I. Characterization of IL4/STAT6 rearranged nuclear receptor signaling and macrophage angiogenic activity in bone marrow derived macrophages

Retinoid X receptors (RXRs) are general heterodimerization partners of lipid-sensing nuclear receptors such as peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), retinoic acid receptors (RARs) and Vitamin D receptor (VDR). Therefore, we aimed to investigate the IL-4-induced changes in genome-wide RXR binding in unstimulated and IL-4-stimulated mouse bone marrow-derived macrophages (BMDMs) using chromatin immunoprecipitation assays with sequencing (ChIP-seq). We found more than 4500 new RXR-bound genomic regions in macrophages following 24 hours IL-4 exposure compared to unstimulated BMDMs. To further characterize the epigenetic features of these genomic regions, we examined chromatin openness with Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) methods. We observed that chromatin accessibility was significantly increased around new RXR-bound genomic sites in IL-4-exposed BMDMs. Taken together, these findings suggest the rearrangement of RXR-cistrome during alternative macrophage activation though IL-4-induced opening of new latent enhancer set.

Next, we wanted to identify the heterodimerization partner(s) of RXRs at the newly bound genomic regions, therefore we performed *de novo* transcription factor binding motif analysis. We could detect significant enrichment of IL-4/STAT6 signaling pathway-induced PPARγ-binding DR1 *de novo* motif. In agreement with this finding, we observed IL-4-induced PPARγ-binding at these regulatory regions. Interestingly, the IL-4-induced RXR-binding proved to be STAT6 and PPARγ-dependent using STAT6 and PPARγ-deficient BMDMs. These results indicate that the IL4-induced rearrangement of genome-wide RXR-binding needs the presence of STAT6 and PPARγ transcription factors.

In order to further characterized the functional properties of IL-4-expanded RXR/PPARγ cistromes, we wanted to study the ligand responsiveness of 2554 RXR/PPARγ-bound de novo enhancers using global run-on sequencing (GRO-seq) and RNA Polymerase II-pS2- specific ChIP-seq methods. Unexpectedly, both methods showed that the small fraction of RXR/PPARγ-bound *de novo* enhancers was sensitive to RXR ligand LG268 and/or PPARγ ligand RSG. Based on RNA Polymerase II-pS2-specific ChIP-seq experiments, we identified 85 highly permissive, 72 RXR ligand dominant, 111 PPARγ ligand dominant and 52 repressed RXR/PPARγ heterodimer-bound de novo regulatory regions in same subTADs with ligand responsive gene bodies. We found that the signal intensity of RNAPII-pS2 at the NR ligand responsive enhancers-associated gene bodies almost fully recapitulated the changes we observed on the enhancers except for PPARγ ligand dominant enhancers, which exhibited LG268-mediated induction as well, but the gene bodies were more responsive to RSG. The repressive effects of the ligands were not significant on the regulatory regions near repressed genes, but they showed a trend towards repressed RNAPII-pS2 binding. Taken together, these results raised the possibility that the RXR/PPARγ heterodimers act mainly ligand-independent manner at the *de novo* enhancers.

Next, we carried on the investigation of functional role of ligand insensitive alternative macrophage polarization-specific RXR/PPARγ heterodimers applying coactivator P300 and cohesin complex member RAD21-specific ChIP-seq as well as ATAC-seq methods. We observed that IL-4-induced P300 and RAD21 bindings were partially abolished in PPARγ

deficient macrophages under the ligand insensitive PPARγ-bound regulatory regions. In addition, IL-4-mediated enhancement of chromatin accessibility also showed PPARγ dependency at these genomic regions. Taken together, these findings suggest that PPARγ plays important role in the development of alternative macrophage polarization-specific enhancer composition in a ligand-independent manner.

Next, we wanted to examine that the newly deposited RXR/PPARγ heterodimers had roles in directing the progression of the cells as transcriptional memory marks. Contrary to the IL-4-activated transient STAT6 transcription factor binding, RXR/PPARγ heterodimer binding showed its maximum following long-term (24 hours) IL-4 stimulation and it was retained after wash-out of IL-4. In addition, we observed that the second IL-4 stimulation led to the elevated IL-4 responsiveness including increased STAT6, P300 and RAD21 binding as well as enhancer RNA expression at the ligand insensitive RXR/PPARγ heterodimer-bound genomic regions. The elevated IL-4 responsiveness proved to be completely PPARγ dependent suggesting that PPARγ is an important player in the transcriptional memory of alternatively polarized macrophages.

To investigate the biological consequence of PPARγ-directed transcriptional memory, we performed mRNA sequencing (RNA-seq) and RNA Polymerase II-specific ChIP-seq experiments. Our RNA-seq analysis identified 235 genes that were exclusively induced upon the second IL-4 stimulation and showed PPARγ dependence. Our RNA Polymerase II-specific ChIP-seq experiments demonstrated that the majority of these genes was completely insensitive to either PPARγ and RXR agonists, or to the PPARγ antagonist. Among these genes, many cell-extracellular matrix interaction-linked KEGG pathways including focal adhesion, extracellular matrix receptor interactions, regulation of actin cytoskeleton, and tight junction were significantly overrepresented. Therefore, we tested the contribution of these changes in an *in vitro* ‘‘scratching assay’’ and found that re-epithelialization by HREC (human retinal endothelial cell) cells were significantly diminished if they have received supernatant from double-stimulated PPARγ-deficient macrophages compared to wild-type counterparts. Taken together, our results suggest that macrophages received repeated IL-4 stimulation may secrete factors that can potently enhance cell growth and/or migration in a PPARγ-dependent, but ligand-independent, manner.

Note: As we described in the grant, we hypothesized that IL-4 priming facilitates RXR agonist-induced expression of pro-angiogenic factor VEGFα in the alternatively polarized macrophages. However, our RT-qPCR-based results did not confirm our assumption. Therefore, we did not perform ELISA and CAM assays in this alternative macrophage polarization system.

II. Characterization of IL4/STAT6 mediated active repression and anti-inflammatory activity in macrophages

In this subproject, we aimed to study the IL-4/STAT6 signaling pathway-activated alternative macrophage polarization-specific transcriptional program. Therefore, we performed STAT6 and RNA Polymerase II-specific ChIP-seq experiments in IL-4-stimulated and unstimulated BMDMs for the investigation and characterization of IL-4-regulated STAT6 cistrome. As

expected, STAT6 binding was negligible in unstimulated BMDMs. Remarkably, upon IL-4 stimulation STAT6 recruitment was observed at 20119 genomic regions. The binding of STAT6 was dramatically induced during the first hours of IL-4 stimulation, but it was reduced nearly to the basal level between 6 and 24 hours of IL-4 treatment. Comparing the STAT6 cistrome to the RNA Polymerase II positive genomic regions revealed that 60.5% of STAT6 peaks (12175/20119) overlapped with RNA Polymerase II positive genomic regions in non-polarized and/or short (1 hour) IL-4-treated macrophages. We classified the RNA Polymerase II positive STAT6 peaks based on IL-4-dependent regulation of RNA Polymerase II binding. We identified 2677 STAT6-bound genomic regions were associated with rapid IL-4-induced decrease of RNA Polymerase II binding suggesting that IL-4-induced STAT6 binding indeed results in repression at these genomic loci.

To study the IL-4/STAT6 signaling pathway-activated alternative macrophage polarization-specific transcriptional program, we performed RNA-seq experiments in wild-type and STAT6 deficient BMDMs at different time points following IL-4 stimulation. We identified four IL-4-induced gene clusters based on expression dynamics and fold induction. Furthermore, we could find one IL-4-repressed gene cluster with 538 genes. Repression by IL-4 was already observed after 3 hours IL-4 stimulation and showed STAT6-dependency. Interestingly, rapid IL-4-induced reduction of RNA Polymerase II binding was also detected at gene bodies of IL-4-repressed genes. We decided to assign ″repressor″ STAT6-bound genomic regions to the IL-4-repressed genes, using RNA Polymerase II binding as a reporter of enhancer activity. We predicted the topologically associated domains in which gene regulation might take place by STAT6, using macrophage-derived CTCF and RAD21 ChIP-seq data sets, utilizing a previously described algorithm. We found that "repressor" STAT6 peaks were tightly associated with the IL-4-repressed gene cluster. These results suggest a tight connection between the direct STAT6-dependent repression of histone H4 acetylation and IL-4/STAT6 signaling-mediated control of neighboring gene expression at the same topological domain.

Next, we wanted to further characterize the IL-4/STAT6 signaling pathway-mediated direct transcriptional repression. Therefore, we performed new H3K27Ac, p300, PU.1, JUNB, CEBPα, HDAC1, HDAC2 and HDAC3-specific ChIP-seq data sets. Similarly to the RNA Polymerase II binding, significantly attenuated H2K27Ac enrichment was detected at the ″repressor″ STAT6 peaks-associated genomic regions in 1 hour IL-4-treated BMDMs. In addition, reduced coactivator p300, and lineage determining factor PU.1, CEBPα and JUNB binding were also observed at these genomic regions following 1h IL-4 exposure. Although, HDAC1, HDAC2 and HDAC3 binding were also detected at the STAT6-repressed enhancers in unstimulated macrophages, but the IL-4 stimulation could not influence their occupancies. Taken together, these findings suggest that the STAT6-mediated direct transcriptional repression is associated with reduced coactivator and lineage determining transcription factor binding.

The constitutive, IL-4-independent binding of classical HDACs such as HDAC3 at the STAT6-repressed enhancers raised the possibility that IL-4-induced changes in the activity of these enzymes might play a role in the IL-4/STAT6 signaling pathway-mediated repression. Therefore, we decided to reanalyze the publicly available HDAC3 deficient and wild-type alternatively polarized macrophages-derived microarray data sets (Mullican et al.: Genes and Development, 2011) focusing on the role of HDAC3 in IL-4- mediated repression. We found 1628 IL-4-repressed genes in wild-type BMDMs and identified 371 genes containing IL-4-

repressed gene cluster, which showed attenuated repression in HDAC3-deficient macrophages following IL-4 treatment. Using our ChIP-seq data sets, we identified 325 STAT6-repressed enhancers in the sub-topologically associated domains of IL-4/HDAC3-repressed genes. These enhancers were bound by HDAC3, but HDAC3 occupancy was not altered by IL-4 stimulation. These results indicate that the presence of HDAC3 at the IL-4/STAT6-repressed enhancers is required for the IL-4-induced repression of a specific subset of genes.

To investigate the biological importance of IL-4/STAT6 signaling pathway-mediated transcriptional repression, we performed in silico pathway analyses using different algorithms including KEGG pathway analysis and IPA. Among the IL-4-repressed genes, we identified some significantly overrepresented inflammation-linked signaling pathways such as NOD-like receptor signaling and Toll-like receptor signaling pathways. Therefore, we wanted to determine whether prior activation of IL-4/STAT6 signaling can influence the inflammatory program of macrophages and we performed RNA-seq as well as RNA Polymerase II and NFkB-p65-specific ChIP-seq experiments on IL-4-pre-treated and LPS activated BMDMs. Our RNA-seq analysis identified 520 genes which showed significantly attenuated LPS-responsiveness following 24 hours of IL-4 pre-treatment. In addition, RNA Polymerase II binding showed a similar pattern to "steady-state" mRNA level at the gene bodies of IL-4 repressed genes suggesting that IL-4 pre-treatment modulates LPS-induced gene expression primarily at the transcriptional level. Based on our STAT6 and NFkB-p65-specific ChIP-seq data sets, we identified 961 overlapping STAT6 and p65 peaks in the sub-topologically associated domains of IL-4-attenuated LPS-responsive genes. 641 out of 961 genomic regions were associated with significantly elevated RNAPII-binding following LPS activation. Intriguingly, 70% (448/641) of LPS-activated enhancers showed significantly reduced basal and LPS-induced RNAPII binding following 24 hours of IL-4 pre-treatment. These findings suggest that the activation of IL-4/STAT6 signaling can attenuate the inflammatory response of macrophages through selective, direct repression of a distinct LPS-activated enhancer set.

Next, we wanted to further study the NOD-like receptor signaling pathway in IL-4-primed and LPS-activated murine BMDMs. We observed that basal and LPS-induced Nlrp3 and Il1b expressions were significantly reduced at both mRNA and protein levels in IL-4-primed BMDMs. NLRP3-inflammasome activation-dependent IL-1β secretion and pyroptotic cell death were also significantly attenuated in IL-4-pre-treated wild-type macrophages. Interestingly, IL-4-mediated repression of NLRP3 signaling pathway was completely abolished in STAT6 deficient macrophages.

Finally, we wanted to study whether the inflammatory responsiveness of Nlrp3 and Il1b is influenced *in vivo* by nematode infection triggered alternative macrophage polarization. We applied a Heligmosomoides polygyrus (H. polygyrus) infection-based model and we injected the mice with LPS or Salmonella Typhimurium intraperitoneally 9 days after nematode infection. As expected, the number of alternative macrophage polarization marker Ym1-positive macrophages was highly induced in peritoneal macrophages of H. polygyrus-infected mice. Although Nlrp3 was not induced in the applied experimental system by LPS injection or Salmonella Typhimurium infection, steady-state expression was significantly inhibited by H. polygyrus infection, and the inhibitory effect of nematode infection was sustained in the presence of inflammatory stimuli. Nematode infection did not result in Il1b expression by peritoneal macrophages but both LPS injection and Salmonella Typhimurium infection resulted in a robust induction of Il1b expression. This elevated expression was significantly diminished

in macrophages from H. polygyrus-infected mice. Taken together, these findings indicate that IL-4 priming can partially inhibit the inflammation-responsive NOD-like receptor signaling pathway *in vitro* and *in vivo* through direct STAT6-mediated transcriptional repression of its key genes including Nlrp3 and Il1b.

III. Characterization of IL4/STAT6 mediated post-transcriptional repression and regulation of macrophage viability

Our preliminary data showed that IL-4/STAT6 signaling pathway directly induces the anti-proliferative miR-342-3p and its host gene EVL expressions in murine and human macrophages. Therefore, we wanted to explore the functional effect of miR-342-3p on macrophage proliferation and/or apoptosis in a mouse macrophage cell line RAW264.7 transfected with miR-342-3p or miR-negative control miRNA mimics. Following transfection, cell numbers were determined at various time points by propidium iodide staining of permeabilized adherent cells. The cell number of miR-342-3p overexpressing macrophages showed 40 and more than 80 percent reduction at 24 and 48 hours after transfection, respectively, as compared to the miR negative control-transfected cells. To confirm our results resazurin reduction and neutral red uptake-based assays as independent in vitro cell viability analyses were also applied. MiR-342-3p reduced viable macrophage cell numbers by 40 and 55% at 48 hours post-transfection in the resazurin and neutral red uptake assays, respectively. In order to assess if the decreased cell viability observed is the result of impaired progression of the cells through the cell cycle or it is due to increased cellular death we performed cell cycle analysis by PI staining and a necrosis/apoptosis assay by Annexin V/PI double-staining of the miR-342-3p and miR-negative control-transfected macrophages. Analysis of cell cycle distribution by Hoechst staining and slide-based imaging cytometry revealed that miR-342-3p overexpression was associated with a slight but not significant increase in the number of cells in S-phase. However, miR-342-3p overexpression significantly increased the number of both Annexin V positive/PI negative early and Annexin V/PI double positive late apoptotic cells. From these results we concluded that miR-342-3p regulates macrophage cell numbers via induction of apoptosis.

In order to study the molecular background of pro-apoptotic function of miR-342-3p in macrophages, we combined transcriptomic, computational and biochemical approaches. Based on our microarray analysis, 2640 downregulated and 2341 upregulated genes were identified in mi-342-3p overexpressing macrophages compared to negative-control-transfected cells. Using a complex miRNA target identification algorithm, we identified 813 predicted miR-342-3p target genes which were downregulated in pre-miR-342-3ptransfected cells. Interestingly, the gene set of downregulated potential miR-342-3p target genes contained 23 genes from negative regulators of apoptosis Gene Ontology (GO) category. Finally, we proved with the usage of biochemical approaches that the anti-apoptotic Bcl2l1 is a direct target gene of miR-342-3p. Taken together, these findings suggest that miR-342-3p induces apoptotic cell death in macrophages via repression of anti-apoptotic gene network.