Antigen-specific oral tolerance for the treatment of inflammatory and allergic diseases

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Genetically Modified *Lactococcus lactis* for Delivery of Human Interleukin-10 to Dendritic Cells

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Abstract

The effective ability of dendritic cells (DC) to initiate different types of T cell responses upon different maturation conditions makes this cell-type an important target for innovative strategies requiring either polarized immunity (i.e. in vaccination or cancer) or tolerance (i.e. in chronic inflammatory diseases). We describe a novel DC targeted strategy to induce T-cell mediated tolerance by genetically engineered non-pathogenic Lactococcus lactis expressing human IL-10 (L.lactis\textsuperscript{IL-10}). Monocyte-derived DC were incubated with viable L.lactis or L.lactis\textsuperscript{IL-10} and maturation factors (MF; IL-1β, TNF-α and LPS) and used to stimulate highly purified naïve T cells to assess the nature of adaptive immune responses generated. T cells generated by mature DC exposed to L.lactis\textsuperscript{IL-10} markedly suppressed the proliferation of bystander T cells. The regulatory T cells generated by L.lactis\textsuperscript{IL-10} were dependent on full maturation of DC, as DC exposed to L.lactis\textsuperscript{IL-10} in the absence of MF did not induce suppression. Furthermore, both L.lactis\textsuperscript{IL-10}-matured DC and regulatory T cells showed enhanced production of IL-10, which was instrumental in the induction, but not the function, of regulatory T cells. These data further indicate that delivery of anti-inflammatory and immune-stimulatory agents by food-grade bacteria may be a promising strategy in the therapy of intestinal mucosal disease.

Introduction

Dendritic cells (DC) are short-lived regulators of the adaptive immune system controlling both peripheral tolerance and immune activation[1]. Immature DC continuously repopulate the mucosal tissue of the gastrointestinal tract and are able to sense luminal microbial antigens via transepithelial dendrites[2]. Upon activation by pathogens and through environmental signals DC undergo a very flexible program of maturation. During this differentiation process, DC loose their endocytic capacity, migrate to secondary lymphoid structures and acquire the ability to induce a wide variety of T cell responses. Active immunity is induced by pathogenic motifs and danger signals in the local environment while in the absence of pro-inflammatory signals or presence of anti-inflammatory signals, like IL-10, DC are tuned for maintaining peripheral tolerance[3,4 and 5]. Because DC instruct many types of lymphocytes they are an important target for several approaches of immuno-therapy including long-term vaccination, graft-versus-host disease, cancer and autoimmune disease[6].
Several strategies have been designed to modulate DC function in order to induce regulatory T cell responses, including DEC-205, Vitamin D3, Lyso-PS, FHA and interleukin-10 (IL-10) [7-11]. The disadvantage of these immunomodulatory agents is that efficacy may be lost in vivo. [12-14]. We have engineered a non-pathogenic bacterium, *Lactococcus lactis*, to secrete human IL-10 (*L.lactis*IL-10), this approach is a realistic and safe therapeutic option in humans [15]. We have examined the immunoregulatory properties of *L.lactis*IL-10 by coculturing human monocyte-derived DC with viable *L.lactis*IL-10. We show that *L.lactis*IL-10 matured DC but not *L.lactis* matured DC produce enhanced levels of IL-10 and prime naïve T cells to become IL-10 producing suppressor T cells.

### Results

**L.Lactis IL-10 primes DC to promote the development of regulatory T cells**

IL-10 is well known for its immunoregulatory effects on DC resulting in low expression of costimulatory molecules and, as a consequence, low T cell stimulatory capacity. Therefore, we investigated to what extend *L.lactis*IL-10 affects DC function and primes DC to promote the development of regulatory T cells from naïve precursors. To this aim, immature DC were activated in the presence of *L.lactis*IL-10, *L.lactis* and/or IL-10 and subsequently co-cultured with naïve T cells to induce the differentiation of effector T cells. These effector T cells were analyzed for their regulatory potency in a T cell suppressor assay. This assay utilizes two cell-cycle tracking dyes to distinguish between DC-primed T cells (effector T cells) and CD4+ peripheral T cells (responder T cells)[16].

The mean fluorescence intensity of responder T cells during culture with effector T cells derived from MF (MF; TNF-α, IL-1β and LPS) matured DC was taken as 100%. Compared to this reference, only 33% of all responder T cells went into cell cycle progression when *L.lactis*IL-10 and MF were present during DC maturation (Figure 1a). When immature DC were activated with *L.lactis* and MF 67% of all responder T cells went into cell cycle progression (Figure 1a). The mean inhibition from five separate experiments was 42% and 72% for *L.lactis*IL-10 and *L. lactis* respectively (Figure 1b). The question remains whether the spatially restricted production of IL-10 is crucial for the induction of regulatory DC or that other factors resulting from genetic manipulation might play a role. Therefore, DC were activated
with \( L.lactis \) in the presence of increasing doses of recombinant human IL-10.

Compared to the reference situation of MF matured DC (taken as 100%), effector T cells from \( L.lactis \) matured DC were able to reduce the proliferation of responder T cells from 100% to 78%. However, increasing doses of recombinant human IL-10 (0.5 ng/ml, 5 ng/ml and 50 ng/ml) increased the suppressor capacity of DC-instructed effector T cells to 67.5%, 46.5% and 48.5% respectively (Figure 1c).

Thus, \( L.lactis \) is able to induce the differentiation of regulatory DC with subsequent generation of T cells that have the capacity to suppress the proliferation of bystander (responder) T cells. The ability of \( L.lactis \) to induce regulatory immune responses is amplified by the presence of IL-10 during DC activation.
**L. lactis**IL-10 induces the generation of IL-10-producing DC and IL-10 producing T cells

We determined the number of bacteria needed for optimal IL-10 induction in DC in our system. To this aim, *L. lactis*IL-10 was grown overnight at 37°C, diluted 1:50 and grown for three hours at exponential growth phase (approximately 1x10^7 cfu/ml). Subsequently, serial dilutions were made and bacteria were grown for four more hours in the absence of DC (Figure 2a). A dilution of 10^5 cfu/ml of *L. lactis*IL-10 produced the highest amount of IL-10 (81 pg/ml). At higher concentrations of bacteria lower concentrations of IL-10 were detected, most likely due to acidification of the supernatant and instability of IL-10 at lower pH.

To elucidate the role of bacterial IL-10, we analyzed the production of IL-10 during the incubation of immature DC and bacteria and after restimulation of mature DC and DC-instructed T cells. Culture supernatants from immature DC were harvested at 4 and 48 hours after activation of the DC with *L. lactis* or *L. lactis*IL-10 to determine the amount of bacterial IL-10 (at 4 hours) and total IL-10 (at 48 hours) produced. Only 130 pg/ml IL-10 was detected during the first four hours of culture with viable *L. lactis*IL-10 while no IL-10 was found in the cultures with *L. lactis* (Figure 2b). Given the short time course, the IL-10 detected must have been secreted by *L. lactis*IL-10. After 48 hours, total IL-10 production was 1200 pg/ml in DC-cultures containing *L. lactis*IL-10 compared to 680 pg/ml in DC-cultures containing *L. lactis* (Figure 2b).

To assess the ability of DC to produce IL-10 upon encounter with T cells we restimulated DC after activation of DC with *L. lactis* or *L. lactis*IL-10. DC activated by *L. lactis*IL-10 produced 3.2 ng/ml IL-10 upon restimulation with CD40L (Figure 2c), but DC activated by maturation factors only produced 0.1 ng/ml IL-10 and DC activated by *L. lactis* produced intermediate amounts of IL-10 (Figure 2c).

The production of IL-12p70 was comparable between various activated DC, ranging from 0.26 – 0.75 ng/ml, and these amounts are significantly lower compared to the production of IL-12p70 by immature DC (3.6 ng/ml) (Figure 2c).

To analyze the cytokine production of *L. lactis*IL-10 activated DC-instructed T-cells, we isolated the T cells from the mixed lymphocyte culture and subsequently stimulated them with CD3 and CD28. Effector T cells primed by *L. lactis*IL-10 activated DC produced 2.4 ng/ml IL-10 while effector T cells co-cultured with *L. lactis* activated DC produced 0.7 ng/ml IL-10 (Figure 2d). The IL-10 production by *L. lactis*IL-10 generated T cells could be reversed by the addition of neutralizing IL-10 antibodies during the activation of DC with *L. lactis*IL-10 (Figure 2d). Moreover, T cells instructed
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by L. lactisIL-10- or L. lactis -primed DC also produced high amounts of IFN-γ (4.2 and 4.3 ng/ml respectively) (Figure 2d).

In conclusion, very low levels of spatially restricted bacterial IL-10 during initial activation of immature DC are sufficient to amplify IL-10 by mature DC and effector T cells.

**The induction of regulatory DC by L. lactisIL-10 is dependent on full maturation of DC**

Maturation of DC is important for migration to regional lymph nodes, MHC-presentation, expression of co-stimulatory molecules and the induction of effector T cell responses. DC matured with L. lactisIL-10 in the absence of MF appeared to lack regulatory capacities as 99% of all responder T cells went into cell cycle progression (Figure 3a). As shown
Cell-based delivery of IL-10

Figure 3. The induction of regulatory DC by L.lactisIL-10 is dependent upon full maturation of DC. a. Immature DC were matured with MF or 1x105 CFU of viable L.lactisIL-10 with and without MF. After 48 hours DC were subsequently cultured with naïve T-cells, these DC-derived T-cells (test cells) were washed and labeled with PKH. Test cells were stimulated with CD3/CD28 (1:5000/1:2000) and after 16 hours CSFE labeled CD4+ T cells (responder cells) were added (T cell suppressor assay). After 5 days T cells were harvested and cell cycle progression analyzed by flow cytometry. Depicted is the cell cycle progression of responder cells stimulated in the presence of MF derived test cells (white histograms) and of responder cells stimulated by L.lactisIL-10 or L.lactisIL-10/MF derived test cells (gray histograms).

b. Immature DC were left unstimulated (gray histogram), stimulated with L. lactisIL-10 (continuous line) or with L.lactisIL-10/MF (discontinuous line). Maturation status after 48 hours was assessed by CD83 and CD86 expression.

earlier the presence of MF and L.lactisIL-10 during DC maturation induced effective regulatory T cells as only 39% of all responder cells went into cell cycle progression (Figure 3a). Subsequently, maturation status of DC matured by L. lactisIL-10 in the presence and absence of MF was compared (Figure 3b). Regulatory DC activated by L.lactisIL-10 and MF were CD83+ and CD86high, while DC activated by L.lactisIL-10 alone were CD83- and CD86low (Figure 3b).

Apparently, full maturation of DC activated by L.lactisIL-10 is needed for the induction of regulatory DC and regulatory immune responses.

IL-10 production by regulatory DC is essential for the induction of regulatory immune responses

To delineate the role of IL-10 in the generation of regulatory immune responses blocking experiments with an antibody directed against IL-10
were performed. As discussed earlier, bacterial IL-10 was important for the induction of effective regulatory DC (Figure 4a) and addition of αIL-10 during initial activation of immature DC reduced the suppressor activity (Figure 4a). When αIL-10 was added to the mixed culture of \textit{L.lactis}\textsuperscript{IL-10} induced regulatory DC and naïve T cells the inhibition was lost and all (100%) responder T cells went into cell cycle progression (Figure 4a). However, αIL-10 had no inhibitory effect on regulatory T cells during the culture of \textit{L.lactis}\textsuperscript{IL-10} induced regulatory T cells and responder T cells as only 52% of all responder T cells went into cell cycle progression (Figure 4a). In addition, neither αTGF-β nor αCTLA-4 were able to abrogate the regulatory activity of \textit{L.lactis}\textsuperscript{IL-10} induced effector T cells during the suppressor-assay (Figure 4b). On the contrary when effector T cells and responder T cells were cultured separately in a transwell system the suppressor function of effector T cells was complete abrogated (Figure 4b).

These results suggest that IL-10 production by \textit{L.lactis}\textsuperscript{IL-10}-primed regulatory DC is essential for the induction of suppressor T-cells while IL-10 production by suppressor T-cells was not required for the suppressive effect.

**Discussion**

IL-10 is a well-known immunomodulator that is able to reduce the maturation and cytokine production of immature DC[17]. Treatment of immature DC with IL-10 results in the induction of antigen-specific anergic T cells[18, 19]. These T cells appear to be able to suppress the activation and proliferation of allogenic T cells in a contact dependent, antigen specific manner that is mainly dependent on CTLA-4 expression but not on IL-10 production by T cells[20].

In the present study we demonstrate that activation of DC with the combination of spatially restricted IL-10 and a non-pathogenic microorganism primes regulatory DC. \textit{L.lactis}\textsuperscript{IL-10} induced regulatory DC, provided they are fully matured, produce high amounts of IL-10 and promote the development of regulatory T cells from naïve precursors. A common function of many different types of regulatory T cells described is the ability to suppress the proliferation and cytokine production of bystander T cells. Here we describe yet another type of regulatory T cells which, in some aspects, is different from those earlier described. These regulatory T cells instructed by \textit{L.lactis}\textsuperscript{IL-10} – exposed DC are able to induce suppression in the absence of DC and/or bacteria and produce large amounts of both IL-10 and IFN-γ. In addition, their effects are not mediated via TGF-β or CTLA-4. Because \textit{L.lactis}\textsuperscript{IL-10} derived effector T
Cell-based delivery of IL-10

It is likely that yet undefined contact-dependent factors mediate their suppressive effect on CD4\(^+\) T cells. Apparently, IL-10 production by regulatory DC is essential for the induction of regulatory T cells while IL-10 production of regulatory T cells was not needed for the suppressive effect. The exact function of IL-10 production by regulatory T cells is not known but might be important for the persistence of regulatory immune responses through feed-back on newly recruited immature DC in the periphery \textit{in vivo}[21].
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*L. lactis* is a food-grade gram-positive bacterium that is used for food fermentation and it is regarded as intrinsically safe for human use. The utilization of *L. lactis* for delivery of proteins [22, 23] has several advantages including the fact that like other lactic acid bacteria, *L. lactis* has intrinsic characteristics able to suppress immune responses via modulation of DC[24]. In our experiments we found that the development of regulatory T cells could be prevented by the addition of anti-IL-10 antibodies during co-culture of *L. lactis*IL-10/immature DC and mature DC/naive T cells. This suggests a possible IL-10 mediated induction of suppressor immune responses by *L. lactis*IL-10.

Lactic acid bacteria are able to temporarily divide and reside within the gastrointestinal tract maintaining a continuous production of specific proteins. Thus, we propose a novel cell-based strategy to target DC function; bacteria are employed to spatially restrict the delivery of recombinant proteins at the cell surface of target cells, in this case DC. Treatment of human beings with recombinant bacteria is a novel and highly promising avenue to treat immune mediated gastro-intestinal disease. A phase I study in patients with Crohn’s disease has shown that genetically engineered bacteria are clinically safe and biologically contained[25].

In conclusion, we provide evidence for a cellular mechanism as to how genetic engineered *L. lactis* may be adapted for the induction of regulatory immune responses in humans. Spatially restricted production of IL-10 by genetically engineered *L. lactis* modulates DC function in a regulatory manner. The observation that genetic modified bacteria are able to deliver biologically active proteins to human cells opens several possibilities to develop other therapeutic strategies. It might for example be interesting to test biologically active proteins not only for immuno-regulation in chronic inflammation but also for growth-regulation in cancer.

**Materials and methods**

**Bacterial strains**

For the generation of *Lactococcus lactis* MG1363 IL-10 (*L. lactis*IL-10) and *Lactococcus lactis* MG1363 (*L. lactis*) the plasmid pOTHY12 was used. For *L. lactis*IL-10, a 1 Kb region including at its 3’ end the constitutive thyA promoter (PthyA) from *L. lactis* MG1363 precedes a fusion gene between the usp45 secretion leader and hIL-10. This leads to a functional
coupling of PthA to usp45-hIL-10, expression of the precursor, correct N-terminal processing of the precursor and secretion of mature hIL-10. In optimised in vitro growth, L. lactis MG1363 pOTHY12 will produce in its culture supernatant approximately 1 µg hIL-10 per 2x10⁹ bacteria. In pOTHY12, the hIL-10 gene is followed by a region identical to the 1Kb segment downstream of the thyA gene. Both L. lactisIL-10 and L. lactis were grown overnight at 37°C (Elbanton incubator) in M17 broth (Difco) supplemented with 0.5% glucose and 50 µg/ml erythromycin (Abbott, Saint-Rémy-sur-Avre, France). Bacteria were diluted 1:50 in IMDM 5% FCS and grown for three hours at 37°C and harvested at exponential growth phase (approximately 1x10⁷ cfu/ml).

**Generation and maturation of DC**

All cultures were performed in Iscove’s modified Dulbecco’s medium (IMDM) with 1% FCS (HyClone, Lagan, UT) and erythromycin (50 µg/ml, Abbott). Peripheral blood of healthy volunteers was used to generate immature DC; monocytes were isolated after Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradient centrifugation. Then, 5x10⁵ monocytes were cultured in the presence of recombinant human (rhu) GM-CSF (500 U/ml; a gift from Schering-Plough, Uden, The Netherlands) and rhuIL-4 (250 U/ml; Pharma Biotechnology). After 6 days, cultures consisted of CD14⁻, HLA-DR⁺, CD83⁻, CD86low and CD40high immature DC. These cells produce high amounts of IL-12p70 upon stimulation with CD40L and are called immature DC. Maturation was achieved at day 6 by adding 1x10⁵ cfu of the above-mentioned bacteria with or without the following stimuli: recombinant human (rhu) IL-1β (5 ng/ml; Boehringer Mannheim, Germany), rhu TNF-α (25 ng/ml; PBH, Hannover, Germany), LPS (Sigma) and rhu IL-10 (Schering-Plough, Uden, The Netherlands). After 4 hours of DC culture with viable bacteria, 50 µl of supernatant was harvested for measurement of bacterial IL-10 (direct cytokine measurement; CLB, Amsterdam, The Netherlands) and gentamycin (86 µg/ml, Sigma) was added to prevent bacterial overgrowth. On day eight (mature) DC were harvested and washed twice, culture of DC on M17 agars confirmed that bacterial killing was complete. Mature DC were stimulated (2 x 10⁴ cells in 200 µl) with one of the following stimuli: IFN-γ (gift from Dr. P van der Meide; U-Cy tech, Utrecht, The Netherlands) and/or CD40L-transfected J558 plasmacytoma cells (gift from Dr. P. Lane, Birmingham Medical School, Birmingham, U.K.). After 48 hours stimulation, supernatants were used for cytokine detection using ELISAs for IL12p70 (R&D Systems, detection limit 31.2 pg/ml) and IL-10 (CLB, Amsterdam, The Netherlands).
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**T cell priming by mature DC**
Mature DC (5x10^3 cells/200 µl) were incubated with 2.5 x 10^4 highly purified CD4^+CD45RA^+CD45RO^- naive T cells (>90% as assessed by flow cytometry) from PBMCs using MACS separation system (Miltenyi Biotech, Germany). When indicated rat-anti-human IL-10 neutralizing IgG1 (1:1000, BD Pharmingen, San Jose, CA, USA) antibodies were added to the mixed lymphocyte reaction, purified rat IgG1 (BD Pharmingen) was used as isotype control. After adequate priming, T cells were further expanded in IMDM 10% FCS containing IL-2 (10 U/ml, Cetus, Emeryville, CA) and IL-15 (10 ng/ml, R&D Systems). When proliferation was halted, T cells were harvested and used for restimulation with CD3 and CD28 or suppressor T cell assay.

**T cell restimulation and cytokine measurement**
T cells (100.000 cell/200 µl) were stimulated with soluble mouse anti human-CD3 and mouse anti human-CD28 (both CLB, Amsterdam, The Netherlands), final dilution 1:1000. After 48 hours stimulation, supernatants were used for cytokine detection using ELISAs for IFN-γ (R&D Systems, detection limit 31.2 pg/ml) and IL-10 (CLB)

**T cell suppressor assay**
Non-proliferating DC primed T cells (test cells) were harvested and washed three times with serum-free IMDM. Test cells (5x10^5) were stained with 3x10^-5 M PKH-26 (Sigma), a red cell cycle tracking dye, for 5 minutes at room temperature according to the manufacturer’s instructions. After thorough washing, 2.5x10^4 T cells were pre-activated overnight with anti-CD3 (1:5000) (CLB, Amsterdam, The Netherlands) and anti-CD28 (0.5 µg/ml) in round-bottom 96-well plates. For transwell experiments, 2x10^6 PKH labelled test cells were put in the upper compartment of a 24-well plate transwell system (Corning Inc.). The following day CD4^+ T cells (responder cells) were labeled with CFSE (0.5 mM, Molecular Probes Inc., Eugene, OR), a green cell cycle tracking dye, for 15 minutes at room temperature. After 5 days the cellular content of CFSE in the responder cells was analyzed by flowcytometry. As indicated, rat-anti-human IL-10 (BD Pharmingen), goat-anti-human TGF-β (R&D, Minneapolis, USA) and mouse-anti-human CTLA-4 (Bioscience) were used as neutralizing antibodies.
Statistics

For comparison of cytokine production a heteroscedastic Student t-test was performed, when multiple groups were present a Kruskall-Wallis One Way Analysis of Variance was performed (SPSS, version 11.01, Chicago, IL, USA). Statistical significance was defined as a p<0.05, confidence interval 95%.

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References

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