Macrophages are critical components of innate immunity as they are implicated in the elimination of invading pathogens and the progression of certain diseases for example cancer. In order to achieve these roles, macrophages undergo the process called polarization through which the cells change their gene expression profile and functional characteristics due to changes in the extracellular milieu of molecules. The net result of polarization is the diverse macrophage subpopulations present in almost each and every organ, which can drastically change under pathophysiological settings. The polarization of macrophages has been shown to contribute to the progression of inflammatory and cancerous diseases, but the underlying molecular mechanisms are not well understood. We aim to understand the molecular details of the polarization process and identify the transcriptional regulators that are required for the completion of polarization. These studies may reveal new druggable targets and allow us to modulate this process in a pathophysiological condition.

To understand the polarization process, we differentiated bone marrow-derived progenitors in the presence of monocyte/macrophage colony stimulating factor (M-CSF) and generated bone marrow-derived macrophages (BMDMs). These BMDMs were then polarized into alternatively polarized macrophages by the addition of interleukin-4 (IL-4) cytokine, which represents the opposite side of the bacterial stimuli triggered classical macrophage polarization, associated with inflammatory conditions. Alternatively, polarized macrophages are involved in the dampening of the inflammatory response, but facilitate certain regenerative processes for example wound healing, muscle regeneration. More transcription factors have been described that can mediate this process, but Signal Transducer and Activator of Transcription 6 (STAT6) is the main initiator of the cellular transition, which will eventually turn on a whole cascade of other transcriptional regulators. One of the members of this cascade is the Peroxisome Proliferator Activated Receptor gamma (PPARg), which is a ligand-activated nuclear receptor and deposited in high numbers to the genome during alternative polarization in a STAT6-dependent manner. PPARg forms permissive heterodimers with the Retinoid X Receptor (RXR), meaning that it can be modulated by small lipophilic molecules from both sides of the heterodimer. According to the literature, PPARg is required for complete alternative polarization, but ligand activation does not capable of activating most of the genes associated with the alternative polarization program. This observation suggests a mechanism, which does not employ ligand activation via the receptor and contradicts with the dogmatic view that PPARg acts in a ligand-dependent manner. Based upon these results, we hypothesized that PPARg is a dominantly ligand insensitive transcriptional regulator of the chromatin structure during alternative polarization.

First, we characterized the PPARg:RXR cistromes in alternatively polarized BMDMs using Chromatin Immunoprecipitation sequencing (ChIP-seq), which identified approximately ten thousand heterodimer-bound genomic regions. As a result of the polarization we observed ~4200 de novo heterodimer-bound regulatory regions, which was associated with a continuously opening chromatin structure during the time course of our experiments determined by Assay for Transposase Accessible Chromatin sequencing (ATAC-seq). Interestingly, we found many PPARg:RXR-bound genes that were not responsive to any of the receptor modulating ligands (Rosiglitazone (RSG) – full agonist of PPARg, GW9662 – antagonist of PPARg), while the best known target genes of PPARg were responsive to these molecules. Is it possible that the vast majority of PPARg-bound sites are not functional and merely the byproducts of the high protein level of the receptor?

Genome-wide analysis of nascent RNA production with Global Run-On sequencing in the presence of the activating ligand (RSG) revealed that indeed the vast majority of PPARg-bound genomic regions are not capable of mediating ligand-dependent transcriptional responses. We have also carried out RNA-polymerase II-specific ChIP-seq experiments in the presence of the activating ligands, which confirmed our findings made at the nascent RNA level. More specifically, out of the ~5300 high confidence PPARg:RXR-bound genomic regions, only 200 responded to the ligands, including the effects of the RXR full agonist LG10268. These results clarified that the phenomenon of ligand insensitive receptor binding to chromatin is prevalent throughout the polarization. What can be the functional relevance of these sites?

To answer this question, we used a myeloid-specific Pparg knockout mouse model system and differentiated BMDMs, which were polarized in the presence of IL-4. First, we tested if the opening of chromatin is affected in the absence of the receptor. ATAC-seq experiments revealed diminished chromatin openness as a result of alternative polarization compared to the wild type controls. Moreover, studying the recruitment of the co-factor/histone acetyltransferase P300 and the architectural protein RAD21 revealed diminished binding of these proteins to chromatin in the absence of PPARg. Altogether, though the ligand activation of the receptor does not always have obvious effects at the transcriptional level it does affect the local chromatin structure.

Gain of function experiments using the wild type and ligand mediated activation function mutant receptor in Pparg KO immortalized BMDMs provided further evidence that ligand activated

transcription is not required for the chromatin opening capacity of the receptor. Consequently, ligand insensitive receptors can modify the chromatin structure, but whether this will have any functional relevance was not clear at this point, so we conducted new experiments to clarify if the altered chromatin structure allows for cellular memory formation.

We set up a model in which we stimulated BMDMs for one day in the presence of IL-4, then we removed the cytokine by washout and incubated the cells for another day, followed by restimulation with IL-4. Interestingly, more genes and their enhancers exhibited memory, manifested in more robust and quicker responses to IL-4 restimulation, including the Arg1 gene, which is one of the marker genes of alternative polarization. In addition, some of the genes exhibited transcriptional memory to IL-4 pre-stimulation were Pparg-dependent, which encouraged us to assess this phenomenon at the global scale by doing RNA-seq.

Genome-wide transcriptomics at the mRNA level shed light on the gene set that exhibited transcriptional memory to IL-4 restimulation in a Pparg-dependent manner. If we considered only those genes, which exclusively upregulated upon restimulation in a Pparg-dependent manner, we discovered a very coherent gene set consisting of 235 genes responsible for the remodeling of the extracellular matrix, which are critical components of regenerative processes. Out of the 235 genes, 211 were completely insensitive to PPARg and RXR ligands. Functional assay of in vitro wound healing confirmed that endothelial cells were able to facilitate quicker wound closure if they received supernatants from macrophages double stimulated with IL-4, the phenomenon which was Pparg-dependent. In addition, in a mouse model of muscle regeneration, sorted macrophages progressively expressed higher and higher levels of the extracellular matrix components and the related enzymes, which coincided with the appearance of PPARg in an IL-4 containing environment. Further investigations are required to conclusively link PPARg to appearance of this gene set in this in vivo model.

To sum up, our work identified PPARg as a ligand insensitive epigenomic regulator of chromatin allowing for the establishment of transcriptional memory, conferring progressive macrophage polarization upon repeated stimulation.

In a follow up set of experiments we focused on understanding the late genomic program of alternative polarization in great detail. Under pathological conditions, alternatively polarized macrophages are contributing to allergic reactions and also reported to be important for the

elimination of multicellular parasites, but similar phenotypes have also been noted in patients with sepsis. In some of these systems, alternatively polarized macrophages have been reported to multiply in the tissue of residence, proved to be important for controlling infections and regeneration.

At the molecular level, macrophage polarization is initiated by transiently binding transcription factors (TFs) at the chromatin level. The open chromatin regions of macrophages contain the binding motifs of PU.1, AP-1, C/EBP and IRF transcription factors, which are all important for macrophage development and function. The combinations of these factors establish the macrophage-specific regulatory element landscape (lineage identity) via collaborative interactions. This pre-formed chromatin environment functions as a molecular landing strip for signal-dependent transcription factors (SDTFs) initiating the polarization process. The early steps of alternative macrophage polarization have been extensively studied, demonstrating that the polarization program establishes *de novo*/latent enhancers, which can mediate new cellular functions and also provide cellular memory. The immediate transcriptional regulator and initiator of alternative polarization is Signal Transducer and Activator of Transcription 6 (STAT6). STAT6 acts in a rapid and transient fashion by translocating into the nucleus upon phosphorylation (ref). DNA binding of STAT6 homodimers alters the expression of hundreds of genes in a relatively short time (60 minutes), including several transcription factors. Due to its transient mechanism of action, the majority of STAT6 is released from the chromatin after 24 hours of cytokine exposure. Therefore, the polarization program enters into a state where STAT6 does not have the capacity to directly control the following molecular events. Several transcription factors have been described as important regulators of alternative polarization including Peroxisome Proliferator Activated Receptor Gamma (PPARg) KLF4, IRF4, MYC and more recently BHLHE40. The latter is reported to control the proliferation of large peritoneal macrophages when IL-4 is abundant. Our recent study suggests that the nuclear receptor PPARg, is a long-term regulator of alternative polarization and provides transcriptional memory but does not possess robust regulatory roles when macrophages transit from the early to late polarization state.

It is important to note here that based on our current view, the above-mentioned transcription factors are acknowledged as purely STAT6 regulated, although their expression levels are rising and maintained during the times when STAT6 is excluded from the nucleus. This apparent contradiction supports the proposal that directly STAT6-controlled transcriptional regulator(s) linchpin(s) exist(s) coordinating late stages of polarization.

Here we took a systematic approach, which uses the combination of mapping genome activity patterns and *de novo* motif analysis. Using the combination of P300 (general co-activator with

histone acetyltransferase activity) and H3K27ac chromatin immunoprecipitation sequencing (ChIP-seq) we defined the early and late genome activity patterns of alternative polarization. By mapping the early and late epigenomic changes of interleukin-4 (IL-4)-mediated alternative macrophage polarization we identified the TF Early Growth Response 2 (EGR2), bridging the proximal (early) and secondary (late) gene expression program of polarization. EGR2 is a directly IL-4/STAT6-regulated TF, responsible for 77% of the induced, polarization gene signature, including a large TF network. Mechanistically, EGR2 binding induced chromatin accessibility, recruited chromatin remodelers, cofactors and RNA-polymerase II for the execution of the late polarization program. Thus, our study identified EGR2, as a STAT6-dependent TF, representing a key link into the secondary transcriptional events of alternative macrophage polarization.