes were aliquoted in Eppendorf tubes and 5 µg/mL tetanus toxoid (TT; Merck Millipore, Billerica, MA) with or without *S. mitis* (MOI 1:1) was added. The suspensions were thoroughly mixed and transferred to 24 well plates (Nunc multidish, ThermoFisher) for 5 d co-culture.

For the target gene approach, $1x10^6$ monocytes were seeded in 24 well plates with or without UV-inactivated *S. mitis*, *S. pneumoniae* (MOI 25:1) or *E. coli* (MOI 1:1). In some cultures the COX inhibitor indomethacin was added (Sigma; 10, 20, 40 or 80 µM) together with *S. mitis*. After 3 d of culture, the cells were collected, washed and analyzed by flow cytometry.

All supernatants that were collected were immediately centrifuged at 4 °C and 10000 g for 10 min to remove residual bacterial cells before they were frozen at – 20 °C for later analysis.

RNA extraction

Total RNA was extracted using the QIAcube and the QIAcube standard RNeasy mini with DNase digestion protocol for the RNeasy mini kit (both from Qiagen, Valencia, CA, USA). Total RNA was eluted in 20 µL nuclease free H_2O and the RNA quantity and purity were determined using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Illumina® bead array

Genome-wide transcriptional analysis was performed using Illumina® bead array technique at the Norwegian Microarray Consortium in Oslo, Norway (project no.: GCF-0090).

Utilizing Illumina® TotalPrep™-96 RNA Amplification Kit, biotin-labeled cRNA was synthesized from 300 ng total RNA by first- and second strand reverse transcription follow by *in vitro* transcription of cRNA. cRNA quantity was determined using a NanoDrop Spectrophotometer while RNA size and quality were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). 750 ng of biotin-labeled cRNA was hybridized to a Illumina HumanHT-12 v4 Expression BeadChip, each of the 12 arrays consisting of more than 47 000 50-mer gene specific bead-linked probes, each bead containing hundreds of thousands of probes of the same sequence. Data from the microarray were filtered using the software program J-express (Dysvik & Jonassen, 2001).

ELISA

PGE2 analyses were performed on supernatants from monocytes exposed to *S. mitis* with or without indomethacin for 3 d using a PGE2 immunoassay (RnD Systems, Minneapolis, MN, USA) carried out according to distributor's instructions. In brief, sample (diluted 2:1) and standard were added to the provided plate together with primary antibodies and incubated for 1 h at RT on a shaker set at 300 RPM. PGE2 conjugate was added to each well and further incubated for 2 h. After four washes, substrate solution was added and the optical density was determined using a Synergy HT microplate reader (BioTek, Winooski, NH, USA) set to 450 nm.

IL-10 was assayed on supernatants from monocytes exposed to *S. mitis* for 3 d using a human standard ABTS ELISA development kit (PeproTech, Rocky Hill, NJ, USA). Some changes to manufacturer's protocol was made as described below. A high binding 96 well flat-bottom microtiter plate (Corning) was coated with capture antibody by overnight incubation at RT. After washing four times in PBST (PBS with 0.05 % Tween-20 (Sigma)), the plate was incubated at RT for 1 h with

blocking buffer (1 % IgG-free BSA (Jackson Laboratories, West Grove, PA, USA) in PBS) before it was washed as before and samples and standard were added. After 2 h incubation at RT on a shaker set at 150 RPM, the plate was washed as before and further incubated for 2 h on a shaker with detection antibody. After washing, the plate was incubated for 30 min with AP-labeled streptavidin (1:10000) before yet another wash and detection using alkaline phosphatase yellow substrate (pNPP; Sigma) and microplate reader.

FACS analysis

Cells were collected and stained with viability dye eFluor780 (ThermoFisher) in PBS to exclude dead cells. After washing in FACS buffer (PBS supplemented with 5 % v/v FBS), cells were labeled for 30 min on ice in the dark with the following antibody panel for the measuring T cell proliferation: CD3-PacBlue (clone OKT3), CD4-APC (clone OKT4; both from Biolegend, San Diego, CA, USA) and CD25-PerCP/Cy5.5 (clone BC96; eBiosciences, San Jose, CA, USA). To assess the surface phenotype of monocytes, the following panel was used: CD14-BV421 (clone HCD14), HLA-DR-BV605 (clone L243), PD-L1-PE/Cy7 (clone 29E.2A3), CCR7-PE (clone G043H7), CD40-APC/Cy7 (clone 5C3), CD80-PE (clone 2D10), CD86-APC (clone IT2.2) and CD83-APC (clone HB15e), all from Biolegend. Data acquisition was performed on an Attune Nxt flow cytometer (ThermoFisher) and analyzed using FlowJo 10.2 software (Three Star, Ashland, OR, USA).

Results

***S. mitis* induces a mixed inflammatory and immunoregulating phenotype in monocytes**

Monocytes were exposed to *S. mitis* and transcriptome analysis was carried out of the immediate (1 h) and late (16 h) responses. The datasets were first filtered for genes with signal intensity (SI) below background (SI < 1000) for unstimulated and stimulated fractions (Fig. 1A).

After exposure of 1 and 16 h, we found that 89 (10 down, 79 up) and 536 (291 down, 245 up) genes were differentially regulated by 2 fold or more in response to *S. mitis*, respectively (Fig. 1B**,**

upper diagram). Fifty genes were mutually regulated at both 1 and 16 h and these comprised both pro-inflammatory (*CCL2, CXCL1, CXCL2, IL1A, IL6, TNF*) and anti-inflammatory mediators (*IL10, SOCS3*) (Fig. 1C). Gene ontology (GO) analysis identified the common genes to be involved in inflammatory responses, responses to lipopolysaccharide, regulation of apoptotic processes, cell chemotaxis and immune responses (Supplementary table 1).

Several other immunoregulatory factors were also differentially regulated after 16 h exposure to *S. mitis*. This included down-regulation of genes encoding HLA class II-molecules (*HLA-DP, HLA-DR*) and proteins involved in their processing (*CD74, HLA-DM*), in addition to cell adhesion molecules (*PECAM1, ITGB2*) (Fig. 1D). Up-regulated genes comprised important genes involved in cell migration (*CCR7, CXCL1, CXCL2, CXCL8, CCL2, CCL3, CCL5, CCL20*) and immune modulatory molecules (*IL1A, IL1B, IL6, IL10, IL23A, IL36G, TNF, PTGES, PTGER4*) (Fig. 1D).

S. mitis inhibits proliferation of T cells in response to unrelated antigen

We have previously shown that *S. mitis* inhibits proliferation of two tetanus toxoid (TT) specific memory Th17 clones in response to their specific antigen [4]. Here, we corroborated this observation using PBMCs from healthy human blood donors. TT was added to CFSE labeled PBMCs with or without *S. mitis* and cell proliferation was measured on day 5 (Fig. 2A). Cells from most donors showed significant inhibition of proliferation of TT reactive CD4+CD25+ T cells when *S. mitis* was added (Fig. 2B) and the difference with untreated cells was statistically significant (Wilcoxon matched-pairs signed rank test; $P<0.05$).

Therefore, we next examined *S. mitis*-mediated differential regulation of selected markers known to be involved in monocyte activation (CD40, CD83), migration (CCR7), and regulation of T cell activation (CD80, CD86, HLA-DR) and inhibition (CD274/PD-L1). Monocytes were exposed to UV-inactivated *S. mitis* for 3 d before they were harvested and analyzed by flow cytometry. For comparison, we included the streptococcal opportunist *S. pneumoniae* and the gram-negative *Escherichia coli* (*E. coli*). *S. mitis* triggered a significantly higher expression of the inhibitory molecule

PD-L1 and cell migratory molecule CCR7 in monocytes as compared with unstimulated cells (Fig. 3). Differences in HLA-DR protein expression did not reach statistically significance, in contrast to what was found at the transcriptional level. As compared with *S. pneumoniae*, however, *S. mitis* stimulation showed significantly increased expression of CD40, CD83, CD80, CD86 and PD-L1, while HLA-DR showed greater down-regulation in response to *S. pneumoniae* (Fig. 3).

In order to determine if the observed differential regulation of the chosen markers was specific for *S. mitis* or for commensals in general, we included a panel of five other *S. mitis* strains and seven other streptococcal species. We did not observe clear trends for either *S. mitis* strains only or commensals (Supp. Fig. 2). The *S. mitis* type strain displayed the strongest up-regulation of inhibitory molecule PD-L1.

Our mRNA data set showed that levels of IL-10 transcripts after both 1 and 16 h exposure to *S. mitis* were increased (Fig. 1D). Therefore, we measured the IL-10 levels in the supernatants of monocytes exposed to *S. mitis* for 3 d. We found significantly higher secretion of IL-10 in response to *S. mitis* compared to unstimulated cells and cells exposed to *S. pneumoniae* (Fig. 4).

PGE2 secretion by monocytes exposed to *S. mitis* regulates PD-L1 expression

In our microarray data set, we observed increased transcription of both *PTGER4*, the gene that encodes a PGE2 receptor that mediates immunosuppressive functions, and *PTGS2*, the gene encoding cyclo-oxygenase 2 (COX2; Fig. 1D), the enzyme responsible for converting PGH2 to PGE2. In the supernatants from monocytes cultured with UV-inactivated *S. mitis* for 3 d we found significantly increased levels of PGE2 (Fig. 5A). By adding increasing amounts of the COX inhibitor indomethacin to cultures of monocytes we observed a dose dependent reduction of PD-L1 in monocytes exposed to *S. mitis* (Fig. 5B). These findings correspond to observations done in macrophages and myeloid-derived suppressor cells [9] and also parallel our previous observation that *S. mitis* induces secretion of PGE2 from human oral keratinocytes [10].

Discussion

S. mitis colonizes all niches of the oral cavity and is therefore one of the microbes that will encounter the underlying tissues in the case of wounding in the oral cavity. As *S. mitis* does not survive for long in blood [1], the most relevant site where *S. mitis* can affect inflammation and immunity lies within the oral tissue itself. Monocytes are important in surveillance of the blood stream as well as of the lymphoid and non-lymphoid tissues in steady state [5]. They also, however, migrate into infected tissues, and participate in the inflammatory cascade and immune activation [11]. Depending on the encountered antigen, monocytes can participate in either immunoactivation or immunosuppression [4, 12, 13]. Therefore, in this study, we studied the effect that the oral commensal *S. mitis* can have on monocytes.

First, we exposed cultures of monocytes to *S. mitis* and performed a transcriptome analysis of the cells. The data indicated that *S. mitis* can induce local monocyte-mediated inflammation by activating an array of inflammatory mediators (IL-1β, IL-6, IL-23, IL-36γ, TNF) that are essential for triggering the acute phase response and activate tissue-resident immune cells. Transcription of chemoattractants that recruit monocytes, T cells and neutrophils to the effector site (CCL2, CCL3, CCL5, CCL20, CXCL1 and CXCL2) was also increased. However, the concomitant finding of increased secretion of IL-10 and PGE2 and expression of PD-L1 implies that *S. mitis* can induce a regulatory loop that regulates neutrophil activation and dampens the severity of the triggered immune response, as was observed in the case of *Toxoplasma gondii* infection [13].

The biological effects of PGE2 are diverse and dependent on the differential expression of its four receptors, EP1-4 [14]. PTGER4 encodes the EP4 receptor that mostly promotes anti-inflammatory effects of PGE2 [14, 16]. Recently, however, EP4 was also proposed to mediate pro-inflammatory effects as it is shown to be directly involved in inhibition of IL-10 while triggering IL-23 secretion in human tolerogenic dendritic cells [17]. In contrast, we found that *S. mitis* triggers increased secretion of PGE2 and IL-10 as well as increased transcription of EP4 and IL-23. In T cells, PGE2 both inhibits proliferation [18] as well as promotes maturation and activation of Th17 cells

through EP4-mediated IL-23 expression in dendritic cells and IL-23R expression in T cells [19]. IL-23 transcription was found to be increased in the monocytes exposed to *S. mitis* and this might be related to the increased transcription of PTGER4 as EP4 is directly linked to IL-23 production [17]. IL-23 is also an important pro-inflammatory cytokine known for its role in the generation and maintenance of IL-17-producing T cells and neutrophil recruitment through the IL-23/IL-17 axis. In addition, EP4-mediated IL-23 up-regulation is accompanied by down-regulation of IL-12 [17] and this concurs with results from our microarray set where transcription of *IL12* was inhibited. In light of this, our earlier findings of a *S. mitis*-induced memory Th17 response [4] together with the current findings of an *S. mitis*-mediated secretion of PGE2 and transcription of *PTGER4*, *IL23, IL1B* and *IL6*, indicate that *S. mitis* can promote Th17 responses. The transcription of Th1 (IL-12), Th2 (IL-4) and Treg (TGFβ) polarizing cytokines was below background levels in both unstimulated and *S. mitis*-stimulated monocytes (Supp. Fig. 3).

Yet another role for PGE2 and EP4 is regulation of CCR7 [17, 20], a chemokine receptor involved in migration to secondary lymphoid organs (SLOs). The currently observed increased expression of CCR7 and PD-L1, and the simultaneous deactivation of *PECAM1* and *ITGB2* in response to *S. mitis*, indicates that monocytes may migrate to the SLOs and promote tolerance.

We have previously reported that *S. mitis* mediates inhibition of TT specific memory Th17 clones and we presently have extended this observation using PBMCs from healthy human blood donors. We observed a significant reduction in proliferation of CD4+CD25+ T cells when *S. mitis* was added. In light of our current findings, this phenomenon can in part be explained by the PGE2-mediated regulation of PD-L1 combined with increased secretion of IL-10. In addition, when we added COX inhibitor to the cultures of monocytes exposed to *S. mitis*, we observed a reduction of PD-L1 levels below that of basal levels was observed. This shows that PGE2 is involved in the regulation of immunosuppressive PD-L1 in monocytes, similar to what has been observed in macrophages and myeloid-deriver suppressor cells [9]. The combined finding of increased PGE2 secretion in monocytes exposed to *S. mitis* and reduced T cell proliferation in PBMCs exposed to TT

and *S. mitis* as compared to TT alone, indicates that *S. mitis*-induced PGE2 secretion is essential for the inhibitory properties of *S. mitis* and may be one of the mediators that promote commensal tolerance.

Taken together, our findings indicate that *S. mitis* can trigger a form of self-regulation that agrees with its commensal character, through activation of genes encoding chemotactic factors involved in recruitment of immune cells to the site of infection, while at the same time dampening the severity of the response by inhibiting neutrophil activity and T cell proliferation through expression of IL-10, PGE2 and PD-L1. Future perspectives involve studying the direct effects of *S. mitis* on T cells and considering how *S. mitis* can be harvested and utilized in personalized therapeutic approaches.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure legends

Figure 1. *S. mitis* induces a mixed inflammatory and immunoregulatory phenotype in monocytes. Monocytes were exposed to UV-inactivated *S. mitis* and analyzed by transcriptome analysis after 1 and 16 h (N=3). (A) Comparison of microarray expression values of genes differentially regulated by 2 fold or more after 16 h exposure to *S. mitis* relative to unstimulated monocytes. SI: signal intensity. (B) Venn diagrams of genes differentially regulated by *S. mitis* after either 1 h or 16 h or jointly regulated by 2 (top), 5 (middle) and 10 (bottom) fold relative to unstimulated monocytes. FC: fold change. (C) Heat map of the 50 jointly regulated genes after 1 and 16 h exposure to *S. mitis* showing signal intensities. U: unstimulated; Sm: *S. mitis*-stimulated. (D) Heat map of fold change regulations of a set of selected immunologic markers in monocytes exposed to *S. mitis* relative to unstimulated cells.

Figure 2. *S. mitis* inhibits tetanus toxoid-induced T cell proliferation. PBMCs from healthy human blood donors were labeled with CFSE and cultured in the presence of TT with or without *S. mitis*. Proliferation of CD4+CD25+ cells was measured on day 5 (N=7). (A) Dot plot of one representative donor. (B) Proliferation of CD4+CD25+ cells in healthy donors. Data are presented as percent proliferating cells relative to unstimulated cells. Asterisk indicates statistically significance (Wilcoxon matched-pairs signed rank test; $P<0.05$).

Figure 3. Effect of *S. mitis* on expression of surface markers on monocytes. Monocytes were exposed to UV-inactivated *S. mitis* for 3 d and analyzed by flow cytometry for differential regulation of selected surface markers (N=3-11). Asterisks indicate statistically significance (Paired samples t test; *$P<0.05$; **$P<0.005$). U: unstimulated; Sm: *S. mitis*; Sp: *S. pneumoniae*; Ec; *E. coli*.

Figure 4. *S. mitis* exposure induces IL-10 secretion in monocytes. Monocytes exposed to UV-inactivated *S. mitis* for 3 d show significantly higher IL-10 secretion compared to unstimulated cells and cells exposed to *S. pneumoniae* (N=4). Asterisks indicate statistically significance (Paired samples t test; *$P<0.05$; **$P<0.005$). U: unstimulated; Sm: *S. mitis*; Sp: *S. pneumoniae*; Ec; *E. coli*.

Figure 5. *S. mitis*-induced PD-L1 expression on monocytes is regulated in a PGE2-dependent manner. (A) PGE2 concentration was determined in supernatants of monocytes exposed to *S. mitis* for 3 d. (B) PD-L1 expression was determined by flow cytometry on monocytes exposed to *S. mitis* with or without indomethacin (µg/mL) for 3 d (N=2). Data are shown as mean fluorescence intensity (MFI). U: unstimulated; Sm: *S. mitis*; Indo: indomethacin.

Supplementary Figure 1. Graphical abstract. *Streptococcus mitis* was exposed to human blood monocytes and analyzed by transcriptome analysis (1 and 16 h) and flow cytometry (3 d). Levels of PGE2 and IL-10 in supernatant were measured on day 3. Red: up-regulation; green: down-regulation; circles: secreted molecules; diamonds: surface molecules; rectangles: genes.

Supplementary Figure 2. Characterization of differential regulation of surface markers in response to different streptococcal species and strains. Monocytes were exposed to a panel of UV-inactivated bacteria for 3 d and analyzed by flow cytometry for differential regulation of selected surface markers (N=8-12). (MFI: mean fluorescence intensity; T: type strain; Sm: *Streptococcus mitis*; Sp T4: *Streptococcus pneumoniae* TIGR4; Ec: *Escherichia* coli; LPS: lipopolysaccharide; S.int: *S. intermedius*; S. mut: *S. mutans*; S.ora: *S. oralis*; S.sal: *S. salivarius*; S.san: *S. sanguinis*; S.sob: *S. sobrinus*; S.gor: *S. gordonii*.)

Supplementary Figure 3. *S. mitis* promotes a Th17 polarizing cytokine profile. Human blood monocytes exposed to *S. mitis* for 1 and 16 h showed no transcription of neither Th1, Th2 nor Treg polarizing cytokines IL12, IL4 and TGF, respectively. The genes encoding Th17 polarizing cytokines IL1B, IL6 and IL23 showed strong activation in response to *S. mitis*.