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Macrophage plasticity

Alternatively activated macrophages alter their phenotype *in vivo*

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Abbreviations

AAMΦ…………………………………………………………Alternatively activated macrophage

Ab……………………………………………………………………………………………Antibody

CAMΦ……………………………………………………………Classically activated macrophage

coMΦ……………………………………………………………Co-infection-elicited macrophages

FACS………………………………………………………Fluorescence-activated cell sorting

Fig………………………………………………………………………………………………Figure

GAPDH…………………………………………Glyceraldehyde-3-phosphate dehydrogenase

IFN-γ…………………………………………………………………………………Interferon- γ

IL……………………………………………………………………………………………Interleukin

iNOS…………………………………………………………………Inducible nitric oxide synthase

LPS……………………………………………………………………………………Lipopolysaccharide

MΦ…………………………………………………………………………………………Macrophage

NeMΦ……………………………………………………………Nematode-elicited macrophages

PBS-MΦ………………………………………………………………………PBS-elicited macrophages

PCR………………………………………………………………………Polymerase chain reaction

PEC……………………………………………………………………………Peritoneal exudate cells

Relm -α……………………………………Resistin-like molecule- α (also known as FIZZ1)

RT…………………………………………………………………………………Room temperature

SalMΦ……………………………………………………………Salmonella-elicited macrophages

TNF- α ……………………………………………………………………Tumour necrosis factor- α

4cMΦ………………………………………………………………………IL-4c-elicited macrophages

YM…………………………………Secretory protein produced by activated macrophages

Abstract

Macrophages have a well known bactericidal function in the immune system. We also know that they play a role in tissue repair, parasite clearance and allergy. Classically activated macrophages will be producing pro-inflammatory cytokines including TNFα, IL-12, IL-1 and IL-6 and nitric oxide. Alternatively activated macrophages instead express Relm -α and YM-1 and instead of nitric oxide they make arginase and they produce proline and polyamine for tissue repair.

With the discovery of the alternative activated macrophages, the question came how these two develop; do they have plasticity abilities or are they derived from different monocytes? Several *in vitro* experiments have shown that the macrophages are able to change their phenotype from classical to alternative activated, but how do they change *in vivo*? Infection of C57BL/6 mice with *Salmonella typhimurium enterica* serovar Typhimurium and/or *Heligmosomoides polygyrus (bakeri)* gave us alternatively and classically activated macrophages. LPS and/or IFNγ and IL-4 and/or IL-13 were used for culturing *in vitro* as classical and alternative stimuli respective. After infection or culturing we looked at the characteristics of specific activation markers; iNOS, CD86 and CD40 as classical activation markers and YM-1 and Relm -α as alternative activation makers.

After 7 days of infection analysis with FACS and ELISA showed us that *H. polygyrus gyrus* infection increased the expression of Relm -α and YM-1 on macrophages, while it suppressed the expression of CD86. Infection with *S. typhimurium* increased the expression of iNOS on macrophages. Co-infection showed a dominating T_H2 response more similar to the *H. polygyrus* infection.

For culturing we used non-activated and IL-4c elicited macrophages. LPS and/or IFNγ induces a similar expression of classical activation markers in both macrophages, meanwhile the IL-4c elicited macrophages still express Relm -α and YM-1 .

For the *in vivo* study we transferred traceable cells from IL-4c injected mice to *S. typhimurium* infected mice. The macrophages acted similar to the *in vitro* experiment. This study shows us that macrophages are able to express classically activated markers after being alternatively activated. This could be used in control of inflammatory responses, cancer and other studies.

1 Introduction

Macrophages (MΦ) play an essential role in the immune system. For a long time we have known that classically activated macrophages (CAMΦ) produce pro-inflammatory mediators and clear out pathogens and apoptotic cells by phagocytosis and production of anti-microbial effector molecules (e.g. reactive oxygen or nitrogen species). More recently, studies have shown that MΦ also can adapt a phenotype rather associated with tissue repair, tumor progression, hypersensitivity and parasite infections and are called alternatively activated macrophages (AAMΦ) (Gordon 2003, Gordon & Martinez 2010, Stout *et al*. 2005).

During a bacterial infection the MΦ are stimulated with IFNγ secreted by T-helper lymphocytes and lipopolysaccharide (LPS) from the bacteria cell wall. The MΦ will then produce pro-inflammatory cytokines including TNF, IL-6 and IL-1 and nitric oxides (NO) to clear the microbes away (Gordon 2003) (fig. 1). This also causes tissue damage and as in many biological systems there is a counter mechanism, the AAMΦ. These macrophages have among other properties a tissue repair function (Gordon 2003). The AAMΦ phenotype is induced by interleukin (IL) 4 and IL-13 secreted by T helper 2 (T_H2) cells. It is characterized by the expression of distinct cytokines and receptors, which are markedly different to those normally found on CAMΦ. Expression of murine macrophage mannose receptor (Stein & Keshav 1992), Relm -α and YM-1 (Raes *et al*. 2002, 2003, Gordon 2003, Weng *et al*. 2007) are all increased when stimulated with IL-4. Pro-inflammatory cytokines IL-1, TNFα, IL-6, IL-12 and MIP-1α are suppressed by IL-4, giving the MΦ an anti-inflammatory phenotype (Varin & Gordon 2009) (fig. 1). Studies have shown an ongoing T_H2 response will reduce protection against bacterial infections (Finkelman *et al*.1997). Therefore it is important to understand how the immune system balances its reaction in a situation with two different pathogens.

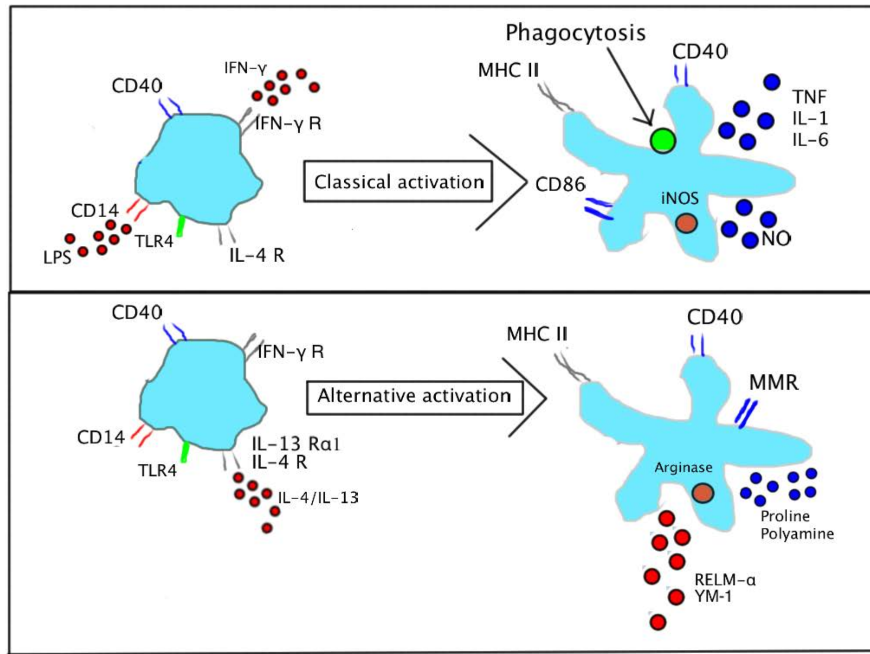

Figure 1. Classical vs. alternative activation of MΦ. IFNγ and LPS induce classical activation while IL-4 and IL-13 induce alternative activation**.** CAMΦ produce pro-inflammatory cytokines such as TNF, IL-1 and IL-6 and they express iNOS and produce nitric oxides to kill bacteria. AAMΦ are associated with wound healing, because they produce proline and polyamine and they secrete anti-inflammatory cytokines, Relm -a and YM-1 . Instead of expression iNOS they express arginase.

This brings us to the question how these MΦ act when both types of activation are needed directly after each other. Are there different populations of MΦ, can they change phenotype or do they renew faster when needed? This has been studied the last years *in vitro*. Mice have been infected with nematodes to get AAMΦ, or stimulated with thioglycollate to get inactivated MΦ and compared their phenotypes. These cells have then been stimulated with different stimuli to see their ability to change. MΦ derived from nematode infected mice have increased production of TNFα, IL-6 and NO after stimulation with LPS/IFNγ (Mylonas *et al* 2009). Stout *et al.* and others have shown that MΦ can change their phenotype when stimulated with the opposite stimuli *in vitro* and *ex vivo* (Stout *et al*. 2005, Mylonas & Nair 2009). However, all of these studies were either performed *in vitro* or the MΦ have been removed from their originally activating environment before re-stimulation with the opposing activating agent. For further research and therapies it is therefore interesting if this also happens *in vivo*.

We used two models of *in vivo* activation through infection with *Heligmosomoides H. polygyrus gyrus (bakeri) (H. polygyrus)* and *Salmonella typhimurium enterica* serovar Typhimurium (*S. typhimurium*) to induce AAMΦ and CAMΦ, respectively. *S. typhimurium* gives a well characterized model of human typhoid fever and *H. polygyrus gyrus* gives a good T_H2 response (Rosenberger *et al*. 2000, Maizels *et al*. 2011). So both have good reasons to be used in this model. *H. polygyrus* enters the body as stage 3 larvae (L3) and invades the duodenal wall for the first 7 days. Around the 8th day of infection they re-emerge into the lumen as adult worms, mate and produce eggs (Maizels *et al*. 2011). *S. typhimurium* is normally ingested orally and penetrates the intestinal mucosa and spreads via the lymph nodes to the spleen and liver (Rosenberger & Scott 2000). We inject the bacteria interperitoneally, sidestepping the initial crossing of the mucosa for experimental reasons, but the bacteria will also spread to the lymph nodes, spleen and liver. So both pathogens infect the small intestine affecting the surrounding peritoneal cavity and therefore create an area of strong T_H2 or T_H1 inflammation, respectively.

We test first if the reactions we get in C57BL/6 are useable for the following experiments. C57BL/6 mice are also know to express a predominant T_H1 response, so we do not know in what degree the alternative activated MΦ will be represented (Finkelman *et al*. 1997). We looked at the MΦ phenotype induced by these pathogens and then how the MΦ act when the mice are infected with both pathogens simultaneously.
To further address whether MΦ change their activation phenotype *in vivo* we made use of a transfer-model of activated cells, where AAMΦ from a T_H2- setting were transferred into *S. typhimurium* infected hosts and monitored for changes in their activation phenotype.

1.1 Aims

The overall aim of this project was to characterize model systems to determine, whether macrophages do change their activation phenotype during sequential co-infection with opposite polarizing agents *in vivo*.
To get answer on our main question we had several sub sub queries.
- Which markers of alternative or classical activation are expressed by macrophages during infection with *H. polygyrus* or *S. typhimurium*?

- Which of these allow clear distinction of the two activation phenotypes by FACS.
- Which activation phenotype do macrophages adopt in the mixed T_H2/T_H1-setting of co-infection?
- Do macrophages change their activation phenotype *in vivo* when exposed to opposing polarizing agents?

2 Materials and Method

2.1 Animals and infection

The animals used for this study were C57BL/6 and B6 DsRed female mice aged 8-10 weeks at the beginning of the study. The mice were housed and bred in our pathogen-free facility, except for the co-infection. These mice were located in our "dirty" facility. A licensed postdoctoral research fellow performed all the treatments on the living mice and was present when the peritoneal exudate cells (PEC) and spleens were isolated. Furthermore all work was approved by the University of Edinburgh Ethical Review Committee and performed in accordance with the UK Animals (Scientific Procedures) Act of 1986.

The pathogens were *Heligmosomoides polygyrus (bakeri)* and *S. typhimurium enterica* serovar Typhimurium (SL3261). The infectious dose of *H. polygyrus was* determined by counting the L3-stage larvae in 20 µl stock-solution prepared from feces from infected animals in duplicates (kind gift of Prof. Rick Maizels). The stock-solution was then diluted to a final concentration of ~200 L3 in 200 µl and instilled per oral gavage.

Overnight-cultures of *S. typhimurium* (kind gift of Prof. David Gray) were prepared in duplicate using 1 pipette-tip of frozen bacterial stock in 20ml LB-medium and cultured for approximately 18 hours at 37°C. The culture was then diluted 1:50 in Dulbecco's-Phosphate Buffered Saline (D-PBS, Sigma-Aldrich, Gillingham, UK) and 200 µl were injected intraperitoneal. Infectious dose was measured retrospectively by spreading the inoculum on LB-agar-plates in a serial dilution and cultured over night at 37°C and counting the colonies.

2.2 Extraction of PEC

The peritoneal cavity was flushed 3 times with 3 ml of RPMI-1640 supplemented with HEPES (Life Technologies, Glasgow, UK), 1% Penicillin/Streptomycin (P/S; Life Technologies) and 0.2% normal mouse serum (NMS; AbD-Serotec, Oxford, UK) to harvest the peritoneal exudate cells (PEC) and secreted cytokines. The first 3ml of lavage of each mouse was collected separately and centrifuged at 300g for 5 minutes at 4°C (Sorvall Legend RT, Buckinghamshire, UK) (the settings were the same in the

rest of the paragraph), 1 ml of the supernatant was taken of for further analysis with ELISA. The remaining PEC of the first, second and third lavage were then pooled and centrifuged. The supernatant was removed and the cells re-suspended in 1ml red blood cell lysis buffer (Sigma-Aldrich) (if necessary) for 2 min at room temperature. Adding 10 ml of RPMI supplemented with HEPES and 1% P/S stopped the reaction.

2.3 Determination of bacterial load in infected animals

The spleen was removed from each mouse and put into 4-5ml RPMI (+HEPES) 0.2% NMS and stored on ice. The spleens were then sterile mashed in 2ml D-PBS through gauze. 100 µl of this cell solution was plated on LB-agar plates in a serial dilution, in D-PBS. The plates were incubated for approximate 20 hours at 37°C to be able to count the number of colony forming units (cfu).

2.4 Cell counting

The PEC were centrifuged at 300 g for 5 min at 4°C (Sorvall Legend RT) and the supernatant removed. 2ml of RPMI (+HEPES) 1% P/S medium was added and 5 µl of the PEC diluted in 5ml CASYton (Roche Applied Science, Burgess Hill, UK) and were counted with help of a CasyCounter (CASY® 1, Roche Applied Science). The PEC were centrifuged and diluted in a final volume of RPMI (+HEPES) 1% P/S to get $4*10^6$ cells/ml or $5*10^6$ cells/ml.

2.5 Macrophage activation & treatment

To alternatively activate the MΦ *in vivo*, 100 µl IL-4complex (IL-4c) was injected twice per mouse at day 0 and day 2. The IL-4c contained 5 µl rIL-4 (1mg/ml; Peprotech EC, London, UK) and 2.8 µl anti-IL-4 (11B11, 9mg/ml; BioXCell, West Lebanon, NH) per mouse per injection incubated for 10min on ice prior to dilution with 92.2 µl D-PBS. The same solution was used for both injections and stored at 4°C between the injection days.

For stimulation with different activation agents, 100 µl of ~$5*10^6$ cells/ml PEC were cultured on 24-well cell culture plates (Corning, Amsterdam, NL) overnight at 37°C in 400 µl RPMI-1640 supplemented with HEPES, 1% P/S, 1% L-glutamin and 5% fetal calf serum (FCS; Life Technologies).

The next day the plates got washed three times with warm D-PBS to remove non-adherent cells. A total of 500 µl stimuli (treated) or supplemented media (untreated) were added to the cells for 24 hours at 37°C. LPS (*Escherichia coli* 0111:B4; Sigma-

Aldrich) and LPS/IFNγ were used for classical activation at a concentration of 100 ng/ml and 20 ng/ml respectively. IL-4 (20 ng/ml) and IL-13 (20 ng/ml) were used for alternative activation.

The supernatant was collected and frozen at -20ºC for further ELISA analysis. The cells were washed once with D-PBS and the cells were lysed with 500 µl/well Qiazol® lysis reagent (Qiagen, Crawley, UK) and stored at -70ºC.

2.6 Enzyme Linked Immuno Sorbent Assay

One milliliter from the first 3ml of lavage and the supernatant of the in vitro cultured cells were used for ELISA. All dilutions are described in table 1.

50 µl coating antibody per well on a 96 well Maxisorp™ plate (Nunc, Thermo Scientific, Roskilde, DK) was incubated over night at 4°C.

The coating antibodies included anti-Relm -α (αRELM-α; Peprotech, 500-P214), αYM-1 (DuoSet ELISA, DY2446, R&D Systems®, Abingdon, UK), αIL-12P40 (551219; BD Bioscience, Oxford, UK), αTNF-α (DuoSet ELISA Development kit, DY410, R&D Systems®), αIL-6 (BD Bioscience, 554400), and αIL-10 (BD, Bioscience 551215)(**Table 1**).

Afterwards the plate was washed 3 times with wash-solution (PBS-T; 0.05% Tween-20 (Sigma-Aldrich) in PBS) and 250 µl/well block buffer (10% newborn calf serum (NCS; Life Technologies) in PBS) added and incubated for 2 days at 4°C to prevent unspecific binding of the cytokines. Then the plate was washed 4 times with wash-solution and 50 µl of the samples added to separate wells. Two standard curves were made with a total volume of 50 µl/well in 16 wells and the corresponding diluted standard. The dilutions were 1:2 with standard:corresponding buffer, starting with 50 µl pure standard and ending with 1:16384.The plate was incubated over night at 4°C.

The standard included recombinant Relm -α (rRELM-α; Peprotech, 450026), YM-1 (Homemade, pleural lavage from *Litomosoides sigmodontis* infected mice), rIL-12p40 (Peprotech, 210-12), rTNF-α (DuoSet ELISA, DY410), rIL-6 (Peprotech, 216-16), and rIL-10 (Peprotech, 210-10)(**Table 1**).

Plate was washed 5 times with wash-solution and 50 µl/well of diluted detection antibody was added for 2 hours at room temperature.

The detection antibodies included biotinylated αRELM-α (aRELM- α biot; Peprotech, 500-P214Bt), αYM-1 biot (R&D SYSTEMS, BAF2446), αIL-12P40 biot (Biolegend,

San Diego, CA, 505302), αTNF-α biot (DuoSet ELISA, DY410), αIL-6 biot (BD Bioscience, 52201), and αIL-10 biot (Pharmingen, 554423)(**Table 1**).

Plate was washed 6 times with wash-solution and 50 µl streptavidin conjugated to horseradish-peroxidase (1:200 in 1% BSA in PBS; DuoSet, R&D Systems®) for YM-1 and TNFα and for IL-6, IL-10, IL-12P40 and Relm -α, 50 µl peroxidase-labeled streptavidin (0.5 mg/ml, 1:1000 in 10% NCS in PBS; KPL) per well was added and incubated for 1 hour at 37°C.

Plate was washed 6 times with wash-solution and 50 µl/well TMB substrate (KPL) was added, allowed to develop a yellow color (approx. 2-5 min) and 100 µl/well 0.18M H_2SO_4 added to stop this reaction. The absorbance was measured using a plate-reader (Molecular Devices, EMAX) at 450 nm and the background signal obtained at 595 nm subtracted.

Table 1. Antibody dilutions.

Cytokine	**Coat**	**Standard**	**Detect**
Relm **-α**	1 in 100 in 0.06M Carbonate buffer (NaH_2CO_3/Na_2CO_3, 2.5/1) pH9.6	1 in 250 in 1% BSA in PBS	1 in 200 in 10%NCS in PBS
YM-1	1 in 720 in PBS	1 in 250 in 1% BSA in PBS	1 in 500 in 1% BSA in PBS
IL-12P40	1 in 500 in 0.2M Na_2HPO_4 pH6.5	1 in 1000 in 10% NCS in PBS	1 in 1000 in 10% NCS in PBS
TNF-α	1 in 180 in PBS	1 in 100 in 1% BSA in PBS	1 in 180 in 1% BSA in PBS
IL-6	1 in 500 in 0.1M Na_2HPO_4 pH12	1 in 200 in 10% NCS in PBS	1 in 1000 in 10% NCS in PBS
IL-10	1 in 250 in 0.2M $NaHPO_4$ pH6.5	1 in 500 in 10% NCS in PBS	1 in 250 in 01% NCS in PBS

2.7 FACS

100 µl (~5*10^6 cells/ml) of PEC were used for the FACS staining and prepared in 96 well v-bottomed plates (Anicrin, Scorze, I). Isotype-control samples were made by pooling 20 µl per sample in every group and the single stain controls by taking the

remaining samples, pool them and take 100 µl/well. The cells were spun at 300g for 5min at 4°C (Sorvall Legend RT), the plate was inverted to get rid of the supernatant and vortexed to re-suspend the pellet (the same centrifuge method and settings were used throughout the FACS staining). The cells were washed twice with D-PBS and centrifuged. Cells were incubated at RT for 10 min in 10 µl LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (dilution 1:500 in D-PBS; Life Technologies) followed by incubation at 17-30 min at 4°C in 10 µl blocking buffer (10% mouse serum, 1% αCD16/32 in FACS buffer (D-PBS supplemented with 0.5% bovine serum albumin (BSA; Sigma-Aldrich), 2mM EDTA (Life Technologies)). 20 µl of the surface stain was added on top and incubated for 30 min on ice with the Abs of interest at the appropriate dilution. The cells were washed and centrifuged twice in FACS 150 µl buffer and incubated in 100 µl 1:3 Foxp3 staining buffer set (1:3 (fixation concentrate: dilutent); eBioscience) over night at 4°C.
Cells were centrifuged and then washed and centrifuged twice with 150 µl 1x permeabilisation buffer (eBioscience, 10x concentrate diluted 1:10 with dH_2O) and incubated at RT for ~30 min with 20 µl of the appropriate Abs for intracellular staining. The cells were washed and centrifuged once with 150 µl 1x permeabilisation buffer and incubated with 50 µl of the second intracellular staining for ~45 min on ice. Finally the cells were washed and centrifuged twice with 150 µl permeabilisation buffer and taken up in 100 µl FACS buffer. FACS analysis was performed with the FACSCanto II (BD Biosciences).
The data was analysed with FlowJo software (ver. 9.5.2; Tree Star, Inc. Ashland, OR).
All the incubation was performed in the dark.

The antibodies included for the surface stain were: αCD86 PE (1:60 or 1:120), αCD19 APC (1:120), αI-A/I-E AF780 (1:240), αI-A/I-E eFluor 780 (1:120), αCD19 AF700 (1:120), αCD19 AF780 (1:240 or 1:120), αI-A/I-E AF700 (1:120), αF4/80 PE/Cy7 (1:120) all purchased from eBioscience. αSiglecF PE (1:120) purchased from BD Pharm. αI-A/I-E PerCP (1:240), αCD11c APC/Cy7 (1:120), αLy6G AF647 (1:120), αI-A/I-E Pacific Blue (1:240), αCD19 APC (1:120) purchased from BioLegend. αLy6G APC (1:120) purchased from AbD Serotech. αCD40 FITC (1:60) purchased from BD Bioscience

The corresponding isotypes included: ratIgG2a FITC (1:60; Biolegend), ratIgG2a PE (1:60 or 1:120; eBioscience).

The first intracellular stain included: purified αRELM-α (1:100, Peprotech), αYM-1 biotinilated (1:40; RnD Labs), αiNOS FITC (1:100; BD Pharm).
The isotypes were: rabbit IgG (1:100), goat IgG biotinilated (1:40), mouseIgG FITC (1:100; BD Pharm).
Detection was carried out by staining with αrabbit IgG PacificBlue® (1:300; Zenon® Pacific Blue™ Rabbit IgG Labelling Kit, Life Technologies) and streptavidin PerCP (1:300; Biolegend)

2.8 RNA extraction

500 µl/well of Qiazol® lysis reagent (Qiagen) was added to the samples and incubated for 10min at RT. Samples were stored at -70°C in nuclease free tubes (Molecular Bioproducts) until further use. 100 µl chloroform (Sigma-Aldrich) was added and the tubes shaken vigorously by hand for ~15 sec and incubated at RT for 2min. The RNA was separated by spinning down cell debris and DNA at 12000 g for 20 min at 4°C (Heraeus Fresco17). The aqueous phase (~250 ul per sample) was collected into new nuclease free tubes, which already contained ~2 µl glycoblue (Ambion). 250 µl isopropanol (Acros Organics) was added to the tubes and inverted 13 times and incubated at RT for 2 min. Centrifuged at 12000 g for 10 min at 4°C to separate the RNA from the isopropanol. The isopropanol was poured off and the tubes tapped dry. The samples were then washed three times with 0.5 ml 70% molecular grade ethanol (Sigma-Aldrich), centrifuged at 7800g for 5 min at 4°C. After each wash, the ethanol was poured off and tapped dry. After the last wash and drying, the samples were centrifuged up to 9600 g and the remaining ethanol was removed with a 200 µl pipette. The tubes were dried up side down for about 30 min until all the ethanol had vaporized. The RNA was diluted in 50 µl nuclease free water (Ambion). Samples were stored at -70°C until further use.
The concentration of RNA in the samples was measured by using a spectrophotometer (NanoDrop2000; Thermo Scientific, Wilmington, DE).

2.9 Quantitative Reverse Transcription PCR

The concentration of the RNA was adjusted to ~150 ng in 12.25 µl nuclease free water (Ambion) in PCR tubes. 1 µl of oligodT (0.5 µg/µl; dT_{15}; Roche Applied Science) was added and this was incubated for 5 min at 70°C and then transferred on ice and incubated for at least another 5 min. To turn the RNA into cDNA, a reverse transcription-mix was added to the samples (Table 2) and incubated at 37°C for 1 hour. To stop the reaction the samples were incubated at 70°C for 10 min and 80 µl nuclease free water was added. Thereafter the samples were frozen at -20°C.

Table 2 RT-mix

	Per sample
5*RT-buffer (Bioline)	4 µl
dNTP's (10x mix, 10mM each; Promega)	2 µl
RNase inhibitor (Rnasin, Promega)	0.5 µl
MMLV-RT enzyme (Bioscript; Bioine)	0.25 µl

On the day of the PCR, the samples were thawed and a standard-solution was made by pooling 5 µl from every sample. Semiquantitative comparisons of amplified products were performed based on the crossing points obtained for each sample related to this serially diluted cDNA-pool run in parallel. Final arbitrary units of the standard curve were undiluted, 1:5, 1:25, 1:125 and nuclease free water. The mastermix was made up out of 490 µl premastermix (LightCycler® 480 SYBR Green I master, Roche), 29.4 µl forward primer, 29.4 µl reverse primer and 240 µl H_2O (LightCycler®, Roche). 8 µl of the mastermix and 2 µl of each sample were added to wells of a 96 well PCR plate (Light Cycler®, Roche). After denaturation of the cDNA at 95°C for 5 min, 50 cycles of 10 sec at 95°C, 10 sec at 60°C and 10 sec at 72°C. PCR quality control was performed by melt curve analysis. The PCR-reaction was done on the LightCycler® 480II (Roche Applied Science).
The relative expression of the genes was than calculated compared to GAPDH, our housekeeping gene, by this equation:

$$Relative\ expression = \frac{conc.target\ gene}{conc.GAPDH}$$

Table 3. Primer sequences and product size

	Forward sequence	**Reverse sequence**	**Tm (for/rev)**	**Amplicon length**
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG	55/55	
TNF-α	GGAAATAGCTCCCAGAAAAGCAAG	TAGCAAATCGGCTGACGGTGTG	59.6/ 62.9	583
IL-10	ACTGCACCCACTTCCCAGT	TTGTCCAGCTGGTCCTTTGT	60/60	103
Arginase 1	GTCGTGGGGAAAGCCAAT	GCTTCCAACTGCCAGACTGT	59/60	109
Relm -α	TATGAACAGATGGGCCTCCT	GGCAGTTGCAAGTATCTCCAC	59/59	107
YM-1	ACCTGCCTCGTTCAGTGCCAT	CCTTGGAATGTCTTTCTCCACAG		

2.10 Statistics

Standard statistical tests were used to analyze the data with Prism 5 for mac (version 5.0d; GraphPad Software) (Table 4).

Table 4. Statistical tests for the respective experiments

		Analysis	**Post-test**	**NB**
Exp 1	ELISA	One-way ANOVA (Kruskal-Wallis test)	Dunn's Multiple Comparison Test	
	FACS	One-way ANOVA	Dunn's Multiple	

		(Kruskal-Wallis test)	Comparison Test	
Exp 2	ELISA	Two-way ANOVA (Bonferroni multiple comparisons)	Bonferroni multiple comparisons	TNFa & IL-6 missed values, no tests performed
	FACS	T-test	Mann Whitney test	
	RNA	Two-way ANOVA (Bonferroni multiple comparisons)	Bonferroni multiple comparisons	Excluded PEC
Exp 3	FACS	One-way ANOVA (Kruskal-Wallis test)	Dunn's Multiple Comparison Test	
Exp4	FACS	One-way ANOVA (Kruskal-Wallis test)	Dunn's Multiple Comparison Test	
	FACS DsRed	T-test	Mann Whitney test	

3 Results

3.1 Characterization of classical and alternative activation markers in *S. typhimurium* and *H. polygyrus* infected mice.

To identify suitable markers of MΦ activation for FACS-analysis and test their behavior in our infection-models C57BL/6 mice were either infected with *H. polygyrus or S. typhimurium.* 7 days after the infection the PEC of all mice, were harvested and analyzed by FACS. MΦ were defined as F4/80 Hi, CD19-, single live cells. I naïve mice approximately 5-10% of MΦ express Relm - α. Infection with *H. polygyrus* did not increase this proportion of MΦ expressing Relm -α, but *S. typhimurium* infection did suppress its expression compared to naïve MΦ (fig. 2). In contrast significantly more Nematode elicited macrophages (NeMΦ; ~10%) expressed YM-1 compared to the naïve MΦ (1-2%), but not compared to the Salmonella elicited macrophages (SalMΦ; ~3%; fig. 2).
iNOS is constitutively expressed by almost all MΦ in naïve and *S. typhimurium* infected mice (~98-100%; fig. 2) but the mean fluorescence intensity (MFI) shows us that the population of MΦ, present during infection with *S. typhimurium* expressed more iNOS per cell (fig. 3,4). In contrast infection with *H. polygyrus* marginally, albeit significantly, reduced the proportion of MΦ expressing iNOS (~93%), but did not alter the average expression level of iNOS per MΦ (fig. 2 & 3).
Similarly the majority of MΦ in all the three groups expressed CD86 (fig. 2), but *H. polygyrus* infection suppressed the average amount of CD86 expressed on the individual MΦ as assessed by MFI (fig. 3, 4). CD40 was expressed equally between the two infections and thereby not a good marker for further experiments (fig. 2).

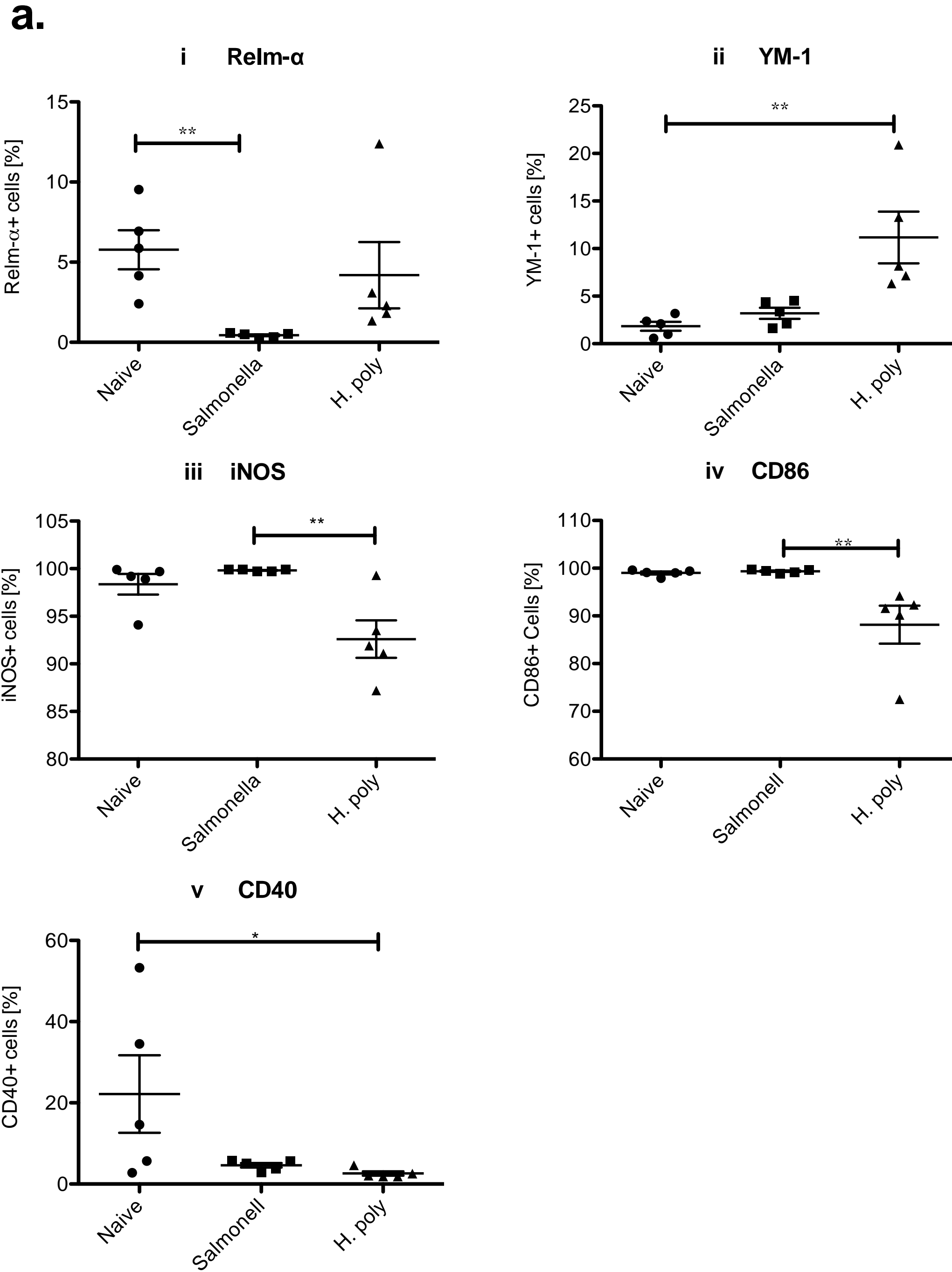

Figure 2. FACS analysis of peritoneal MΦ isolated from naïve mice (circles) or 7 days after *S. typhimurium* (squares) or *H. polygyrus* (triangles) infection. Data are presented as percent positive of F4/80Hi, CD19-, single live cells. **: $p< 0.01$, *: $p<0.05$

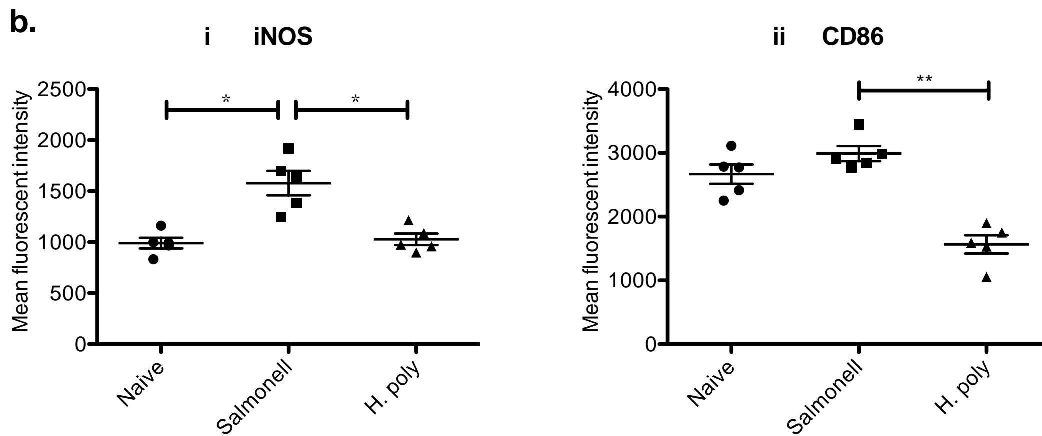

Figure 3. FACS analysis of peritoneal MΦ isolated from naïve mice (circles) or 7 days after *S. typhimurium* (squares) or *H. polygyrus* (triangles) infection. Data are presented as mean intensity of F4/80Hi, CD19-, single live cells. **: p< 0.01, *: p<0.05

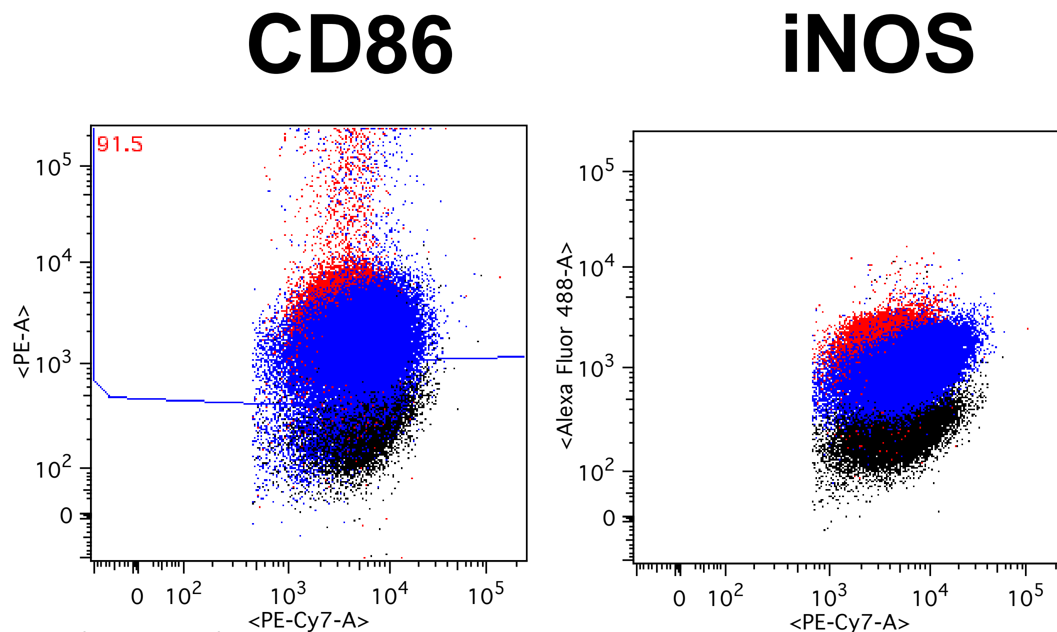

Figure 4. FACS analysis of peritoneal MΦ isolated from 7 days after *S. typhimurium* (blue) or *H. polygyrus* (red) infection compared to isotype-control stained cells (black). Data are presented CD86 and iNOS mean fluorescence intensity of F4/80Hi, CD19-, single live cells.

Another way of getting to know the characteristics of the MΦ is by looking at the cytokines they secrete. Doing ELISA on the lavage fluid we collected from the peritoneal cavity therefore gives another way to determine the activation phenotype of the MΦ.

ELISA analysis for Relm -α, YM-1 showed that *H. polygyrus* infection induces a Th2 response. In contrast to the previous data obtained by FACS *H. polygyrus* infection had significantly increased the production of Relm -α (10-15ng/ml) compared to the

naïve and *S. typhimurium* infected samples (0-5ng/ml). YM-1 showed the same as in the flow cytometry results, it has increased expression after *H. polygyrus* infection. The ELISA for TNF-α and IL-12p40 did not show any differences between the two infections. The values for TNF-α were all below the detection limit. IL-12p40 had a slightly increased expression in both infections, so no distinction between the two was possible (fig. 5).

Taken together the MΦ isolated from *S. typhimurium* or *H. polygyrus* infected mice showed enhanced expression of classical or alternative activation markers. However, while expression of Relm - α and YM-1 allows a clear distinction of positive versus negative cells, only a small proportion of MΦ isolated from *H. polygyrus* infected mice could be clearly identified as alternatively activated. Expression of iNOS and CD86 on the other hand was clearly enhanced by infection with *S. typhimurium*, but the FACS-staining did not allow to clearly distinguish separate populations.

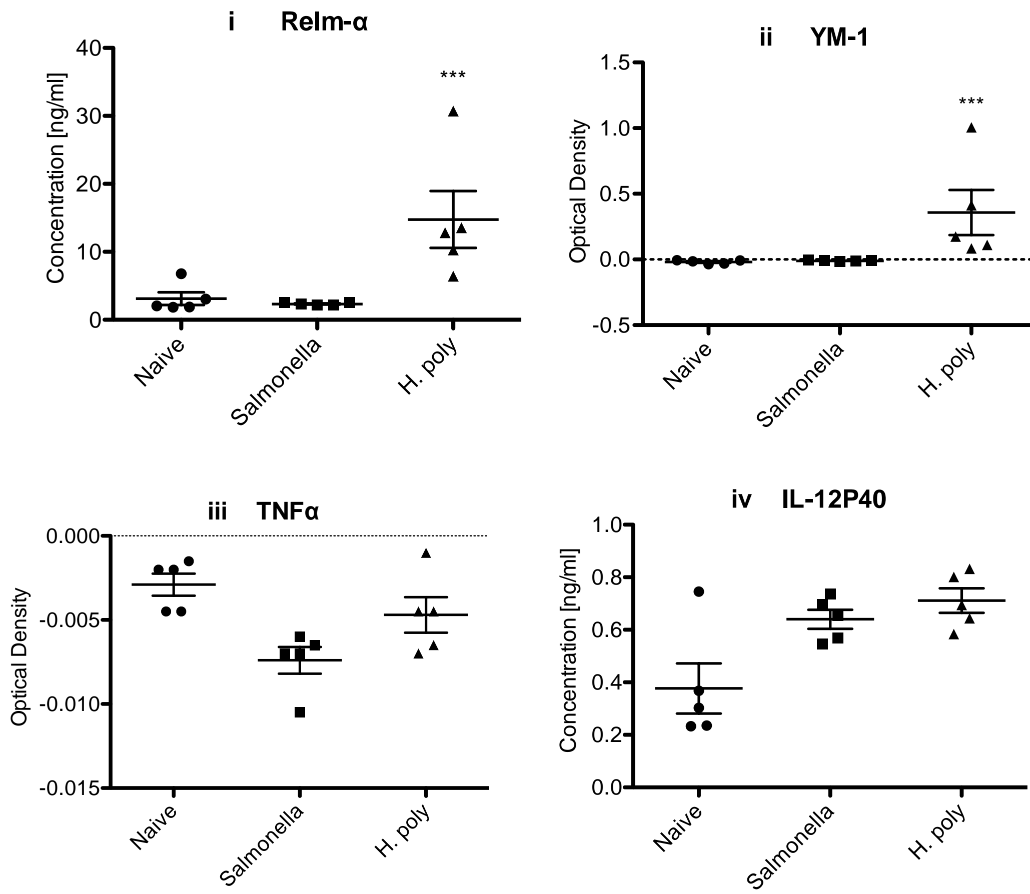

Figure 5. ELISA analysis of the lavage fluid of naïve mice (cirlces) or 7 days after *S. typhimurium* (squares) and *H. polygyrus* (triangles) infection for Relm -a (i), YM-1 (ii), TNF-a (iii) or IL-12p40 (iv). Data is presented as concentration or optical density (minus the background) and each data point represent the results from individual animals. ***: $p<0.001$

3.2 Characterization of classical and alternative activation markers in IL-4c injected mice

In the previous section we could demonstrate that the percentage of alternatively activated MΦ found after *H. polygyrus* infections was relatively low (~10%). To be able to reliable track changes in activation phenotype *in vivo* a better model of *in vivo* alternative activation was needed.

Siam Gordon has shown that IL-4 induces alternative activation in MΦ (Gordon 2003). We used IL-4 complexed to an anti-IL-4 Ab to allow slow release of active IL-4 *in vivo* and get fully alternatively activated MΦ in C57BL/6 mice. As control we injected PBS under the same conditions.

FACS was done on the PEC cells to see their status of activation.

FACS data of F4/80 Hi, CD19-, single live cells, and Relm -α, YM-1 , iNOS and CD86, showed that there was a certain degree of alternative activation, but not as much as hoped for. Relm -α and YM-1 both were expressed more by the IL-4c stimulated macrophages (4cMΦ) than by the PBS stimulated macrophages (~5%; PBS-MΦ) with ~50% of 4cMΦ expressing Relm -α and 40% expressing YM-1 . Similar to the previous infection experiment CD86 was suppressed. In this case even iNOS was slightly suppressed in 4cMΦ (fig. 6). Thus, using IL-4c results in stronger alternative activation than infection with *H. polygyrus* and is therefore preferable for use in transfer experiments.

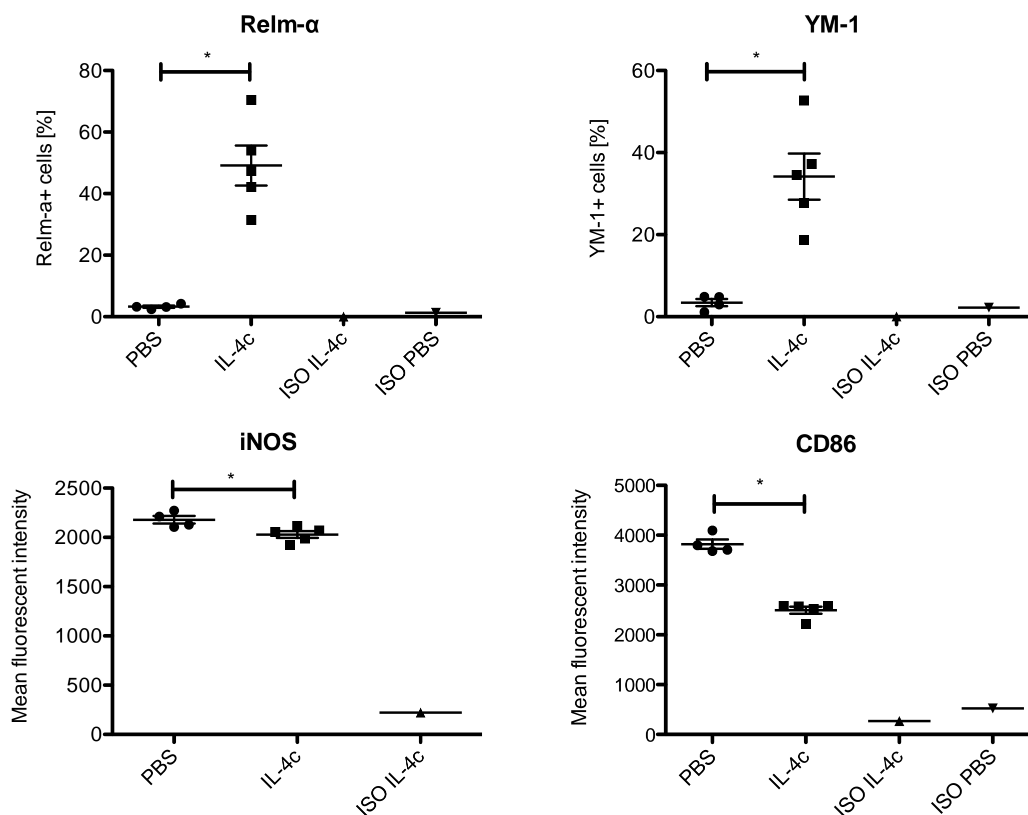

Figure 6. FACS analysis of peritoneal MΦ isolated from PBS (circles) or IL-4complex (squares) injected mice. Data are presented as percent positive or mean fluorescence intensity of F4/80Hi, CD19-, single live cells. *: $p<0.05$

3.3 Capability of expressing classical activation markers after stimulation with LPS and/or IFN-γ *in vitro*

To address whether 4cMΦ exhibited a similar plastic phenotype as previously observed for NeMΦ (Mylonas & Nair 2009), these 4cMΦ and PBS-MΦ were then cultured with classical activation agents (LPS and/or IFN-γ) to determine their ability to adjust their phenotype *in vitro*.

The PEC were purified by adherence to cell-culture plastic for 24 hours and then cultured for 24 hours with classical and alternative activation agents. The released cytokines in the supernatant of the cultured cells were measured with ELISA. Many of the values were out of linear range, so no statistical tests could be done between the values that were out of range. This is not a problem, because we mainly wanted to see if the cytokines were produced at all and not the quantity. The PBS-MΦ produced a lot less cytokines than the 4cMΦ, because the 4cMΦ were represented at a higher ratio in the PEC and adhered better to the tissue culture plastic than the PBS-MΦ. The alternative activation cytokines, Relm -α and YM-1 were highly expressed in all the 4cMΦ, showing that they were still alternatively activated. The analysis was performed 48h after isolation of the cells so the cells could have secreted cytokines that were made before the stimulation and stored inside the cell, rather than still actively producing these cytokines. The values were mostly out of range, so any differences between the different stimuli are not measurable. More interesting were the expressions of the classical activation cytokines. After stimulation with LPS or LPS/IFN-γ, the 4cMΦ expressed cytokines characteristic for classical activation, in this case we measured IL-10, IL-12p40, IL-6 and TNF-α. Also here, we have the problem of the values falling outside the linear range, so any differences in expression between LPS or LPS/IFN-γ stimulated MΦ are not accurate (fig. 7).
Thus, similar to the data described for NeMΦ (Mylonas & Nair 2009) 4cMΦ were able to respond to classically activating stimuli and produced pro-inflammatory cytokines. Due to the limitations of the ELISA-assays and to be better able to compare 4cMΦ with PBS-MΦ, we looked at the mRNA-expression of Relm -α, YM-1 , IL-10, TNFα and Arignase in these cells.

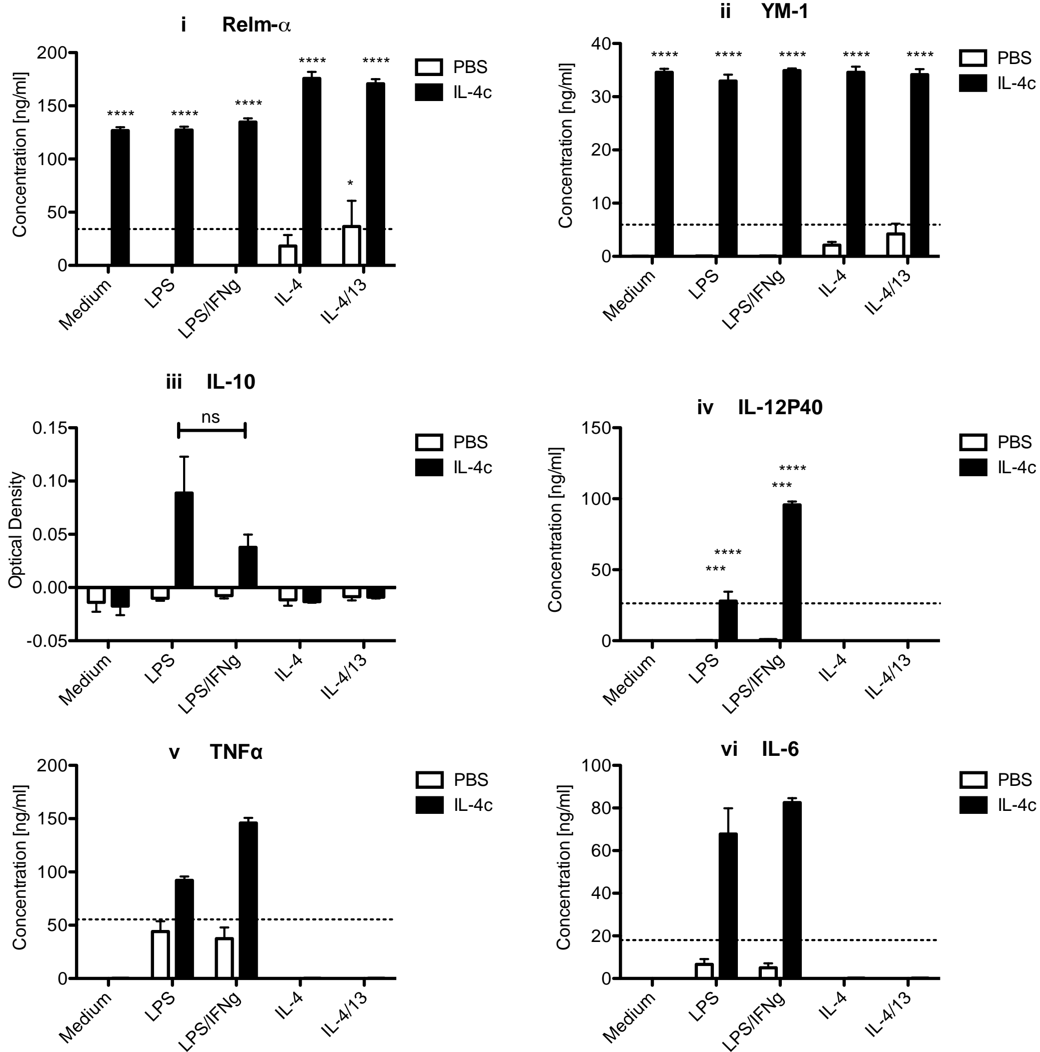

Figure 7. ELISA analysis on the supernatant of the cultured cells. Cells were cultured with LPS, LPS/IFNγ, IL-4 or IL-4/13. ****: $p<0.0001$, ***: $p<0.001$, ns: p< non significant.

We looked at the ability of the 4cMΦ to express certain cytokines and enzymes compared to PBS-MΦ. In the PEC groups we have a mixture of all the cells from the peritoneal cavity, while in the stimulated groups we have washed away the majority of non-macrophages. Differences in quantity of the cells/MΦ between treatment groups do not matter here, because we analyzed at the relative expression compared to GAPDH, our household gene, which should be equally expressed in all cells. The expression of the alternative activation cytokines, YM-1 and Relm -α, were increased when stimulated with IL-4 or IL-4/13 in both 4cMΦ and PBS-MΦ. The 4cMΦ expressed even more YM-1 and Relm -α then any other group. Due to the log-

scale it looks like LPS and LPS/IFNγ suppresses the expression of Relm -α, but this is not significant (fig. 8). Treatment with IL-4 alone or in combination with IL-13 led to higher expression of these markers than in cell analyzed straight *ex vivo* (PEC).
When stimulated with LPS and IFNγ, 4cMΦ produced more IL-10 than the PBS-MΦ. Even with just LPS, the levels were higher, but not statistically significant (fig. 8).
Expression of TNF-α is increased equally in both groups when stimulated with classical activation agents, especially LPS/IFNγ. IL-4 and IL-4/13 have no effect on the expression of TNFα (fig. 8).
Arginase is expressed in Th2 response, and this was seen here as well. The alternative activation agents both increased the expression of arginase in both groups, but more in the 4cMΦ. LPS alone did not suppress the expression of arginase by 4cMΦ, but LPS/IFNγ suppressed the expression down to a similar level as the PBS-MΦ (fig. 8).
Overall these data confirm our previous findings using ELISA. 4cMΦ are equally capable to respond to classically activating stimuli than PBS-MΦ while retaining expression of alternative activation markers.

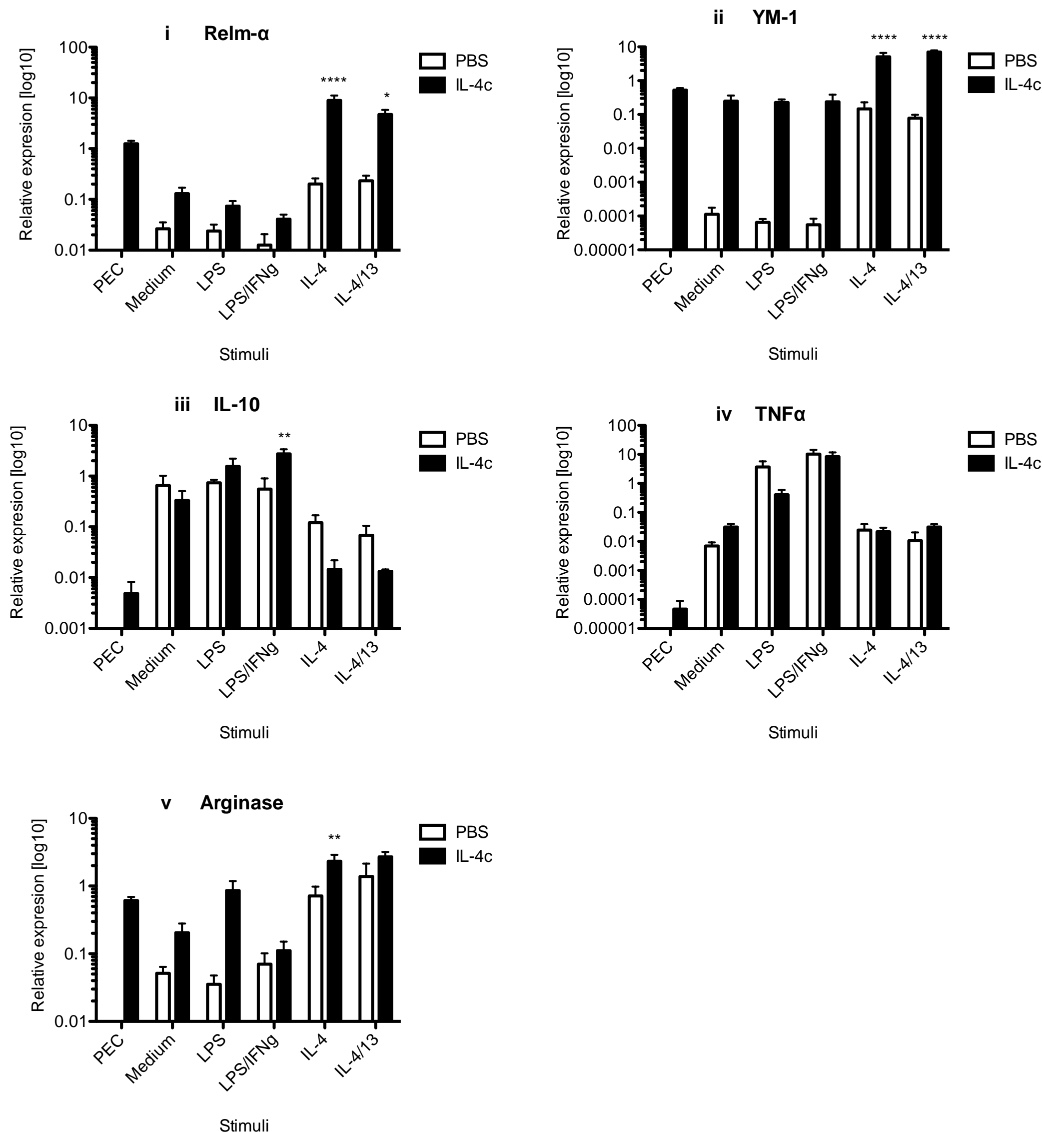

Figure 8. Analysis of mRNA in the cells before and after 24 hour stimulation with LPS, LPS/IFNγ, IL-4 or IL-4/13. Data presented as the relative expression to GAPDH.

3.4 Macrophages tend to adopt an alternatively activated phenotype in a mixed T_H1/T_H2 environment

We have shown now that the MΦ are capable of altering their phenotype from alternative to classical activated *in vitro*. The question now was, when they are in a co-infection and get stimulated with both Th1 and Th2 cytokines, what phenotype they take on. Even though the single infections didn't give us a very distinctive difference for the classical activation markers, we used *H. polygyrus* and *S. typhimurium* for our co-infection.

The infection load of *S. typhimurium* did not differ between the single and co-infected mice (fig. 9). The weight of the mice shows us how the impact of the infection on the mice. Not statistically shown, but the *H. polygyrus* in the co-infection seems to protect the mice from weight loss compared to the single infection with *S. typhimurium* (fig. 9).

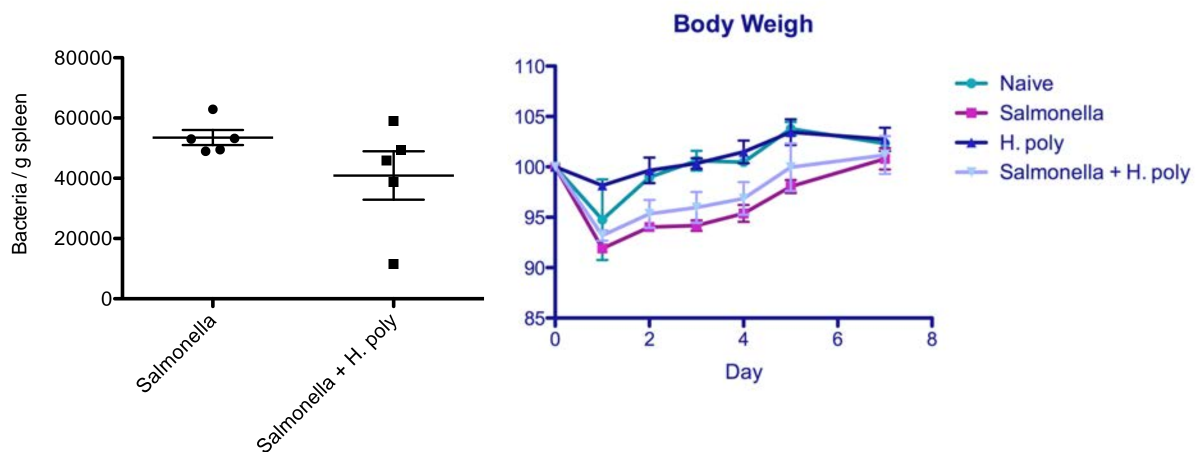

Figure 9. On the left: Bacterial count per gram spleen in mice that have been 7 days infected with *S. typhimurium* or *S.* typhimurium and *H.*poly. On the right: Body weight over 7 days from the naïve (circle) or infected with *S. typhimurium* (square), *H. polygyrus* (triangles) or *S. typhimurium* and *H. polygyrus* (inverted triangle). Data presented as percentage of day one.

To identify MΦ we gated on dendritic cells-, lymphocyte- & eosinophil-, CD19-, single live cells. With this gating we measured CD86 and YM-1 expression.
For the analysis of iNOS & Relm - α we gated on lymphocyte- & eosinophil-, CD19-, neutrophils-, single live cells. Thus, dendritic cells are potentially included in the remaining population for these markers.
The response to *S. typhimurium* suppressed the expression of Relm -α, while the NeMΦ showed an increase in Relm -α. MΦ isolated from co-infected mice (coMΦ) showed an intermediate response, but alternative activation was dominant. The suppressive effect of *S. typhimurium* was overridden by the enhancing effect of *H. polygyrus* (fig. 10).
Infection with *H. polygyrus* induced increased expression of YM-1; this was not altered by the co-infection. Single infection with *S. typhimurium* had no effect on YM-1 (fig. 10).

iNOS expression per cell, as assessed by MFI, was increased in the *Sal*MΦ, as in the first experiment. The coMΦ in contrast showed about the same MFI as the NeMΦ. *H. polygyrus* single infection and the co-infection both showed a decrease of CD86 expression compared to the *Sal*MΦ. Thus, similar to the results of the alternative activation markers, where the Th2 response seemed dominant (fig. 10).
We compared YM-1 + MΦ and YM-1 - MΦ for CD86 expression in NeMO and coMO, to see if the decrease in expression was due to alternative activation. There is no difference between the YM-1 + and YM-1 - MΦ in *H. polygyrus* and the co-infection, so the alternatively activated cells did not express CD86 different from the non-alternatively activated cells in the same mice (fig. 10).

Taken together the MΦ isolated from *S. typhimurium* and/or *H. polygyrus* infected mice showed enhanced expression of classical or alternative activation markers as in the previous experiment for the single infections. However, the co-infections showed a dominating T_H2 response for the markers.

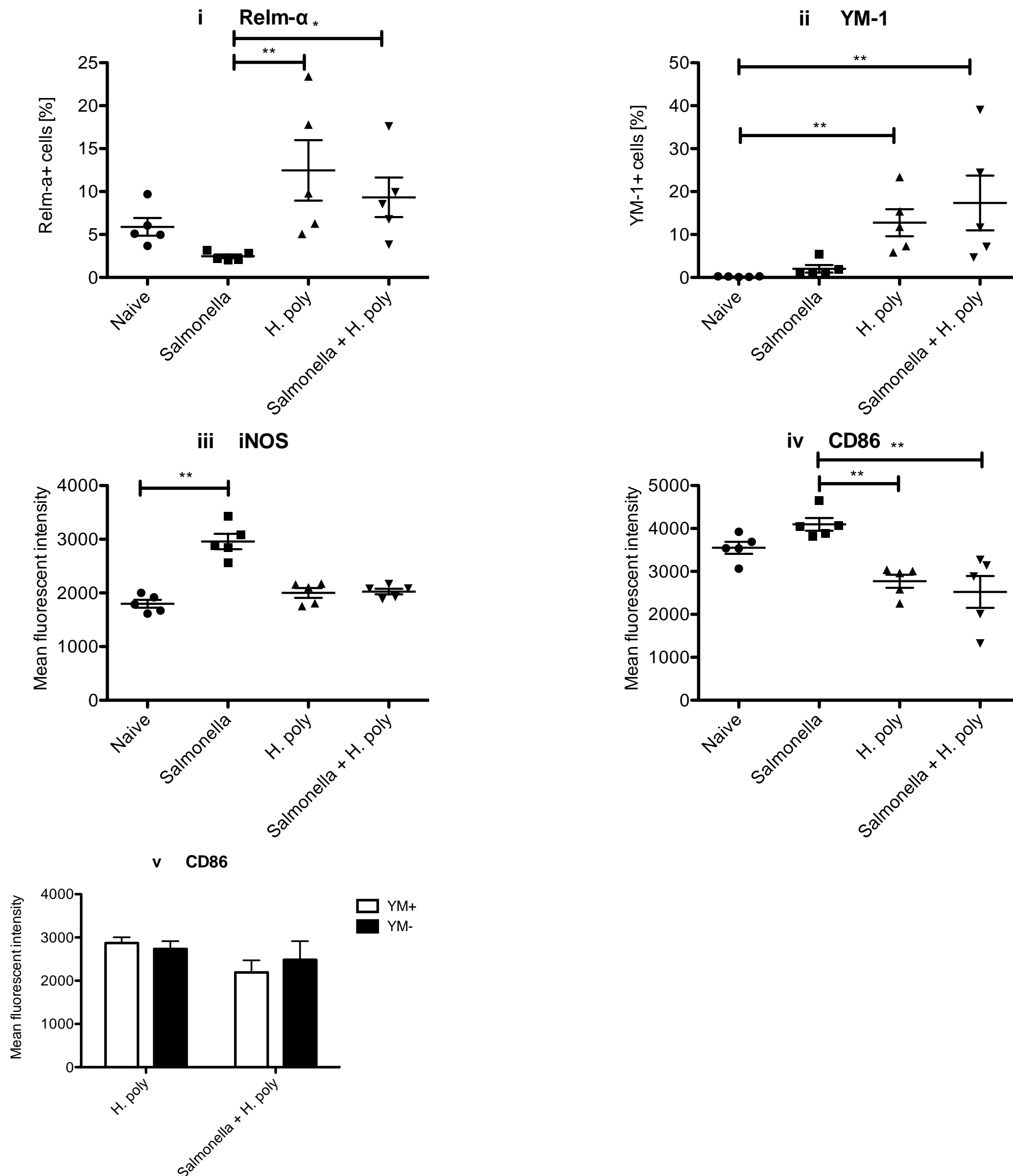

Figure 10. FACS analysis of peritoneal MΦ isolated from naïve mice (circles) or 7 days after *S. typhimurium* (squares), *H. polygyrus* (triangles) or *S. typhimurium* and *H. polygyrus* (inverted triangles) infection of the co-infected mice, gated on dendritic cells-, Lymphocyte- & Eosinophil-, CD19-, single live cells. Data presented as percentage positive cells and mean fluorescent intensity. **: p< 0.01, *: p<0.05

3.5 IL-4c injected DsRed-cells change phenotype when transferred into *S. typhimurium* infected mice

As described above MΦ have the ability to change their phenotype from alternative to classical activation *in vitro*. More interesting is if they behave similar *in vivo*, when there are many other factors involved and the cytokine environment is much more

complex. Therefor we injected B6 DsRed mice with IL-4c as described in materials and methods and transferred the PEC cells to naïve and *S. typhimurium* infected C57BL/6 mice. DsRed mice are genetically engineered mice expressing a red fluorescent protein (DsRed) in every cell. Thus the transferred cells can be easily identified in FACS due to their red fluorescence.

To verify the alternative activation status of the DsRed cells prior to transfer, a proportion of these cells was analyzed by FACS. Gating on MHC+F4/80+, CD19-, single live cells showed us how much the MΦ were alternatively activated before they were injected into the C57BL/6 mice. Similar to the second experiment, ~50% of the 4cMΦ expressed more Relm -α and ~40% expressed YM-1. iNOS was suppressed as before. The differences between the PBS-MΦ and 4cMΦ were clear enough to see a possible difference after the transfer (fig. 11).

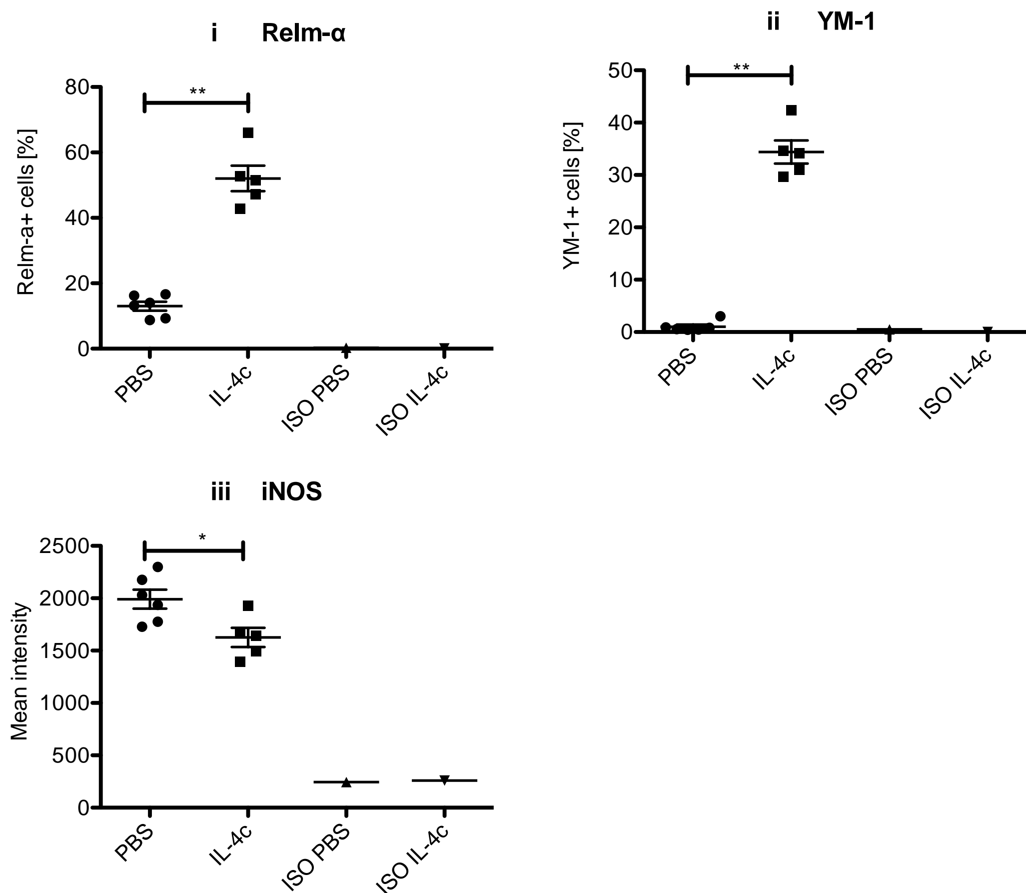

Figure 11. FACS analysis of peritoneal MΦ isolated from DS-red mice injected 24 hours with PBS (circle) or IL-4c (squares) prior to transfer. Data presented as percent positive and mean intensity of MHC+F4/80+, CD19-, single live cells. **: p< 0.01, *: p<0.05

The PEC isolated 24 hours after transfer from the C57BL/6 mice were gated the same as described above including a gating for the DsRed (transferred) cells (PE-A+). The gating on the transferred cells was difficult, because the fluorescence wasn't as strong as expected. In figure 12 you can clearly see the transferred cells, but they have lost their fluorescence and therefore they are really hard to gate on. The gating used includes only a very small proportion of the transferred cells, so we don't included the non-transferred cells.

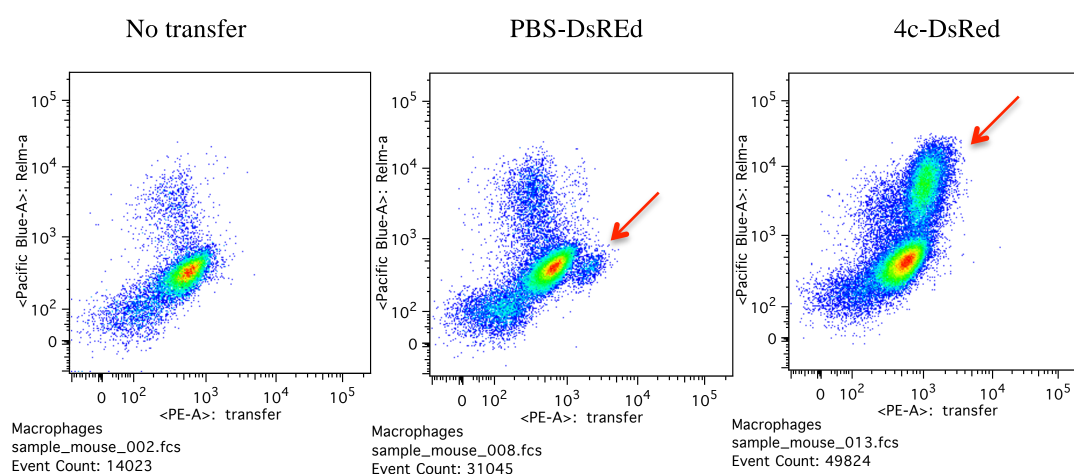

Figure 12. FACS staining of peritoneal MΦ isolated from naïve mice (mouse_002), naïve mice injected with PBS induced DsRed Cells (mouse_008) and naïve mice injected with IL-4c induced DsRed Cells (mouse_13). Data presented as Relm -α+, PE-A+ (Ds-red cells), MHC+F4/80+, CD19-, single, live cells. Gating is not shown, but the transferred cells are clearly seen in the second and third graph (red arrow). The host cells do not express Relm -α in such a distinction as seen in the first graph. Gating is not possible as the cells are barely traceable in the second graph. The staining for YM-1 gave similar graphs (not shown).

The expression of Relm -α was still maintained in 4c-DsRed MΦ following transfer into naïve mice (fig. 13). In the *S. typhimurium* infected mice the expression had decreased, but was not totally gone. As expected there was no enhanced expression detectable in the transferred cells from PBS injected mice (fig. 13).
YM-1 expression by 4cMΦ was similarly down regulated when injected into *S. typhimurium* infected mice. The level of YM-1 in both *S. typhimurium* infected groups is about the same (fig. 13).
iNOS was expressed most in the transferred PBS-MΦ injected into *S. typhimurium* infected mice, as expected. Even the 4cMΦ expressed an increased amount of iNOS,

not as high as the PBS injected mice, but high enough to be able to say the express a classical phenotype (fig. 13).

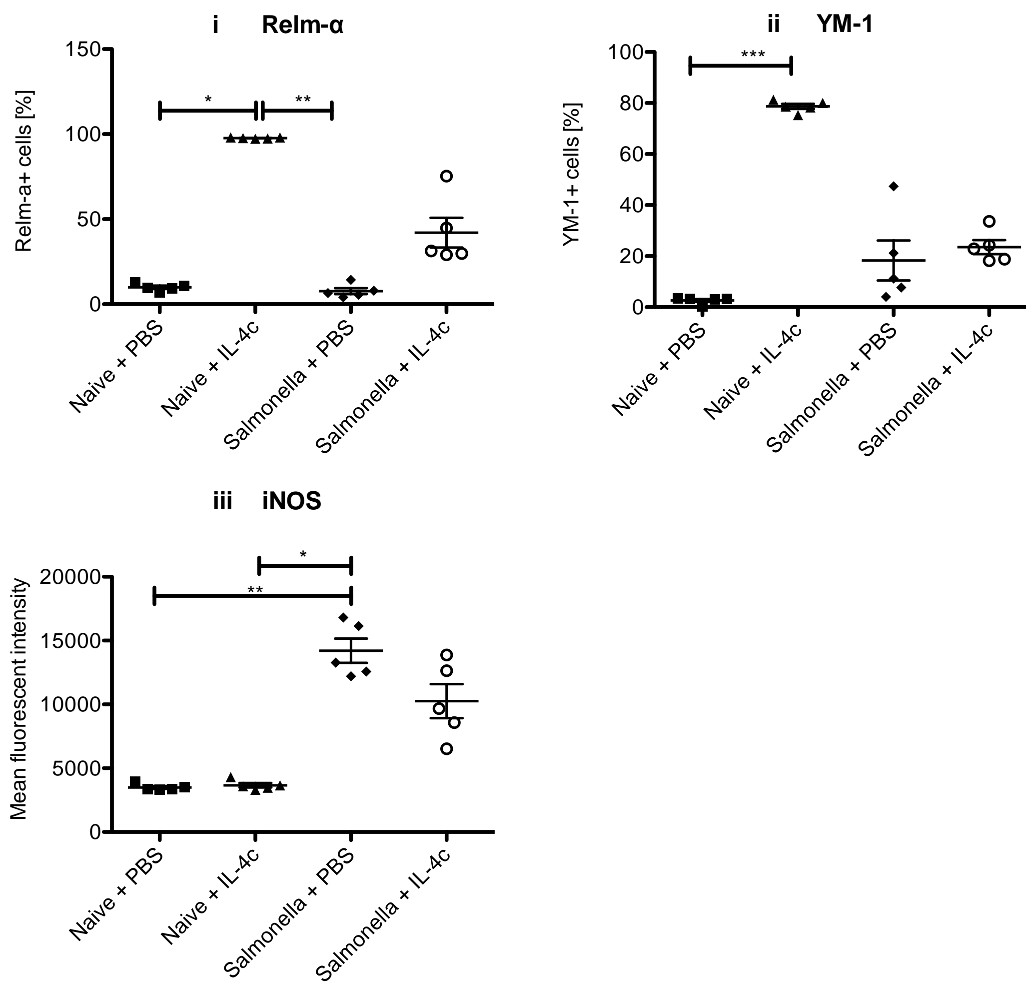

Figure 13. FACS analysis of peritoneal MΦ isolated from 7 days infected with *S. typhimurium* or naive C57BL/6 mice, 24 after injection with DsRed cells. Naïve mice got injected with PBS induced DsRed Cells (squares), naïve mice injected with IL-4c induced DsRed Cells (triangles), *S. typhimurium* infected mice with PBS induced DsRed Cells (diamonds) and *S. typhimurium* infected mice with IL-4c induced DsRed Cells (open cirlces). Data presented as percentage positive and mean fluorescent intensity for PE-A+ (Ds-red cells), MHC+F4/80+, CD19-, single, live cells. ***: $p<0.001$, **: $p< 0.01$, *: $p<0.05$.

4 Discussion

In this study we looked at the ability of MΦ to change activation phenotype in vivo and what activation phenotype they express when they are in a co-infection. Through all the experiments we observed that MΦ are able to change their phenotype. In this study we mostly looked at the ability to change from alternatively activated to classically activated, but also how MΦ act when they are in an environment with both activation agents.

We tested the MΦ first for activation markers after infection with *H. polygyrus* and *S. typhimurium*, to be sure that the infection model we wanted to use worked. *H. polygyrus* is a mouse parasite and should induce a strong T_H2 response (Maizels *et al*. 2011). This means that the MΦ should be alternatively activated, but in our study this isn't the case. Previous experiments have shown that YM-1 and Relm -α are distinct markers of alternative activation (Raes *et al*. 2002, Gordon 2003). Both *Trypanosoma congolense* and *Trypanosoma brucei brucei* infection showed increased levels of YM-1 and Relm -α in infected animals compared to none infected (Raes *et al*. 2003, 2002). *H. polygyrus* induces the expression of Relm -α and YM-1 in peritoneal MΦ, but not as much as in other experiments (Raes *et al*. 2003, 2002). *S. typhimurium* on the other hand suppressed Relm - α, and did not change the expression of YM-1. So there is a distinction possible between MΦ isolated from the two infection groups based on the alternative activation markers, but we wanted to see if the MΦ can change there phenotype, therefore we needed to be able to see a difference in classical activation markers as well. iNOS, CD86 and CD40 are all markers that should be up regulated in a Th1 environment (Gordon 2003, Gordon & Martinez 2010, Rosenberger & Scott 2000). Virtually all MΦ expressed CD86 and iNOS even in naïve mice. Consequently infection with *S. typhimurium* could not enhance the proportion of MΦ expressing either of these markers. However, when the level of expression of these markers within individual MΦ was analyzed (using MFI), salMΦ showed significantly enhanced expression of both markers. *H. polygyrus* on the other hand suppressed the expression of iNOS and CD86 on the percentage of MΦ, so a distinction between CAMΦ and AAMΦ using FACS-analysis can be made. This combination makes the model not optimal, but still usable for the co-infection.

Weng *et al* . showed that when mice get infected with *Citrobacter rodentium* and 7 days later they also get infected with *H. polygyrus* , they will show an intermediate phenotype and express both AAMΦ and CAMΦ (Weng *et al*. 2007). In our experiment when the mice got infected with both *H. polygyrus* and *S. typhimurium* the MΦ expressed a stronger alternative than a classical activated phenotype. The difference is that in our study we infected the mice at the same time with both pathogens. So depending on when the host gets infected, the immune response reacts different. We show that when the host gets simultaneously infected with two opposite immune response inducing pathogens, the MΦ expressed an intermediate, but alternative dominant phenotype in our model. Some of the coMΦ got alternatively activated, but they expressed Relm -α and YM-1 in a lesser degree then the NeMΦ. There is no evidence of classical activated MΦ if we look at iNOS and CD86.

To get more distinctive alternative activated MΦ we injected mice with IL-4c. This is supposed to give a strong expression of Relm -a and YM-1 (Jenkins *et al*. 2011). CD86 was suppressed, similar to the *H. polygyrus* infection, iNOS also was a little bit more suppressed, and so the distinction between PBS-MΦ and 4cMΦ was visible. Even here the percentage of MΦ expressing Relm -a and YM-1 was not as high as we expected, but distinct enough to use in the transfer experiment and *ex vivo* stimulation with different activation agents.

The 4cMΦ were cultured in different stimuli and we looked at the cytokines released by them after 24 hours in the stimuli. Due to the concentrations we used at the ELISA many of the values are out of linear range, so the results show us where we got secretion of certain cytokines, but not how much more in relation to the different treatment groups.

IL-4 and the combination of IL-4 and IL-13 give us an increased expression of Relm -α and YM-1 both in the supernatant and as mRNA. This is consistent with previous studies (Mosser 2003, Loke *et al*. 2002, Gordon 2003). These T_H2-type cytokines induce alternative activation of the MΦ.

Mylonas *et al.* showed that the iNOS expression was induced when NeMΦ were stimulated with LPS and/or IFNγ, we didn’t look at iNOS, but our T_H1-cytokines show the same pattern (Stout *et al*. 2005). When stimulated with classical activation agents, we get a higher expression of the classical cytokines by the 4cMΦ then the PBS-MΦ as it seems in the ELISA data. The mRNA shows us that this is not totally

true, the 4cMΦ expressed the T_H1-cytokines, but only IL-10 when stimulated with LPS/IFNγ is expressed more than in the PBS-MΦ.
Consistent with a previous report by Stout *et al* . , we got an increased release of TNFα in the 4cMΦ, when stimulated with LPS compared to the non-IL-4c stimulated cells (Stout *et al*. 2005).
Mylonas *et al* . also showed that the combination of LPS and IFNγ give a more distinct classical activation then only one of them (Mylonas & Nair 2009). Our study shows the same for most of the T_H1-cytokines.
In arginase 1 we see the opposite, when stimulated with LPS/IFNγ, the expression is suppressed more than with single stimulation with LPS. Compared to Relm -α and YM-1 the expression in 4cMΦ, when stimulated with the alternative activation agents, has not increased more then the PBS-MΦ. This is also shown in Mylonas study (Mylonas & Nair 2009).

To look at the change of phenotype *in vivo*, we transferred the DsRed cells from PBS or IL-4c injected mice into *S. typhimurium* infected mice. This was done at the 6th day of *S. typhimurium* infection. IFNγ production start two days after infection with *S. typhimurium* (Muotiala & Makela 1990), and there was already LPS from the *S. typhimurium*, so the same stimulatory agents as the *ex vivo* experiment are present in the mice. Unfortunately it was hard to detect the DsRed MΦ in the C57BL/6 mice, so the gating is only done on small percentage of the MΦ. This could explain the suddenly higher expression of Relm -α and YM-1 after transfer. The ones that were detectable showed a distinctive pattern in expression of Relm -α, YM-1 and iNOS in the different groups.
In the *ex vivo* experiment both mRNA for Relm -α and YM-1 are more or less suppressed when stimulated with LPS/IFNγ. In the *in vivo* experiment we get similar results, Relm -α and YM-1 are both expressed less in cells transferred into *S. typhimurium* infected mice than in naïve mice. This tells us that the alternative activated state of the MΦ has decreased since the transfer. An increase in expression of iNOS confirmed that the MΦ have started to become classically activated.

We only looked at the ability of MΦ to change from alternative to classical activated; in the future it would be interesting to look at the other way around as well. Also we used IL-4c to induce the alternative activation state, so the MΦ show an alternative

activated phenotype, but normally they will have been stimulated with a lot of other cytokines as well, so the experiment should be redone with a real parasite.
Interesting is also to look at the ability to clear a bacterial infection when there is a parasite infection simultaneously, because IL-4 inhibits clearance of *S. typhimurium* infection; IL-4 deficient mice can resist the bacteria better than wild type mice (Mittrucker & Kaufmann 2000). We showed that previous stimulated MΦ express TNFα and IL-10 almost in the same amount as non-activated MΦ *ex vivo*. This tells us that there should not be a problem clearing the infections simultaneously or after each other, cause the phenotype is right.
Another study has also shown that C57BL/6 clear *Leishmania major* infections without any problem, while BALB/C mice develop a chronic infection (Finkelman *et al.* 1997). These mice make a predominantly T_H1 and T_H2 response; so their immune cells have different phenotypes. The plasticity of the MΦ in the different mice is also interesting to look at.
This study shows a strong evidence of MΦ plasticity. It is important for the future treatments and diseases to understand how much plasticity the MΦ have and how they differentiate.

5 Acknowledgements

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