INTRODUCTION

It is clear now that a key cell type behind both the acute and reparatory phase of inflammation is the macrophage, which contributes to inflammation by many ways. However, all these functions cannot be assigned to a single macrophage cell type. In fact macrophages are plastic, heterogeneous and represent various, functionally diverse and sometimes opposing phenotypes. This diversity is best described by the M1/M2 macrophage polarization paradigm, a concept developed to deal with the functional diversity in terms of opposing immune functions brought about by T helper 1 and T helper 2 cytokines. This concept resembles to the T cell polarization paradigm. There is very little known about the factors and interactions determining M2 differentiation and function. Indeed, only fragmented pieces of information are available on both the regulatory and the effector aspects of M2/alternative activation. There is also a significant gap in our understanding of the commonalities and differences of the mouse and human systems, which are particularly relevant from a clinical point of view. IL-4 and IL-13 signaling has been shown to be important for cell type specification, and one of the transcriptional mediators of these cytokines, Signal Transducers and Activators of Transcription (STAT) 6 proved to be indispensable for alternatively activated macrophage differentiation[9]. Importantly, some recent work also connected the nuclear hormone receptor, PPARγ (Peroxisome Proliferator-activated Receptor) to M2/alternative activation. These receptors form heterodimers with the RXR (Retinoid X Receptor) receptor and are lipid ligand induced transcription factors activated by modified fatty acids and regulating a wide array of metabolic processes in macrophages including lipoprotein uptake, processing, efflux and lipid antigen presentation. They have also been shown to have anti-inflammatory activities by interfering with proinflammatory transcriptional processes. However, their interaction with other transcriptional pathways and contribution to M2/alternative activation is largely unknown. Therefore, understanding the regulatory processes of M2/alternative activation and the effector functions bringing about the immune phenotype could be particularly useful for finding novel and innovative ways to inhibit tumor progression, and represent a novel approach for inflammation control. 2. Hypotheses and key questions

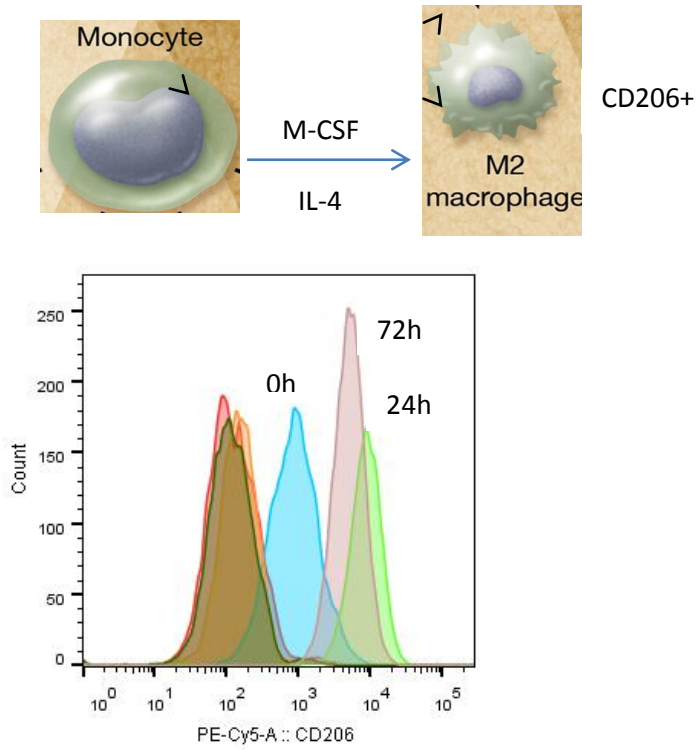
Central hypothesis: The development and function of the M2 cellular phenotype at the gene expression level is mediated by the cistromic interactions of lineage specific (RXRa) and lipid ligand inducible (PPARγ) transcription factors.

RESULTS AND DISCUSSION

1. Monocytes treated with IL-4 differentiate into M2 macrophages

In order to get insights concerning how IL-4 driven genomic and transcriptomic changes relate to macrophage genomic events in human CD14+ monocyte-derived macrophage-like cells (hMQL), first CD14+ monocytes were isolated from peripheral blood and treated according to the experimental design to perform ChIP-Seq and RNA-Seq studies. First, IL-4 responsiveness was confirmed by extracting the expression pattern the M1 and M2 universal marker CD206 compared to untreated cells. Three time points were used: 0h (untreated), 24h and 72h IL-4 treatment. As shown in Figure 1, the universal macrophage marker CD206 is strongly increased in the cell pool after 24h and the effect of IL-4 is visible up to 72h of treatment. We attempt to study other macrophage markers (CD163, CD209, CD16) but this experiments were inconclusive (low quality antibodies for FACS). This data demonstrates the placticity of the *ex-vivo*-derived hMQL to differentiate towards both M1 and M2 macrophages phenotypes and justify the validity of this model in our studies.

Figure 1.



2. Chromatin immunoprecipitation experiments show enrichment of known PPARg and RXRa targets

Chromatin immunoprecipitation experiments were carried out using Mandrup's method. We performed two independent ChIP experiments using PPARg or RXRa antibodies. Figure 2a and 2b show the enrichement of known targets for both transcription factors for the two independent ChIP experiments respectively.

Figure 2a.

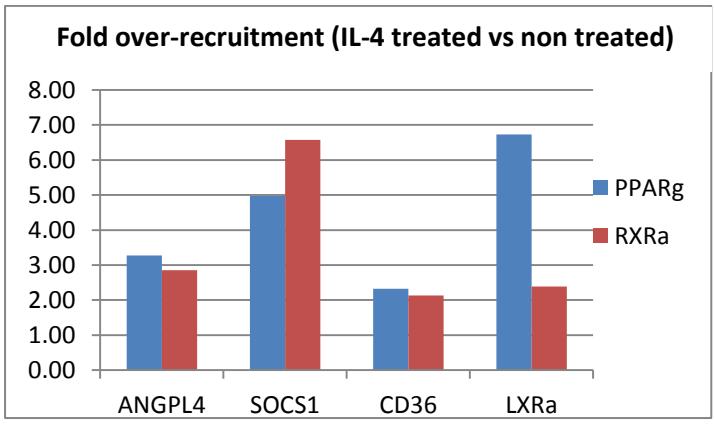
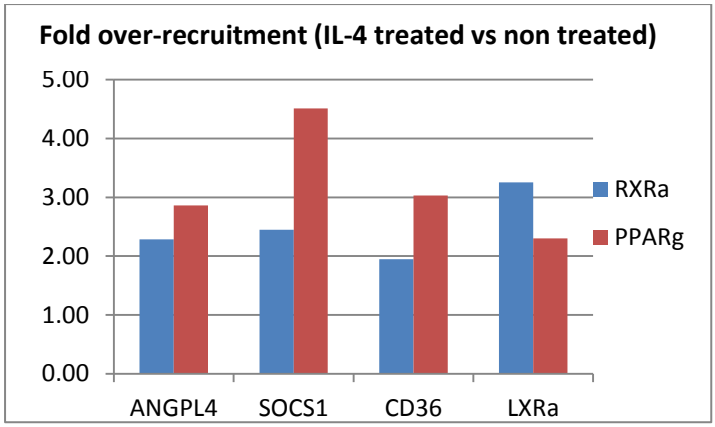


Figure 2b.



Indeed, the promoter regions of ANGPL4, SOCS1, CD36 and LXRa were found to be enriched in these experiments, which proves that upon IL-4 stimulation, PPARg and RXR are over-recruited to these genomic regions. The over-recruitment was found to vary from 2 to about 7 fold.

3. PPARg targets studied by transcriptomics

In order to study the expression of PPARg targets, we performed a transcriptomic study using IL-4 stimulation, and also the treatment with the PPARg agonist Rosiglitazone or the PPARg antagonist GW9662 (Figures 2a and 2b).

Figure 3a.

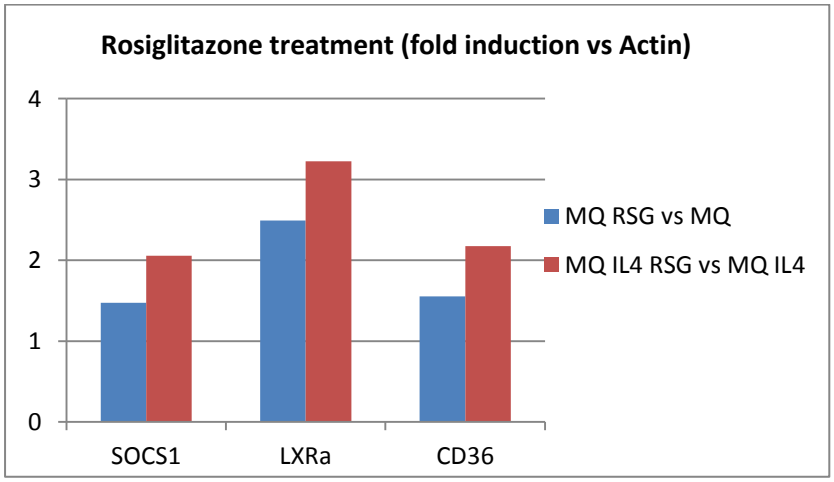
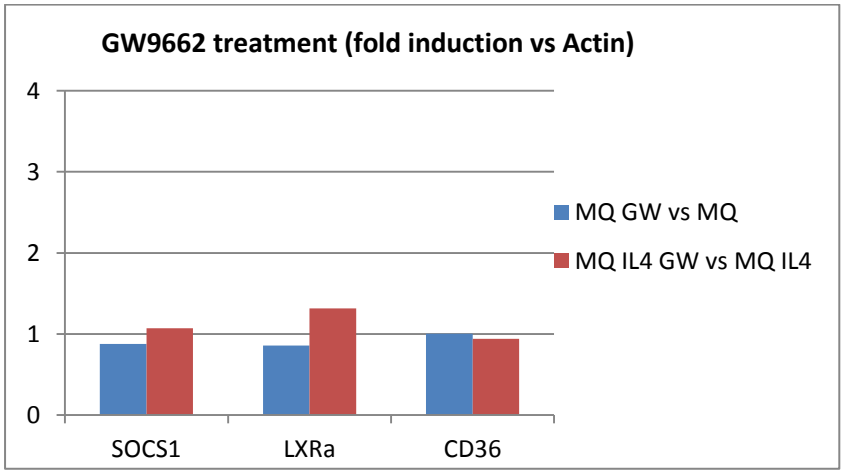


Figure 3b.



We found the activation of SOCS1, LXRa and CD36 upon IL-4 stimulation and the over-activation of these factors upon Rosiglitazone treatment. Coherently, the treatment with GW9662, a PPARg antagonist, overrides the activation observed with both IL-4 and Rosiglitazone.

ChIP-Seq results: the PPARg targets

We sent our immunoprecipitated for deep sequencing. The fragment size analysis of the samples showed a Gaussian around 1kb. The libraries were made and deep-sequenced. Table 1 shows the strongest peaks found for the immunoprecipitation using anti-PPARg antibody on chromatin extracted from IL-4 treated monocyte-devided machrophages.

Table 1.

Chromosome	Starting Pos.	Ending Pos.	Promoter	Peak Strenght
chr6	119558467	119558758	MAN1A1	117.92
chr20	26189800	26190268	LOC284801	71.88
chrX	136520305	136520590	ZIC3	71.65
chr14	88237501	88237924	GALC	70.21
chr9	42415925	42416205	ANKRD20A2	69.24
chr22	18717986	18718307	GGT3P	63.92
chrY	10025064	10025384	TTTY23B	47.46
chr2	33141209	33141697	LINC00486	44.54
chr5	178012450	178012831	COL23A1	42.95
chr16	14580781	14581070	PARN	40.11
chr17	45266414	45266683	CDC27	35.55
chrY	10031294	10031819	TTTY23B	35.23
chr13	28033625	28034167	MTIF3	33.36
chr1	120543871	120544144	NOTCH2	33.28
chr2	133023076	133023677	MIR663B	33.09
chr9	44992423	44993386	FAM27C	33.01
chrY	10019810	10020082	TTTY23B	33.01
chrY	10037707	10037994	TTTY23B	29.21
chr9	67974363	67974627	ANKRD20A1	28.35
chr17	45234187	45234520	CDC27	27.83
chr2	133025578	133025874	MIR663B	27.54
chr2	95475009	95475269	ANKRD20A8P	27.5
chr6	169241091	169241382	SMOC2	26.87
chr2	133029568	133029980	MIR663B	26.14
chr22	20656273	20656522	ZNF74	24.92
chr20	26188828	26189196	LOC284801	24.2
chr22	23827754	23828019	IGLL1	23.14
chr17	45214544	45214729	CDC27	23.08
chr17	41400214	41401277	TMEM106A	21.78
chr12	61865683	61865851	FAM19A2	21.06
chr16	50816904	50817163	CYLD	20.42
chr22	20689541	20689805	ZNF74	20.08

chr2	133016683	133017022	MIR663B	19.7
chr2	133022584	133022820	MIR663B	19.43
chr17	45249202	45249471	CDC27	19.04
chr17	45258842	45259082	CDC27	18.76
chr12	52908418	52908763	KRT5	18.56
chr22	18685535	18685778	USP18	18.14
chr22	21679561	21679758	POM121L8P	18.01
chr20	29653058	29653581	MLLT10P1	17.98
chr22	21522494	21522666	BCRP2	17.98
chr20	29646197	29646652	MLLT10P1	17.88
chr10	103124534	103124753	BTRC	17.26
chr1	165721610	165721770	TMCO1	16.89
chr12	38448751	38448996	ALG10B	16.59
chr11	77597437	77597788	INTS4	16.39
chr1	227700816	227701071	ZNF678	15.76
chr2	87623719	87624056	LINC00152	15.27
chr2	37053313	37053474	VIT	15.14
chr2	37053313	37053474	STRN	15.14

MAN1A1

Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA is an enzyme that in humans is encoded by the *MAN1A1* gene encoding a class I mammalian Golgi 1,2-mannosidase which is a type II transmembrane protein. This protein catalyzes the removal of 3 distinct mannose residues from peptide-bound Man(9)-GlcNAc(2) oligosaccharides and belongs to family 47 of glycosyl hydrolases (Bause et al. 1993).

GALC

gene encodes an enzyme called galactosylceramidase. Through hydrolysis, this enzyme uses water molecules to break down certain fats called galactolipids, which are found primarily in the brain and kidneys. Within cells, galactosylceramidase is found in enzyme-filled sacs called lysosomes where it hydrolyzes specific galactolipids, including galactosylceramide and psychosine. Galactosylceramide is an important component of myelin, the protective covering around certain nerve cells that ensures the rapid transmission of nerve impulses (Wenger et al., 2000).

More than 70 *GALC* gene mutations that cause Krabbe disease have been identified. The most common mutation in affected individuals of European ancestry deletes a large segment of the *GALC* gene (written as 30-kb del). Other mutations insert additional DNA building blocks (base pairs) into the *GALC* gene, delete a small number of base pairs from the gene, or replace a single base pair with an incorrect base pair. These mutations severely reduce the activity of the galactosylceramidase enzyme. As a result, certain galactolipids such as galactosylceramide and psychosine cannot be broken down and accumulate in cells that make myelin. Research suggests that psychosine accumulation is toxic and damages myelin-producing cells, causing the loss of myelin. Without myelin, nerves in the brain and other parts of the body cannot function properly, leading to the signs and symptoms of Krabbe disease.Some individuals with late-onset Krabbe disease have a particular mutation in one of the two copies of the *GALC* gene in each cell. This mutation replaces one of the building blocks (amino acids) used to make the galactosylceramidase enzyme. Specifically, the amino

acid glycine is replaced with the amino acid aspartic acid at position 270 in the enzyme (written as Gly270Asp or G270D). The second copy of the *GALC* gene usually has a different mutation, such as the large 30-kb deletion. The Gly270Asp mutation probably allows some activity of the galactosylceramidase enzyme, which delays onset of the disease (Zaka et al. 2004).

COL23A1

This is a member of the transmembrane collagens, a subfamily of the nonfibrillar collagens that contain a single pass hydrophobic transmembrane domain (Banyard et al., 2003).

PARN

The protein encoded by this gene is a 3'-exoribonuclease, with similarity to the RNase D family of 3'-exonucleases. It prefers poly(A) as the substrate, hence, efficiently degrades poly(A) tails of mRNAs. Exonucleolytic degradation of the poly(A) tail is often the first step in the decay of eukaryotic mRNAs. This protein is also involved in silencing of certain maternal mRNAs during oocyte maturation and early embryonic development, as well as in nonsense-mediated decay (NMD) of mRNAs that contain premature stop codons. Alternatively spliced transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Aug 2008]. GeneCards Summary for PARN Gene PARN (Poly(A)-Specific Ribonuclease) is a Protein Coding gene. Diseases associated with PARN include Pulmonary Fibrosis And/Or Bone Marrow Failure, Telomere-Related, 4 and Dyskeratosis Congenita, Autosomal Recessive 6. Among its related pathways are Deadenylation-dependent mRNA decay and Wnt / Hedgehog / Notch. GO annotations related to this gene include *nucleic acid binding* and *RNA binding*. An important paralog of this gene is PNLDC1. 3-exoribonuclease that has a preference for poly(A) tails of mRNAs, thereby efficiently degrading poly(A) tails.

CDC27

The protein encoded by this gene shares strong similarity with Saccharomyces cerevisiae protein Cdc27, and the gene product of Schizosaccharomyces pombe nuc 2. This protein is a component of the anaphase-promoting complex (APC), which is composed of eight protein subunits and is highly conserved in eukaryotic cells. This complex catalyzes the formation of cyclin B-ubiquitin conjugate, which is responsible for the ubiquitin-mediated proteolysis of B-type cyclins. The protein encoded by this gene and three other members of the APC complex contain tetratricopeptide (TPR) repeats, which are important for protein-protein interactions. This protein was shown to interact with mitotic checkpoint proteins including Mad2, p55CDC and BUBR1, and it may thus be involved in controlling the timing of mitosis. Alternative splicing of this gene results in multiple transcript variants. Related pseudogenes have been identified on chromosomes 2, 22 and Y.

MTIF3

Human mitochondrial translational initiation factor 3 (IF3(mt)) has been identified from the human expressed sequence tag data base. Using consensus sequences derived from conserved regions of the bacterial IF3, several partially sequenced cDNA clones were identified, and the complete sequence was assembled in silico from overlapping clones. IF3(mt) is 278 amino acid residues in length. MitoProt II predicts a 97% probability that this protein will be localized in mitochondria and further predicts that the mature protein will be 247 residues in length. The cDNA for the predicted mature form of IF3(mt) was cloned, and the protein was expressed in Escherichia coli in a His-tagged form. The mature form of IF3(mt) has short extensions on the N and C termini surrounding a region homologous to bacterial IF3. The region of IF3(mt) homologous to prokaryotic factors ranges between 21-26% identical to the

bacterial proteins. Purified IF3(mt) promotes initiation complex formation on mitochondrial 55 S ribosomes in the presence of mitochondrial initiation factor 2 (IF2(mt)), [(35)S]fMet-tRNA, and either poly(A,U,G) or an in vitro transcript of the cytochrome oxidase subunit II gene as mRNA. IF3(mt) shifts the equilibrium between the 55 S mitochondrial ribosome and its subunits toward subunit dissociation. In addition, the ability of E. coli initiation factor 1 to stimulate initiation complex formation on E. coli 70 S and mitochondrial 55 S ribosomes was investigated in the presence of IF2(mt) and IF3(mt) (Koc and Spremulli, 2002).

NOTCH2

This gene encodes a member of the Notch family. Members of this Type 1 transmembrane protein family share structural characteristics including an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats, and an intracellular domain consisting of multiple, different domain types. Notch family members play a role in a variety of developmental processes by controlling cell fate decisions. The Notch signaling network is an evolutionarily conserved intercellular signaling pathway which regulates interactions between physically adjacent cells. In Drosophilia, notch interaction with its cell-bound ligands (delta, serrate) establishes an intercellular signaling pathway that plays a key role in development. Homologues of the notch-ligands have also been identified in human, but precise interactions between these ligands and the human notch homologues remain to be determined. This protein is cleaved in the trans-Golgi network, and presented on the cell surface as a heterodimer. This protein functions as a receptor for membrane bound ligands, and may play a role in vascular, renal and hepatic development. Two transcript variants encoding different isoforms have been found for this gene.

GENERAL DISCUSSION AND CONCLUSIONS

In this work we have performed and controlled Chromatin immunoprecipitation experiments using PPARg and RXRa antibodies as well as expression experiments aiming to subsequently perform ChIP- and RNA-Seq experiments. Among the known targets of PPARg, several of them were found to be enriched in our ChIP experiments, but unfortunately none of them gave a strong signal in the ChIP-seq experiment, neither for PPARg nor for RXRa. The strongest peaks in our ChIP-seq experiments correspond to genes associated with diverse functions but they are difficult to be discussed in the context of the M2 phenotype. We have recently modified the immunoprecitipation protocol aiming to perform ChIP-seq experiments providing us with reliable data concerning the targets of PPARg and RXRa in monocyte-derived machrophages.

The analyses used will provide us with a very large amount of systematic information on the induced and repressed genes in the context of M2 macrophages differentiation and STAT6 and PPARγ activation along with data on the total genome-wide transcriptional activity and binding sites of key transcription factors (PPARγ, RXRa). We anticipate to find hundreds of regulated genes and hundreds if not thousands of genomic binding sites for each transcription factor. It will be particularly intriguing to assess the cistromic (sum and overlap of genomic binding sites) interactions between the various pathways, such as the relationship between STAT6 and PPARγ, or these two factors and RXRa at the genome level. Novel binding motifs might also be identified. Genes with novel regulatory or effector functions will be selected and functionally analyzed.