Macrophage activation phenotypes in type 1 diabetes pathogenesis and therapy

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Abstract

Macrophages are an important key effector cell in the immune system which can practically be found in every tissue. Macrophages have for a long time been considered a population of cells only responsible for pro-inflammatory responses and anti-microbial activities. But over the past decade, many have come to realize the amazing plasticity of macrophages in response to different stimulations. The anti-microbial and pro-inflammatory macrophage is known as classically activated macrophages but newly discovered phenotypes have been revealed named wound-healing macrophages and regulatory macrophages. Through systematic screening we have identified an inducible macrophage activation state which has both wound-healing and regulatory capabilities activated by the novel cytokine combination IL-4/IL-10 with or without TGF-β.

Introduction

The immune system is a highly sophisticated multicellular defense which protects the host from diverse types of invaders. The protection is maintained by two systems, the innate and the adaptive system. The macrophage is one important immune effector cell which has a critical function in both of these systems and is also found in almost every tissue. They have the ability to recognize danger signals through the toll-like receptors (TLRs) which are one type of pattern-recognition receptor (PRR). Macrophages also have the capability to produce reactive oxygen species (ROS), such as nitric oxide (NO) which is another defense mechanism against intracellular infectious bacteria. They also produce a variety of different cytokines and chemokines which orchestrate the immune response at the site of inflammation. Furthermore, the macrophages is one of the professional antigen-presenting cells (APCs), which can process antigen and present it to T-helper cells (Th cells) through the MHC class II receptor complex. Macrophages are also involved in the regular ‘cleaning’ process, which includes phagocytosis of apoptotic bodies, cellular and extracellular debris during tissue remodeling but also the uptake of erythrocytes, approximately $2 \times 10^{11}$ erythrocytes each day which equals to almost 3 kg of iron per year (1). These different functions are mediated by membrane molecules such as different scavenger receptors, integrins and cytokine/chemokines receptors on the macrophages.

Macrophages have for a long time been accepted to be a cell that is mainly involved in pro-inflammatory responses. This phenotype is identified by different markers such as high expression of the enzyme inducible nitric oxide synthetase (iNOS) which leads to NO, the respiratory burst, the secretion of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin(IL)-1, but also the ability to engulf, process and present antigens
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to T cells (1) (2). This phenotype is also known as \textit{classically activated macrophages or type I macrophages}. The polarization is activated by two signals, one cytokine receptor stimulus such as via TNF-\(\alpha\) or interferon gamma (IFN-\(\gamma\)) receptors and a secondary signal mediated by for instance TLR stimuli such as lipopolysaccharides (LPS) or CpGs (2).

Many reports have recently revealed that there is high plasticity in the macrophage population upon non-inflammatory activation. These different cell phenotypes have received a lot of attention during the last decade. The phenotype has for a long time been known as \textit{alternatively activated macrophages or type II macrophages}. They are recognized by the secretion of anti-inflammatory cytokines such as IL-10 and/or transforming growth factor beta (TGF-\(\beta\)), low or no secretion of IFN-\(\gamma\), TNF-\(\alpha\) and IL-1, lower expression of MHC class II and iNOS, higher expression of the membrane receptors CD163 and CD206, improved phagocytosis but also higher expression of the enzyme arginase which produces L-ornithine, a precursor molecule for collagen production (2) (3). The alternative activation of macrophages is not as well defined as for their classically activated counterparts. The first findings showed that IL-4 (4) has the ability to turn inactivated macrophages into alternatively activated macrophages, but it was later determined that IL-4 in combination with IL-13 had an enhancing effect (2). Further investigations have demonstrated that there are a variety of different stimulations which can induce other forms of alternative phenotypes on macrophages, indicating that there is variability within the alternative activated macrophage phenotype. Stimulation with glucocorticoids (e.g. dexamethasone), the immune-suppressive cytokine IL-10, IL-4, immune complexes in combination with TLR stimuli, and TGF-\(\beta\) (1) (2) (3) can at least promote two distinct polarization states, \textit{wound-healing macrophages} and \textit{regulatory macrophages} (1). How stable the different phenotypes are or if it is possible to switch between them has not yet been clarified (5) but they all have a unique activation profile as evident in Figure 1. It has for a while been confusing to use the term \textit{alternatively activated macrophages} which indicates that there is only one alternative activation pathway to classical activation, which has now been proven not to be the case. Thereby these new definitions are a better way of describing the “alternative” macrophage phenotypes.

In the case of tissue injury IL-4 may be released, two early sources for this cytokine being basophils and mast cells. This will turn inactive tissue macrophages into wound-healing macrophages which will induce high expression of the enzyme arginase, thereby contributing to the production of the extracellular matrix. Upregulation of some scavenger receptors is also apparent, two of these being the mannose receptor CD206 and the haptoglobin/hemoglobin clearance receptor CD163 (6). Phagocytosis is also increased in wound-healing macrophages, a requirement for clearance of all the cell debris that is released during tissue injury. Wound-
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Wound-healing macrophages fail to present antigen to T cells, do not produce pro-inflammatory cytokines and have very low or no NO secretion (2) (1).

Figure 1 – Visualization on the plasticity of macrophages. The faded colors in the circle represent more diffuse phenotypes of macrophages that still need to be characterized. Classically activated macrophages are the most well defined phenotype of macrophages today, secreting high levels of pro-inflammatory cytokines and having the ability to present antigen to T cells and stimulate them through co-stimulatory receptors. Wound-healing macrophages which are also known as alternatively activated macrophages have the ability to produce many precursor molecules which are important for the extracellular matrix. They cannot present antigen due to low levels of the MHC class II complex. One good marker is the high expression of the mannose receptor CD206. Regulatory macrophages are the phenotype that is most difficult to detect due to few biomarkers, but IL-10 secretion is at the moment the best marker. They have the ability to modulate the immune response and also to suppress an ongoing, or to prolong inflammation. They can present antigen and also express co-stimulatory molecules such as CD80 and CD86. Reports have revealed another important role, which is their ability to induce regulatory T cells with antigen specificity (Modified from ref.).

Regulatory macrophages mainly arise from stress signals occurring during the adaptive immune response. It has been proposed that glucocorticoids such as dexamethasone can directly or indirectly induce regulatory macrophages (1) and it has been shown that IL-10 is a very potent candidate to induce the activation of this phenotype. Other data indicate that stimulation with prostaglandins, apoptotic cells and ligands for G-protein-coupled receptors can activate regulatory macrophages (1) (2). Unlike the wound-healing macrophages, regulatory macrophages do not produce resources for the extracellular matrix. They secrete high amounts of the anti-inflammatory cytokines IL-10 and TGF-β, have a higher expression of MHC class II and the co-stimulatory receptors CD80 and CD86 which are important for the activation of T
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cells (1) (3). They also have higher expression of FcγRIIb receptor, which has an immunoreceptor tyrosine-based inhibition motif (ITIM) that has anti-inflammatory effects, and this phenotype is thus associated with low or no secretion of pro-inflammatory cytokines (3). Regulatory macrophages have an overall suppressive phenotype and generate an anti-inflammatory milieu at the site of activation, but they are very hard to detect in vitro and especially in vivo. Characterization of tumor-associated macrophages (TAMs) has revealed that they have a phenotype reminiscent of regulatory macrophages (7) and they suppress infiltrating leukocytes in the tumor environment.

Another important aspect of regulatory macrophages is the indication that they have the ability to induce regulatory T cells (Tregs) (8). The mechanism underlying this pathway is not known but IL-10 or TGF-β seem to have important roles. Tregs have the competence to suppress inflammation through the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β, but they can also suppress in an antigen-specific pattern via the T-cell receptor (TCR) (9). This is a very powerful tool in autoimmune diseases such as type 1 diabetes (T1D) and multiple sclerosis (MS) because the Tregs have the ability to suppress specific pathogenic T cell responses at the site of inflammation. Regulatory macrophages can process specific antigens and present this to naïve T cells that will then turn into antigen-specific Tregs. These cells can then be transferred into the host and have an anti-inflammatory and suppressing effect which can reverse a disease. This is a novel mode of therapy that has been utilized in experimental autoimmune encephalomyelitis (EAE) (10), a good model of the human MS disease, similar therapy has also been demonstrated in type 1 diabetes (9).

T1D is a disease in which the β-cells in the pancreas become a target for leukocytes. The β-cells produce insulin, which is an important molecule in the body. The insulin hormone is one of the components that regulate glucose levels in the blood by promoting the body’s cells to increase the intake of glucose. If there is no insulin in the blood then glucose will accumulate and can be detected at very high levels in the urine and in the blood. This can lead to different acute complications such as hypoglycemia, ketoacidosis or nonketotic hyperosmolar coma. There is still today some discussion about what the most relevant pancreatic autoantigens are but glutamate decarboxylase 65 (GAD65) and insulin are two commonly accepted antigens. Macrophages are one of the major “badboys” when it comes to autoimmune disease, they are almost in every tissue and can therefore promote inflammation and attract other leukocytes such as pathogenic T cells. But they are also the cells that are involved in tissue destruction, mainly through secretion of ROS.
The study I conducted is a basic characterization of macrophages from a T1D mouse strain, both female and male. Different stimulations have been used and analysis profiles have been contrived to understand the different phenotypes these stimulations induce. The most regularly used animal model for investigation of T1D is the non-obese diabetic (NOD) mouse. These spontaneously develop diabetes when they are about 20 – 25 weeks old, but the insulitis pathology (the infiltration of leukocytes into the pancreas) initiates after approximately 10 weeks. It is therefore recommended that glucose levels in the animals are monitored at least once a week by using glucose sticks. The major reason that this mouse model has been preferred over others such as the BioBreeding (BB) rat is due to the well-known genome, more available monoclonal reagents and also the relative low maintenance cost (11). The first report of the NOD mouse model was published in 1980 by Makino K. et al and it had a immense impact in the field of diabetes research, many believing that a cure for type 1 diabetes would arise from use of this model. But more than 25 years after the discovery of this model there is still no cure for this disease.

Materials and methods

Animals

The female animals were 8 to 12 weeks old NOD mice that were purchased from Jackson Laboratories (Bar Harbor, Maine, USA); 8 to 12 weeks old male NOD mice were an appreciated gift from Petter Höglund at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet. They were kept in the animal facility at the Center for Molecular Medicine, Karolinska Hospital, where the conditions are pathogen-free and the animals had free access to chow and water. All experiments using mice were approved by the ethical committee Stockholm Nord.

Cell cultures

Femurs were extracted from animals and femoral bone marrow cells were collected by flushing through medium with a 21-gauge needle. Single-cell suspensions were prepared and resuspended in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine (all reagents from Life Technologies, Paisley, Scotland) and 20% L929 cell line supernatant which contains MCSF-1. Two femurs were used for one 75 cm² cell culture flask (Invitrogen, Carlsbad, CA, USA) and incubated at 37°C and 5% CO₂ in a humidified incubator for 8 days and for a further 2 days with 10% FCS and without 20% L929 cell culture supernatant. Cells were harvested by using pre-warmed 1xTrypsin (Gibco, Grand Island, NY, USA) and plated at a density of 0.5 x 10⁵ cells/ml in 24-well plates (Nunc, Roskilde,
Denmark). The only exception was when cells were plated for the low density array assays, cell concentrations at 5 x 10^5 cells/ml were used.

Reagents

LPS, fucoidan and dexamethasone were purchased from Sigma (St Louis, MO, USA) and applied at final concentrations of 10 ng/ml, 10 µg/ml and 200 nM, respectively. Vitamin D₃ was purchased from Merck (Whitehouse Station, NJ, USA) and a concentration of 50 ng/ml was used. The recombinant mouse cytokines IL-4, IL-10, IL-13, IFN-γ and TGF-β were purchased from R & D Systems (Minneapolis, MN, USA) and all cytokines were applied at a final concentration of 20 ng/ml except IFN-γ that was used at a concentration of 10 ng/ml. The tumor supernatant was an appreciated gift from Fredrik Eriksson at the Cancer Center Karolinska Hospital, the supernatant being derived from a B16-F10 mouse melanoma cell line obtained from ATCC (Rockville, MD, USA).

Enzyme-linked immunosorbent assay (ELISA) analysis

ELISA kits for detection of secreted TNF-α, IL-6, CCL3 and IL-10 in cell culture supernatants were purchased from eBioscience (San Diego, CA, USA) and used according to the manufacturer’s instructions. In brief, supernatants were collected from macrophage cultures which had been incubated for 3 days at a cell concentration of 0.5 x 10^5 cells/ml with or without the indicated stimulants for the indicated times, were run in duplicates and absorbance was measured at 450 nm and subtracted with the absorbance at 562 nm using a microplate reader (LabSystems, Basingstoke, UK).

Nitric oxide assay

The measurement of nitric oxide in the cell culture supernatants was performed by using the Griess reagent (modified; G-4410 Sigma, St Louis, MO, USA), which provides a surrogate marker and quantitative indicator of NO production. Supernatants from macrophage cultures which had been incubated at a cell concentration of 0.5 x 10^5 cells/ml with or without the indicated stimulants and for the indicated times were analyzed. Samples were plated in duplicate wells of 96-well plates, mixed with an equal volume of freshly prepared Griess reagent and incubated for 15 min at room temperature. Absorbance was measured at 540 nm using a microplate reader (LabSystems, Basingstoke, UK).

Morphological characterization by immunostaining

Cells were cultured on small round glass slides placed in 24-well plates. After treatment with the indicating stimuli or medium alone, fixation of the cells was performed using 4%
paraformaldehyde pH 7.3 in 1x phosphate buffered saline (1xPBS) (Gibco, Grand Island, NY, USA) incubated at room temperature for 30 min. The fixed cells were incubated with *Maackia amurensis* Lectin I (MAL1) conjugated with fluorescein(Vector Laboratories, Burlingame, CA, USA) at a concentration of 20 µg/ml for 1 hour in the dark at room temperature. MAL1 binds to glycoconjugates bearing galactosyl (b-1,4) N-acetylglucosamine structures. The slides were washed with 1xPBS three times each. The nuclei were stained with DAPI (Molecular Probes, Leiden, The Netherlands) diluted 1:2000 and incubated for 30 seconds at room temperature. The slides were washed three times with 1xPBS and three times with distilled water. The slides were mounted with Mowiol (Calbiochem, La Jolla, CA, USA) on rectangular glass microscope slides and analyzed using a Leica DRMBE fluorescent microscope equipped with a DeltaPix CCD camera. The pictures were analyzed and enhanced by using the included DeltaPix software, every picture being treated in the same way.

### RNA isolation, cDNA synthesis and Taqman low density arrays

Stimulated macrophages at cell concentration of 5 x 10^5 cells/ml were subjected to RNA isolation using the RNeasy kit (Qiagen Inc) according to the manufacturer's instructions. After isolation the RNA concentration was measured by spectroscopy (NanoDrop, Rockland, DE). cDNA was synthesized using SUPERSCRIPT™ II (Invitrogen) reverse transcriptase as per manufacturer. For the TaqMan® LDA assay, cDNA were diluted 400x with RNase-free water to end up on a concentration around 50 ng/ml, 50 µl of 2x Universal TaqMan® Mastermix (Applied Biosystems, Foster City, CA) were added to 50 µl of diluted cDNA. The mixture was applied to an LDA plate (Applied Biosystems) containing 45 different macrophage target genes including 3 housekeeping genes (18S, GAPDH and HPRT). The plate was spun for 2 min at 330 G and was sealed with a manual sealer (Applied Biosystems). The RT-PCR analysis was performed in a 7900 HTA (Applied Biosystems) using the default cycling conditions 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Fluorescent signals were collected during the annealing temperature and CT threshold was set at the point where the sample signals were exponential. Data was analyzed with the comparative CT method and sample data was normalized using the geometric mean of three housekeeping genes (18S, GAPDH and HPRT) which had a correlation factor higher than 0.8. All experiments were related to a negative control.

### Functional assays with CSFE staining and fluorescence-activated cell sorter (FACS)

Spleen and lymph nodes were extracted from female NOD mice. Splenocytes and lymphocytes were extracted and pooled together. Erythrocytes were removed using a lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, in MilliQ dH₂O, 0.2 µm filtered) and filtered through a 40
µm cell strainer to get rid of erythrocyte aggregation and debris. The cells were washed with DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (all reagents from Life Technologies, Paisley, Scotland) and spun down at 1200 rpm at 4 °C for 10 minutes. The CFSE stock solution (Sigma, St Louis, MO, USA) had a concentration of 50 mM and was diluted to 5 µM in 10 ml DMEM for 1 x 10⁷ cells/ml. The CFSE-cell solution was incubated for 5 min in a 37 °C water bath. The reaction were stopped by adding 20 ml ice-cold complete DMEM (10% FCS included), this step being repeated 2 times by adding 10 ml ice-cold complete DMEM. Two experimental designs were used, one involved 24 hours pre-activated lymphocytes/splenocytes that had been activated by plate bound anti-CD3 in 24-well plate. The lymphocytes/splenocytes were washed with DMEM and transferred to pre-stimulated macrophages. The macrophages were stimulated for 24 hours with IL-4/IL-10 or IL-4/IL-10/TGF-β in non-coated 24-well plates and then washed with DMEM before the lymphocytes/splenocytes were added. The combined cells were incubated at 37°C and 5% CO₂ in a humidified incubator for 3 days before readout. This experiment is called Pre-Mac/Pre-Tcell. Naïve lymphocytes/splenocytes were used in the second experimental design, the lymphocytes/splenocytes being transferred to an anti-CD3 pre-coated 24-well plate containing pre-stimulated macrophages. The macrophages were stimulated as in the previous experiment, 24 hours with IL-4/IL-10 or IL-4/IL-10/TGF-β and then washed with DMEM before adding the naïve lymphocytes/splenocytes. Note that the plates were coated with anti-CD3 before the macrophages were added to the wells. The combined cells were incubated at 37°C and 5% CO₂ in a humidified incubator for 3 days before readout. This experiment will be called Pre-Mac/Naïve-Tcell. The positive control were naïve lymphocytes/splenocytes added to anti-CD3 pre-coated 24-well plates and the cells were incubated at 37°C and 5% CO₂ in a humidified incubator for 3 days before readout. The wells were coated by using anti-CD3 antibody from BD Bioscience (Franklin Lakes, NJ, USA) and were diluted with 1xPBS to 5 µg/ml and 1 ml were added to each well and incubated for 1 hour at 37°C and 5% CO₂ in a humidified incubator. The wells were washed 3 times with 1xPBS. The readout was performed using a FACScalibur from BD Bioscience. The cells were washed with 1xPBS before analyzed. The data were analyzed using the FlowJo software from TreeStar (Ashland, OR, USA).

**Results**

*Variation in the IL-10 response depends on the stimulation agent and sex*

A panel of 12 different stimulation agents was used to stimulate female bone marrow derived macrophages and for the male counterpart, a panel of 8 different stimulations was used. One of the best biomarkers for anti-inflammatory or regulatory macrophages at the moment is secreted
IL-10, but also the suppressive effects on IL-6, TNF-α and NO. High levels of IL-10 were not been detected from the female strain, the two most prominent stimulations being IL-4/IL-13 and Fucoidan (Figure 2). We did not measure IL-10 in the supernatants that had been stimulated with IL-10 due to the risk for measuring our own added IL-10 reagent. But there is a high probability that IL-10 or IL-10 in combination with IL-4 has enhancing effects on IL-10 secretion (12). Interestingly, the IL-10 levels were lower when the cells were co-stimulated with IL-4, IL-4/IL-13 or TGF-β and LPS/IFN-γ, except for stimulations with fucoidan and the tumor supernatant that still gave high levels of IL-10. Vitamin D3 and the tumor supernatant had no effects on IL-10 secretion during single stimulation but vitamin D3 had a suppressive effect on IL-10 secretion during co-stimulation.

The male IL-10 levels had a different profile relative to the female counterpart. Almost all stimulation agents had an effect on IL-10 secretion (Figure 3), especially dexamethasone. But high levels of IL-10 were also detected in the supernatant from LPS/IFN-γ treated cells. The tumor supernatant treated cells indicated very high levels of IL-10 secretion but the possibility that the IL-10 is from the tumor supernatant itself cannot be excluded. Dexamethasone induced high levels of IL-10 when the cells were co-stimulated with LPS/IFN-γ. Fucoidan stimulated cells secreted high levels of IL-10 during the co-stimulation assay. The only stimulation that had no effect on IL-10 secretion during co-stimulation was IL-4/IL-13.

**IL-4/IL-10 with or without TGF-β has suppressive effects on TNF-α, CCL3 and NO during co-stimulation with LPS/IFN-γ**

Single stimulated female bone-marrow derived macrophages did not secrete TNF-α or NO except for the LPS/IFN-γ and to some extent fucoidan stimulated cells. Macrophages co-stimulated with IL-4/IL-10 and LPS/IFN-γ secreted lower levels of pro-inflammatory agents such as TNF-α and NO (Figure 2) in contrast to the LPS/IFN-γ stimulated cells (classically activated). The suppressive effects were enhanced when TGF-β were added to the IL-4/IL-10 group. There was low or no secretion of TNF-α or NO from IL-4/IL-10 or IL-4/IL-10/TGF-β stimulated cells. Dexamethasone had similar suppressive effect on the TNF-α secretion but lower effects on the NO levels. These data indicate that IL-10 and TGF-β were very important players when it came to the suppressive effects on the pro-inflammatory agents. Another interesting finding was the suppressive effects of vitamin D3 on TNF-α secretion during co-stimulation.

The male derived macrophage data pointed to a similar pattern (Figure 3), no increase of TNF-α or NO during single stimulations except for CCL3 secretion. Macrophage inflammatory protein-1 (MIP-1) also known as chemokine CCL3, is a very pro-inflammatory chemokine and has the
ability to attract T cells to the site of inflammation through vascular cell adhesion and
transendothelial migration. It also enhances the secretion of IFN-γ by T helper cells (13). Single
stimulation with IL-4/IL-10 yielded a small increase of CCL3 relative to the untreated control.

IL-4/IL-10 had the greatest anti-inflammatory effects on the co-stimulated cells. IL-4/IL-10 had
a suppressive effect on the secretion of CCL3 from male bone marrow-derived macrophages
during co-stimulation with LPS/IFN-γ (Figure 3). Interestingly, fucoidan increased CCL3 levels
during co-stimulation to higher levels than with the single LPS/IFN-γ stimulation. Dexamethasone and IL-4/IL-13 had small effects and the tumor supernatant and vitamin D₃ had
almost no or very small effects on the CCL3 secretion during the co-stimulation assay.
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- **IL-10 Secretion**
  - Absolute change
  - Relative change
- **IL-6 Secretion**
- **TNF-α Secretion**
- **NO Secretion**

**Figure 2** - IL-10, IL-6 and TNF-α cytokine secretion and NO production from stimulated female bone marrow-derived macrophages. IL-4/IL-13 have the ability to induce macrophages to produce IL-10, but not during the co-stimulation with LPS/IFN-γ. Fucoidan shows the ability in both single and co-stimulations to induce the secretion of IL-10. The levels of IL-10 are relatively low overall. IL-6 secretion is upregulated for almost all stimulations except IL-10, TGF-β/IL-10 and dexamethasone. Only dexamethasone and vitamin D3 had the ability to influence IL-6 secretion during the co-stimulation. Interestingly, IL-4/IL-10 and TGF-β/IL-4/IL-10 increase the secretion of IL-6 during single stimulations to levels more than half that of the LPS/IFN-γ stimulated cells. TNF-α and NO have basically the same pattern, TGF-β and IL-10 have the best affects on the secretion of these two agents. Dexamethasone has better effects on TNF-α secretion than on NO. Vitamin D3 had suppressive effects on TNF-α secretion. Black bars represent the absolute value and the grey bars represent the change relative to unstimulated control macrophages. Values derive from three separate experiments.
Dexamethasone and LPS/IFN-γ had the greatest suppressive effect during co-stimulation. All of the single stimulation agents except LPS/IFN-γ and tumor supernatant had small suppressive effects on TNF-α secretion. IL-10 and dexamethasone had the best suppressive effect on TNF-α and NO during co-stimulation. The single stimulation agent which had an effect on CCL3 secretion was IL-10, which also had the most suppressive effect during co-stimulation. Black bars represent the absolute values and the grey bars represent the change relative to unstimulated control macrophages. Values derive from triplicates from one experiment.
Both female and male derived macrophages secrete high levels of IL-6

One interesting discovery was the high levels of IL-6 produced by stimulated macrophages. For the female strain (Figure 2), no stimulation agent could decrease the IL-6 levels below that of unstimulated control macrophages. The only stimulation that did not change IL-6 secretion levels appreciably were IL-10, TGF-β/IL-10 and dexamethasone, respectively. To our surprise, IL-4/IL-10 and TGF-β/IL-4/IL-10 increased IL-6 secretion to more than one-and-a-half the levels of LPS/IFN-γ treated macrophages.

**Figure 4** - A. Switching assays in which the pro-inflammatory agent LPS/IFN-γ was applied 24 hours followed by addition of anti-inflammatory/type II cytokines/agents for another 24 hours. LPS/IFN-γ induces a small increase of IL-10 secretion when stimulated for 24 h, and upon restimulation the only upregulating stimulation was IL-4/IL-13 and to some extent fucoidan. NO secretion seems to be unaffected by the switching procedure. B, Switching assays using the anti-inflammatory/type II agents IL-4, IL-10, IL-13 and dexamethasone. IL-10 and dexamethasone prime macrophages to become excellent IL-10 secreting cells when restimulated with LPS/IFN-γ. The cytokines did not affect NO secretion during the first 24 h of stimulation except dexamethasone which decreased NO levels. NO secretion increased dramatically when stimulated for another 24 h with LPS/IFN-γ in all cases. White bars represent 24 h of stimulation with the stimulation agent written prior to the + sign, grey bars represent another 24 h stimulation (48 h accumulated) with the agent represented subsequent to the + sign. The wells were washed three times with DMEM between stimulations, one experiment being performed in duplicate.
The data suggest that it is IL-4 which is responsible for the higher IL-6 levels. The only stimulation agents that had an influence on the LPS/IFN-γ co-stimulated macrophages were vitamin D₃ and dexamethasone. The male derived macrophages could be stimulated so that they secreted lower levels of IL-6 relative to the unstimulated control macrophages (Figure 3). IL-10, dexamethasone, fucoidan and vitamin D₃ had the ability to suppress IL-6 secretion, but only dexamethasone and partially fucoidan could affect IL-6 levels during co-stimulation, the other stimulation agents having no effect at all. The data from both male and female derived macrophages indicates that dexamethasone was the only agent that could suppress IL-6 secretion from LPS/IFN-γ stimulated macrophage from both sexes.

**IL-10, IL-4/IL-13 and dexamethasone prime naïve macrophages to become regulatory macrophages upon stimulation with LPS/IFN-γ**

One assay was designed with macrophages derived from male bone marrow to evaluate how the cells behaved upon switching between pro-inflammatory and anti-inflammatory cytokines during 48 hours (Figure 4), with 24 hour stimulation in each case. IL-4/IL-13 was the only cytokine group that could uphold IL-10 secretion when switching from pro- to anti-inflammatory cytokines was carried out. No measurements of IL-10 were done on IL-10 treated cells for previously described reasons. IL-10 and dexamethasone pre-stimulated macrophages show fascinating potential to become cells which have a high ability to produce and secrete IL-10 upon stimulation with LPS/IFN-γ. IL-4/IL-13 stimulated cells secreted almost the same amounts of IL-10 as did the control group, 24 h DMEM + 24 h LPS/IFN-γ. The NO response was very stable when the cells were stimulated with LPS/IFN-γ. None of the anti-inflammatory/type II agents had the capability to prevent NO secretion during the switching assay, either way. Dexamethasone induced lower NO secretion during the first 24 hours but when switched to LPS/IFN-γ for a further 24 hours the NO secretion reached the same levels as for the other stimulations.

**Macrophages show diverse types of morphologies when stimulated with different stimulation agents**

The female derived macrophages revealed different morphological phenotypes when stimulated with the different agents (Figure 5). The untreated cells had irregular forms that often consisted of 3 nodes, low fluorescence intensity being detected. IL-4 stimulated macrophages were much rounder, were less dendrites but had no or few fibril structures. The cells had higher fluorescence intensity relative to untreated macrophages but also populations of big groups/clusters (clumps). High fluorescence intensity was also detected in the IL-10 treated
cells, with a significant higher expression of fibrils and many pseudopodia reaching out from the cell body. The IL-4/IL-10 treated cells illustrated a morphology reminiscent of a mixture of both IL-4 and IL-10 treated cells. Higher fluorescence intensity, smaller pseudopodia with fibrils but not as much as for IL-10 alone, and cells in big clusters were also observed. The IL-4/IL-13 cells had more irregularly elongated shapes, low fluorescence intensity and no or low levels of fibrils. Big groups were also detected here. The TGF-β stimulated cells had a unique shape, forming very elongated but tubular cells. The macrophages had long pseudopodia but the shapes were very dendritic, had high fluorescence intensity and almost no big groups were evident. The TGF-β/IL-10 stimulated cells revealed a mixed morphology of both IL-10 and TGF-β treated cells. Long tubular shaped pseudopodia with fibrils were evident that at some positions fused together with fibrils from other cells. The fluorescence intensity was not much brighter than for the untreated cells and no big groups were observed. Another blended morphology was TGF-β/IL-10/IL-4 stimulated cells; it had some big groups that were found in different locations, and the groups had several round cells. Dendritic cells with lower levels of fibrils and pseudopodia were also found. Dexamethasone treated macrophages also demonstrated a unique morphology; they were more round but at the same type irregular with some very thin pseudopodia, but many cells had circular bright spots in the cytoplasm, that are evident at the lower left of the dexamethasone picture in Figure 5. Dexamethasone also introduced the formation of cells with bigger nuclei. The fucoidan stimulated cells had a very similar morphology as the untreated macrophages but with lower fluorescence intensity. The tumor supernatant had many similarities to IL-10, with the formation of pseudopodia and fibrils. Both flat and round cells were detected but also big groups were found. The classically activated macrophages i.e. LPS/IFN-γ stimulated cells were bigger but also very flat in the outer parts of the cell body, having a more bright intensity at the core of the body.
Variations were detected in gene expression during single stimulations but patterns could be observed.

A panel of 9 anti-inflammatory genes has been monitored using low density microarrays for female derived macrophages (Figure 6) which had been stimulated with the different agents. IL-4 treated cells clearly reveal wound-healing capabilities due to the extremely high expression of arginase (Arg1). IL-10 and TGF-β in combination with IL-4 had an enhancing effect on Arg1 expression. IL-10 itself did not affect the expression of the anti-inflammatory genes, except for a small decrease of Arg1 but also suppression of CCL22 expression by 10-fold. Vitamin D<sub>3</sub>, IL-4 and IL-10 induced almost a 10-fold increase of the CD163 receptor but IL-4 and IL-10 together increased the expression up to almost 30-fold. IL-4 (with combinations) and LPS/IFN-γ were the
only stimulations that affected the expression of FcγRIIb and SOCS1. The only stimulations that had an effect on IL-10 were TGF-β (with combinations) and LPS/IFN-γ; interestingly, TGF-β suppressed IL-10 secretion by almost 20-fold. A similar pattern was evident with the expression of CLEC4A2. IL-4 (with combinations) induced very small increases of the CD206 receptor, around a 3-fold change, but LPS/IFN-γ, TGF-β and TGF-β/IL-10 decreased this expression, especially LPS/IFN-γ.

The pro-inflammatory panel within the microarray consisted of 13 genes and is also depicted in Figure 6. LPS/IFN-γ clearly increased expression of many important pro-inflammatory genes such as iNOS (NOS2), CIITA (MHC class II), IL-1β, IL-6, TNF-α, CCR7 and CXCL10. IL-4 (with combinations) increased iNOS expression around 10-fold, the only stimulation that did not increase iNOS expression being IL-10 (with combinations). IL-10 itself decreased CCR7 expression more than 30-fold, as did TGF-β and dexamethasone. CIITA expression was extremely decreased by TGF-β/IL-10, dexamethasone and fucoidan. Dexamethasone and vitamin D3 had the highest impact on IL-1β expression, with more than 1000-fold and 40-fold decreases, respectively.

**TGF-β, IL-10 and dexamethasone had important anti-inflammatory roles on the macrophages when they were co-stimulated with LPS/IFN-γ**

Anti-inflammatory/type II stimulation agents were used in combination with LPS/IFN-γ to determine different gene expression profiles and to identify dominating signaling pathways (Figure 7). TGF-β/IL-10/IL-4 clearly increased Arg1 expression almost 200 times, indicating that TGF-β and IL-10 had an enhancing effect on the IL-4-dependent Arg1 expression. TGF-β/IL-10/IL-4 also suppressed LPS/IFN-γ and decreased CD206 expression. IL-4/IL-10 with or without TGF-β increased the expression of FcγRIIb, but interestingly no stimulation agent except dexamethasone could prevent the decrease of IL-10 expression. TGF-β and fucoidan had similar suppressive effects on CD206 expression similar to LPS/IFN-γ. TGF-β decreased the expression of CCL22 more than 50-fold.

IL-4 in combination with IL-10 but also dexamethasone had very suppressive effects on the expression of iNOS (Figure 7). TGF-β decreased the expression of iNOS but not to equivalent levels as did IL-4/IL10. IL-6 expression was increased by IL-10, IL-4/IL-13 and TGF-β, the expression being higher than for LPS/IFN-γ. Dexamethasone and fucoidan were two agents that had major negative effects on IL-6 expression. Another suppressive effect of dexamethasone was on CCR7 expression, which was decreased more than 40-fold. Fucoidan had extreme suppressive effects on TFPI expression, around 2000-fold.
Figure 6 – LDA results from single stimulated female bone marrow derived macrophages, the lines above and below the zero line representing no fold change. The data is from one experiment related to the unstimulated control.
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**Figure 7** – LDA results from co-stimulated female bone marrow derived macrophages, the lines above and below the zero line representing no fold change. The data is from one experiment related to the unstimulated control.
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**IL-10/IL-4 primed macrophages had suppressive effects on differentiating T cells.**

Macrophages that had been stimulated for 24 h by IL-10/IL-4 with or without TGF-β polarized to cells with different suppressive capabilities. T-cells pre-activated with plate-bound anti-CD3 were strongly suppressed by both IL-10/IL-4 and IL-10/IL-4/TGF-β treated macrophages (Figure 8, red) after three days of incubation. The macrophages suppressed the differentiating naïve T-cells more effectively (Figure 8, green); the TGF-β treated macrophages had even higher ability to suppress the differentiating T-cells as can be visualized in both generation 2 and 3 in Figure 8.

![Graphs](image)

**Figure 8** - Functional assay illustrating the suppressive effects of IL-4/IL-10 or TGF-β/IL-10/IL-4 stimulated macrophages (Pre-Mac) on α-CD3 pre-activated (Pre-Tcell, red) or activating (Naive T cell, green) splenocytes/lymphocytes. The graph represents the percentage of CFSE-stained cells in each generation i.e. division.

**Discussion**

The results clearly indicate that IL-4/IL-10 stimulated macrophages have suppressive effects on the secretion of pro-inflammatory agents such as TNF-α and NO independently of the sex. In fact, the male and female derived macrophages both followed the same pattern when it came to cytokine secretion. These data were confirmed by the TNF-α and IL-1β expression levels derived from the LDA assay. IL-10 seems to be the key player when it came to anti-inflammatory properties. Interestingly, TGF-β enhanced the suppressive effects of IL-10 in some cases. TGF-β/IL-10 stimulated macrophages had the highest expression of CIITA, a transcription factor associated with the MHC class II expression. Higher levels of MHC class II is important for the induction and activation of Tregs.

The levels of IL-10 secretion during single and co-stimulation were very low for both sexes. High levels of IL-10 were only detected after priming the cells for 24 hours with IL-10 or IL-4/IL-10
and then re-stimulating the cells with LPS/IFN-γ for another 24 hours. This two-hit stimulation process has also been observed from other groups (1) (12) and it has similarities to how the T cell becomes activated via two signal activation pathway. The major difference between the co-stimulation and the switching assay is the timing. Both the cytokines IL-10 and IFN-γ are added simultaneously during the co-stimulation assay, these two different pathways will then compete to polarize the cells towards one phenotype. The data indicates that IL-10 had a suppressive effect on classically activated macrophages, but the response was still pro-inflammatory. The timing was really important to take into account during the switching assay, the 24 hour IL-10 or IL-4/IL-10 stimulated cells being pre-polarized during the first step and then activated or re-stimulated for another 24 hours with LPS/IFN-γ. The IL-10 and IFN-γ signal cascade will not compete in this scenario; one hypothesis can be that the IL-10 stimulated macrophages have lower levels of IFN-γ receptors or trigger a cascade that inhibits the IFN-γ-mediated signal. LPS, on the other hand activates/stimulates the macrophages by binding to TLR4 and thereby delivers the secondary signal.

The opposite direction of switching, hence anti-inflammatory towards pro-inflammatory (classical), yielded a different profile. The 24 hour LPS/IFN-γ stimulated cells were very stable and did not change their polarization state when re-stimulated with an anti-inflammatory/type II agent for another 24 hours. Our in vivo studies have revealed other interesting results; we have obtained data that indicates the switching of tumor-associated macrophages (anti-inflammatory phenotype) to a more pro-inflammatory response that directly or indirectly suppresses the tumor and reverses the pathogenesis (14).

A molecule that has recently been related to autoimmune diseases is Vitamin D₃ (15). It has been reported that it has various effects on the immune system but our results from female derived macrophages indicates that it mediated significantly lower levels of TNF-α production, it also having suppressive effects on IL-6 and NO. The pattern was not evident in the male derived macrophages. Another effect of Vitamin D₃ was the suppression of IL-1β gene expression, as has also been reported from other groups (14).

The LDA data clearly indicates the wound-healing properties of IL-4 stimulated macrophages, with a high level of Arg1 expression and increased expression of CD163 and CD206. Interestingly, IL-10 in combination with IL-4 revealed a unique profile in the LDA results, the MHC class II expression being unchanged, but with lower expression of IL-1β, TNF-α and NOS2. The LDA results from the co-stimulated cells indicated high Arg1 expression, low NOS2 expression but also low IL-6 expression. This could unfortunately not be confirmed by the protein assay, one reason for this might be due to the high levels of IL-6 that were outside the detection range of the ELISA kit. This profile indicated properties of both a wound-healing and a regulatory phenotype. TGF-β had in some cases an enhancing effect on the anti-inflammatory
properties of the IL-4/IL-10 stimulated macrophages, except for one important cytokine, the IL-10 expression. The IL-10 expression was low for all the different stimulation agents, which can be due to the one hit/one signal procedure.

Another interesting finding was the diverse morphological phenotypes, that was revealed by application of the different stimulation agents. The IL-10 stimulated macrophages formed very dendritic and fibrous cell bodies that may increase the effectiveness to present antigen to T cells by letting them "roll" on the macrophage plasma membrane. These dendritic macrophage morphologies are very similar to the morphology of dendritic cells (DCs) which are the professional T cell activators. This cell body property brings the macrophages one step closer to their brother the DCs, which is a topic that has been brought up again to the table for revision (16).

The functional assay clearly presents the suppressive properties of IL-4/IL-10 stimulated macrophages. IL-4/IL-10/TGF-β stimulated macrophages seem to have a more suppressive effect on pre-activated T-cells, which can be important for scenarios of chronic ongoing inflammation. A future assay which may yield interesting results is to analyze how these macrophages acts upon specific Th1 and Th17 cells but also if there is any difference between IL-4/IL-10 or IL-4/IL-10 + LPS stimulated macrophages.

There is obviously a lot more to learn about these interesting and important immune cells, and while it was my ambition to shed more light onto their nature, I concluded that this is actually more mysterious than I originally thought.
References


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