SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

EGR2 is an epigenomic regulator of alternative macrophage polarization and a nexus modulator of alveolar macrophage-related antifungal immunity

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INTRODUCTION

Macrophages are dominant parts of the innate immune system and create the first line of protection against different pathogens. The adaptive action of macrophages is a result of a rapid and plastic environment-induced response based on special epigenomic, gene expressional and functional program.

The internal surfaces of the lung are directly encountered by inhaled air pollutants and pathogens. The tissue resident alveolar macrophages (AMs) are associated with the bronchoalveolar epithelial lining. Their pivotal roles are the recognition and elimination of pathogens and harmful agents, and parallelly they initiate the inflammatory response. AMs recognize the pathogen-associated molecular patterns (PAMPs) of targets by pathogen recognition receptors (PRRs). Next, AMs internalize the bound particles by phagocytosis and inactivate the microbes in phagolysosomes. The intracellular killing of pathogens based on lysosome acidification, reactive oxygen species (ROS) formation, and enzymatic degradation.

In turn, the induced downstream inflammation-associated signaling cascades through complex transcriptional regulatory steps lead to diverse proinflammatory cytokine production and numerous lipid mediator secretions, which orchestrate the multicellular response of acute inflammation of the lung. Finally, AMs switch to resolving phenotype and take part in the regeneration of injured lung. The coordinated action of immune mechanisms is sufficient to create defense against infective agents. However, the inadequate program of molecular and cellular events leads to manifest diseases such as acute lung injury or pulmonary fibrosis.

In our study we focused on the details of alternative macrophage polarization mechanisms and based on our primary results we followed our work with the examination of Early Growth Response 2 (EGR2) mediated changes in AMs.

THEORETICAL BACKGROUND

Diversity and importance of macrophages

The diversity of macrophages arises from their ability to respond to a wide variety of environmental signals, allowing them to specialize and perform distinct functions in different tissues and contexts.

Tissue-resident macrophages are primarily derived from yolk sac progenitors or fetal liver monocytes during embryogenesis. These cells seed tissues before birth and undergo self-renewal, largely exempt from adult hematopoiesis. In adults, circulating monocytes, derived from bone marrow hematopoietic stem cells (HSC), can be recruited to tissues, where they differentiate into macrophages in response to inflammatory cues.

While there are numerous common treats of different macrophage populations such as initiation of inflammatory response, engulfment of pathogens or debris and resolution of inflammation, based on tissue localization, they fulfill specific functions also.

From the aspect of activation and polarization, macrophages demonstrate extreme plasticity influenced by the cytokine milieu and tissue microenvironment. However, the polarization state of macrophages represents a wide spectrum, canonically the literature distinguishes the two end points, the classical polarization state or “M1 phenotype” via the activation of Toll-Like Receptor (TLR) or different inflammatory cytokine signaling pathways and the alternative polarization state or “M2 phenotype” induced by T helper 2 cell-derived (Th2) IL-4 or IL-13 cytokines. Functionally, classically polarized macrophages exhibit high microbicidal activity through the production of pro-inflammatory cytokines, while the alternatively polarized macrophages act in parasite infections, support wound healing mechanisms, support the resolution of inflammation and maintenance of tissue integrity. The complexity and adaptability of macrophage roles, making them integral players in both maintaining tissue homeostasis and mediating disease pathogenesis.

The differentiation and function of alveolar macrophages

AMs are specialized immune cells located in the alveoli of the lungs. They play a crucial role in maintaining pulmonary homeostasis and defending the respiratory system against inhaled pathogens and particulates.

AMs embryonically derived from fetal liver. During embryonic development the fetal liver monocytes translocate to the parenchyma of fetal lung on embryonic day 13.5-14.5. Here, the local microenvironment leads to the maturation of pre-AM stage which terminates after birth. The terminally mature and functional AMs show the expression of CD45 as a general immune cell marker, the F4/80 and CD11c as a macrophage marker and the SiglecF as a specific AM surface marker protein, however constant downregulation and finally the loss of CD11b expression occurs during the maturation process.

The well-differentiated AMs are capable to supply widespread functions. They provide alveolar clearance through the recognition and elimination of inhaled pathogens, detoxifying air pollutants and regulate the surfactant homeostasis.

In response to lung injury or inflammation, they can enhance their uptake of damaged surfactants and cellular debris, helping maintain alveolar stability and function during the repair process. On the other hand, they fulfill dominant role in other mechanisms of tissue repair and remodeling following lung injury.

Invasive *aspergillosis*

Invasive *aspergillosis* is a severe fungal infection primarily caused by *Aspergillus fumigatus (AF)*, although other species can also be involved. Inhalation of airborne conidia (spores) is the primary route of entry into the human body. The spores are usually cleared by a competent immune system; however, they can germinate and invade tissues in immunocompromised hosts.

The incidence of invasive *aspergillosis* has increased, particularly among patients with hematological malignancies, HSC and solid organ transplants. The lungs are the most affected site, leading to pulmonary invasive *aspergillosis.* Key clinical symptoms include persistent fever despite broad-spectrum antibiotic therapy, pleuritic chest pain, cough, hemoptysis, and dyspnea.

The mainstay of treatment for invasive *aspergillosis* is antifungal therapy, but in some cases the surgical resection of localized sites of infection may be necessary. The prognosis of invasive *aspergillosis* is generally poor without treatment, particularly in severely immunocompromised individuals. The overall mortality remains high, but early diagnosis and appropriate antifungal therapy can significantly improve survival rates.

The Early Growth Response 2 (EGR2) transcription factor

EGR2 is part of the early growth response (EGR) family of zinc-finger transcription factors, which also includes EGR1, EGR3, and EGR4. These proteins bind DNA and regulate gene expression. The target genes are involved in various cellular processes, such as growth, differentiation, and response to environmental stimuli.

EGR2 is critically involved in the development of the peripheral nervous system. It is required for embryonic development and the generation of hindbrain and specifically regulates the expression of genes necessary for the proliferation and differentiation of Schwann cells.

Related to the immune system, we know that EGR2 is especially important in T lymphocytes. EGR2 is expressed during the development of T cells in the thymus, where it takes part in the differentiation of developing thymocytes.

EGR2 shows expression in activated macrophages and a marker transcription factor of AMs. Just a limited number of studies touched slightly the mechanism of action and exact function of this transcription factor in these cell types.

AIMS

In the first part of our study, the general aim was to identify transcriptional regulators in alternative macrophage polarization via unbiased genome-wide profiling.

1. We aimed to map the active enhancers of bone marrow-derived macrophages (BMDMs) in short term and long term IL-4-induced alterative polarization.

2. Based on the action of defined genomic regions we wanted to categorize the temporal dynamic of regulatory events.

3. We planned to find DNA binding transcriptional regulators which fulfill pivotal role in the mediation of genomic changes.

4. We aimed to examine the potential tissue specific importance of the novel found regulators.

Next, after describing EGR2 transcription factor as a candidate modulator of AM phenotype, we expanded our aims by the followings:

1. Analyze the cellular composition of lung in myeloid-specific EGR2 deficient mice.

2. Describe the EGR2-mediated epigenomic, transcriptomic and functional changes in AMs.

3. Put into *vivo* context the EGR2 dependent alterations.

4. Examine the importance of EGR2 in pathological processes in the lung.

MATERIALS AND METHODS

Bone marrow-derived macrophages (BMDM) culture and treatment conditions

Isolation and differentiation of bone marrow progenitors were completed as described previously (Daniel 2014). BMDMs were differentiated by addition of L929 cells' supernatant. On the 6th day, the cells were treated with IL-4 (20 ng/ml) for 1 or 24 hours.

Chromatin immunoprecipitation

The experiment was performed as previously described (Daniel 2014). Ovation Ultralow Library Systems V2 (Nugen) was applied for library preparation. The used antibodies: P300 (sc-585), and H3K27ac (ab4729).

Mouse strains

The *Egr2*$^{fl/fl}$ strain was a kind gift from Patrick Charnays's laboratory (C57BL/6 background). We crossed the lysozyme Cre (LysCre) and *Egr2*$^{fl/fl}$ strain to reach the myeloid specific knock out (KO) condition (*Egr2*$^{fl/fl}$). We used *Egr2*$^{+/+}$ LysCre littermates as controls.

Real-time quantitative polymerase chain reaction for eRNA and mRNA detection

We isolated total RNA of cells by TRIZOL (Invitrogen). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was applied for reverse transcription. The transcript detection was run by SYBR Green (BioRad) intercalation. We calculated the expression values by the delta Ct method. The raw expression values were normalized to Ppia expression.

EGR2-expressing embryonic stem cell-derived myeloid progenitor cell generation

The EGR2-expressing doxycycline-inducible murine embryonic stem cells (ESCs) were cultured in DMEM (Thermo-Fischer Scientific) complemented with 15% Fetal Bovine Serum (FBS) (Thermo-Fischer Scientific), 1000 U/ml Leukemia Inhibitory Factor (LIF)

(Merck), streptomycin (50 μg/ml), and penicillin (100 U/ml) (Merck) in the presence of 200 μg/ml G418.

The embryoid bodies (EBs) were created as previously described (Boto et al. 2021). ESCs were cultured in Iscove′s Modified Dulbecco′s Medium (Thermo-Fisher Scientific) containing 15% FBS, iron-saturated transferrin (200 ng/ml, Sigma), 4.5 mM monothioglycerol (Sigma) and ascorbic acid (50 ng/ml, Sigma). The dissociated cells were cultured for 3 days in alpha-MEM supplemented with 20% FBS, GM-CSF (50 ng/ml, PeproTech, London, UK), and 50 μM β-mercaptoethanol (Merck). The induction of EGR2 was initialized by 1 μg/ml of doxycycline (DOX).

ATAC-seq

25000 cells served as template for ATAC-seq. The nuclei of AMs were isolated by ATAC-LB (10mM Tris-HCl pH7.4, 10mM NaCl, 3mM MgCl2, 0.1% IGEPAL). The isolated nuclei were used for tagmentation by Nextera DNA Library Preparation Kit (Illumina). We amplified the tagmented DNA by Kapa Hifi Hot Start Kit (Kapa Biosystems). We analyzed the quality of fragment distribution of libraries with Agilent Bioanalyzer. The sequencing was performed by HiSeq 2500 platform.

RNA-seq

The cells' total RNA content was isolated by TRIZOL (Invitrogen). Samples with >7 RNA integrity number were processed for library preparation by Ultra II RNA Sample Preparation Kit (New England BioLabs). We generated cDNA by random priming reverse transcription. The samples were sequenced on Illumina NextSeq 500 instrument.

CUT&RUN

80000 AMs were aliquoted per sample. The CUT&RUN was performed by the CUTANA ChIC/CUT&RUN Kit (EpiCypher) according to the manufacturer's instructions.

The DNA was purified by the CUTANA DNA Purification Kit. For library preparation 1 ng of DNA was used and performed by the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs). Illumina NextSeq 500 was used for sequencing with a Mid Output Kit.

Bronchoalveolar lavage (BAL) for alveolar macrophage isolation

The mice were euthanized by isoflurane (Forane, Baxter) inhalation. We washed out the bronchoalveolar space three times with 800 µl of Phosphate Buffered Saline (PBS) solution supplemented with 200 mM Ethylenediaminetetraacetic Acid (EDTA, pH: 7.4) and 1% FBS. The isolated BALF was centrifuged to separate the cellular components. We resuspended the cells in 200 µl of Ammonium-Chloride-Potassium (ACK) lysis buffer to lyse contaminating blood cells. To pellet cells, we centrifuged them with the same settings.

Generation of single cell suspension from total lung for flow cytometric analysis

The animals were euthanized by isoflurane inhalation. The lungs were removed and cut into small pieces on ice, then a Lung Dissociation Kit (Miltenyi Biotec) was applied.

Flow cytometry and cell sorting

The cells were incubated with Fc Receptor Blocking Reagent (Miltenyi Biotec) in 1:100 dilution, then they were stained by antibody mix. In intracellular stainings the AMs were fixed and permeabilized by the FoxP3/Transcription Factor Staining Kit's (eBioscienceTM) 1×Fixation/Permeabilization reagent. The flow cytometry analysis and sorting were performed with Cytek Aurora and FACS Aria III (BD Biosciences) spectral flow cytometers.

Phagocytosis assays

We created a cell culture by plating 100.000 cells at 6-well dishes in 1 mL RPMI medium supplemented with 10% FBS, and 5%-5% of penicillin and streptomycin. When the cells were attached, we applied 1x106 pHrodo-Red labeled zymosan, E. coli, S. aureus or

dextrane bioparticles (Invitrogen) per one well. Upon incubation period we scraped up the attached AMs and measured them by FACS Aria III flow cytometer (BD Biosciences).

Confocal microscopy

For confocal microscopy, we plated 50000 AMs into 8-well chambered coverslips (Ibidi). We added $2x10^6$ Texas-Red labeled zymosan (Invitrogen™, Z2843) per one well. We recorded the confocal images by Zeiss LSM 880 microscope (Carl Zeiss, Jena, Germany).

Cytokine Array

Quantibody® Mouse Cytokine Array 1000 Kit (QAM-CAA-1000) was applied and the experiment was performed by RayBiotech. Hierarchical clustering of log-scaled protein amount (μg/ml) values was carried out by Cluster 3.0 and the non-scaled heatmap was visualized by JavaTreeView.

Fungal strains

AF 293 *Aspergillus fumigatus* strain was applied. It was cultured on a standard nitrate minimal medium at 37°C. To harvest conidia, the fungi were grown for 6 days.

Zymosan-induced *in vivo* lung inflammation model

Egr2$^{+/+}$ and *Egr2*$^{fl/fl}$ animals were anesthetized by the inhalation of isoflurane. 300 μg zymosan bioparticles (Invitrogen) were intranasally administered.

***Ex vivo* zymosan treatment of alveolar macrophages**

We created a cell culture by plating the cells at 24-well dishes in 0.5 mL RPMI medium supplemented with 10% FBS, and 5%-5% of penicillin and streptomycin. When the cells were attached, we applied zymosan bioparticles (unlabeled, Invitrogen) in 50 μg/ml concentration.

***Aspergillus fumigatus* – alveolar macrophage *ex vivo* co-treatment model**

The cells were cultured in RPMI medium containing 5% penicillin, 5% streptomycin and 10% FBS. We treated them with *AF* conidia (10^6/well). We replaced the medium upon 1-hour and we incubated the cells for 6 hours. After scratching the AMs we prepared decimal dilution series. We inoculated the samples in nitrate minimal media agar slants at 37°C and after 48 hours we counted the colony forming units (CFUs).

Time-lapse microscopy

The isolation of AMs, the culture and treatment conditions were the same as in CFU assay. After washing out the *AF* conidia, in every 5 second time-lapse images were snaped by Olympus upright microscope (Tokyo, Japan) using 10x magnitude, 0.25 NA plan achromatic objectives (Carl Zeiss, Jena, Germany), and 2-megapixel UVC USB 2.0 camera boards (Asus Computer International, Fremont, CA, USA).

***Aspergillus fumigatus in vivo* infection model**

As immunosuppression, we administered cyclophosphamide (CP, 250 mg/kg) i.p. The first treatment was 3 days prior infection and we repeated it on day 1 post infection. On the same days, we applied gentamicin prophylaxis (5-mg/kg body weight). We instillated $3.5x10^6$ *AF* conidia per mouse.

Histological analysis

The isolated organs were fixed in 4% buffered paraformaldehyde (PFA, pH7.4) then embedded in paraffin. The sections were visualized by haematoxylin and eosin (H&E), Masson's trichrome, Periodic acid–Schiff (PAS) and Grocott staining using standard methods. The myeloperoxydase (MPO) immunohistochemistry was carried out applying rabbit monoclonal primary antibodies with Envision (biotin-free) Peroxidase-based Detection Kit (Dako) and DAB substrate chromogen (Vector Labs).

Determination of lipid mediators by Targeted Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS)

The total lungs samples were snap frozen in liquid nitrogen. After we put the samples to ice-cold methanol containing deuterated internal standards. The lungs were minced and placed to -80°C for 1 hour. After centrifugation the supernatants went out solid-phase extraction (SPE) using C18 columns (Biotage, preconditioned with washes of methanol and water). Samples were measured applying a high-performance liquid chromatography system (HPLC; Shimadzu, Kyoto, Japan). A Qtrap5500 mass spectrometer (AB Sciex, Framingham, MA) was coupled to the HPLC. Data acquisition was accomplished using Analyst software v.1.7. A scheduled multiple reaction monitoring (MRM) method was used to identify lipid mediators. Identification of lipid mediators in the samples was accomplished using Sciex OS-Q v.1.7. Lipid mediators were quantified by integrating peak areas above the baseline and interpolation based on external standard curves.

Analysis of bronchoalveolar lavage fluid protein contents

The protein level was measured by BCA assay (Thermo Fisher Scientific). We determined lactate dehydrogenase (LDH) activity by LDH Activity Assay Kit (Sigma-Aldrich). The cytokine levels were detected using TNFα, IL-6 (Biolegend), CXCL9 (cat.no. #EMCXCL9), CXCL11 (cat.no. #EMCXCL9), and CXCL13 (cat.no. #EMCXCL13) enzyme-linked immunosorbent assay (ELISA) Kits (Invitrogen).

Seahorse analysis of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR)

XF96 oximeter (Seahorse Biosciences, North Billerica, MA, USA) was applied for the measurement of OCR and ECAR values. AMs were seeded in 96-well plates one day prior to

the experiment. 1 hour prior to the assay, the cell culture medium was replaced with unbuffered medium. The values were recorded after 5 minutes of 30 sec mixing cycle 5 times.

Western Blot

We generated whole cell lysates applying RIPA lysis buffer. The prepared lysates were resolved in a 4-16% gradient Bis-Tris polyacrylamide gel by electrophoresis and transferred to transfer membrane. We blocked the membranes then we probed them with 1:1000 anti-EGR2 antibody (Abcam, ab108399). We washed the membranes and added 1:20000 of anti-Rabbit IgG (R&D Systems, HAF008). After the membranes were incubated with SuperSignal West Pico PLUS Chemiluminescent Substrate.

Data and software availability

The next-generation sequencing data are available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository (GSE151015 and GSE181087).

Statistics

The differentially expressed genes (DEGs) were described applying one-way analysis of variance (ANOVA) test supplemented with a post-hoc Tukey honestly significant differences test. In all other experiments Student's t test was applied. Significant differences between groups were considered if p-value was ≤ 0.05 (Indicated by asterisks: *p ≤ 0.05, **p≤ 0.01, ***p≤ 0.001, ****p≤ 0.0001).

RESULTS

The genome activity patterns of short- and long-term alternative macrophage polarization

To identify the genomic events of alternative macrophage polarization we applied IL-4 treated murine BMDM polarization model. We analyzed the temporal changes of cistrome and transcriptome by unbiased datasets. We used unstimulated BMDMs as control and we detected the short-term polarization after 1 hour and the long-term polarization upon 24 hours IL-4 cytokine treatment. To map active enhancers, we designed a ChIP-seq-based approach. We determined the genomic binging sites of P300, a transcriptional co-activator, and the level of active histone mark H3K27ac. The combined occupancy of these signs represented the active enhancers that we used as a pool of further analysis.

As a first action to get insight into epigenomic events, we defined genomic regions with differential levels of P300 and H3K27ac. We identified nearly similar amount of activated (n=16735) and repressed (n=17582) regulatory elements during alternative BMDM polarization. 14353 genomic sites were unchanged. We clustered the regulatory elements based on activity pattern and we separated them to “early transient”, “early sustained” and “late” categories. These experiments opened the possibility to identify the dominant driver transcriptional regulators which mediates these epigenomic alterations.

The EGR2 is a late-stage regulator of alternative macrophage polarization

To find potential transcription factors mediating different genome activity patterns, we run motif enrichment analysis based on changing genomic regions. Near known macrophage-specific transcription factor binding sites we found motifs associated with undescribed transcription factors which show high specificity to sustained genomic activity. Among these factors the EGR was enriched in both “early sustained” and “late” categories, indicating the

potential significance of EGR transcription factor family in the regulation and maintenance of alternative macrophage polarization.

To determine which member of the EGR family affects the genomic activity, we analyzed the mRNA level of all members of EGRs in unstimulated an IL-4 treated BMDMs. Just the Egr2's mRNA was detectable and showed elevation during alternative macrophage polarization. We measured the protein level of EGR2 which followed the same expressional tendency. We hypothesized that the EGR2 is the modulator of alternative polarization from EGR family based on these results.

Next, we created an EGR2 knock-out (KO) mouse model, specific for myeloid cells to examine the role of this transcription factor in alternative polarization. We generated BMDMs from control and *Egr*$^{fl/fl}$ mice and we treated them with IL-4 in different time point between 0 and 24 hours. Then we measured the expression of alterative polarization marker genes' (*Retnla, Ccl17, Chil3, Chil4, Arg1 and Mrc1*) mRNA level by RT-qPCR. We detected significant repression in all cases in late (12 or 24 hours) time points. Based on this, EGR2 is especially a late-stage regulator of alternative macrophage polarization.

To check the influence of EGR2 in opposite way, we performed gain of function experiments transfecting mouse ESC-derived myeloid progenitors with doxycycline (DOX)-inducible EGR2 genetic construct. First, we confirmed the overexpression of *Egr2* mRNA upon DOX treatment with RT-qPCR measurement, after we measured a similar M2 marker gene set than in the loss of function experiment. The mRNA level of *Retnla, Chil3, Chil4* and *Ccl17* was elevated upon EGR2 upregulation and showed additional induction after 24 hours. These results strengthened evidence of EGR2's role in the regulation of M2 macrophage polarization.

Lung-resident macrophages show high expression of EGR2

We wanted to examine the potential importance of EGR2 in tissue resident macrophages. By analyzing the expression of EGR family members based on already published

RNA-seq datasets (GSE63340) from different tissue resident macrophage population and monocytes, we found that *Egr2* has a relative specific and strong expression in AMs.

Based on publicly available bulk RNA-seq and scRNA-seq data from different stages of AM maturation we reanalyzed the expression values of different transcription factors. We found that the mRNA level of EGR2 is elevated especially at the final stage of differentiation. This intriguing pattern raises the opportunity to the role of EGR2 in the coordination of specific cellular functions through epigenomic regulation of gene expression.

The myeloid-specific EGR2 deficiency leads altered immune cell composition of lung and affects alveolar macrophage phenotype

To test our hypothesis, we started to analyze the changes upon myeloid-specific loss of EGR2 in the lung. First, we compared the total number of different cells isolated from the whole lungs of control and EGR2 myeloid-deficient mice by flow cytometry.

The number of endothelial cells, epithelial cells, and fibroblasts were relatively identical in the lungs of control and EGR2 myeloid KO animals. Within immune cell populations we detected significant reduction in the number of mature AMs and interstitial macrophages, Ly6C^{+} monocytes, NK cells, B cells, CD4+ and CD8+ T cells. Only the DCs showed significant elevation from analyzed populations, the number of Ly6C^{-} monocytes, neutrophil granulocytes, eosinophils and γδ T cells were roughly identical. Our results show that EGR2 influences via direct and indirect ways the composition of myeloid and lymphoid immune cells, but not affect the presence and number of non-immune populations in the lung.

Next, we prepared formalin-fixed, paraffin embedded histological samples from EGR2 KO and WT lungs. We applied H&E and MPO staining for better distinguishment of cellular components. The general structure of lung was intact in both genetic backgrounds. We observed difference in the ratio of MPO positive AMs between the WT and EGR2 lungs. The lungs

isolated from EGR2 deficient animals represent a higher number of AMs within the same area of tissue. This finding seems to be opposite as the tendency of flow cytometry measurements. To solve the discrepancy, we examined the maturity status of AMs localized in the bronchoalveolar space by quantifying the expression of CD11b, CD11c, F4/80 and SiglecF proteins as well-known markers that represent different stages of AM differentiation.

The terminally differentiated AMs are positive for the standard immune cell marker CD45, and show high expression of F4/80, CD11c, and SiglecF. The expression of CD11b reduces during AM maturation. We defined the AMs from whole lung homogenate, and we also isolated them by BAL to avoid the potential mistakes that could occur because of unexpected dysregulation of standard markers.

In the $CD45^+$ cell population of control and EGR2 deficient lungs the AM subpopulation was separable based on F4/80 and CD11c expression in both conditions. The number of AM were significantly elevated in *Egr2*$^{fl/fl}$ lungs. We selected this population and analyzed the SiglecF and CD11b expressions. In *Egr2*$^{fl/fl}$ samples we detected a near total loss of $SiglecF^+$ subgroup of AMs and the ratio of $CD11b^+$ cells were significantly higher. We isolated the bronchoalveolar CD45+ cells and stained them with the same markers. These results were the same as previous ones from total lung homogenates. The myeloid-specific loss of EGR2 does not block the differentiation of AMs totally but leads to a modulated maturation phenotype.

To characterize the general metabolic functionality of EGR2 deficient AMs we used Agilent Seahorse XF Analyzer-based experiments, and we determined the mitochondrial oxygen flux and ECAR. We *ex vivo* measured the metabolic processes. In basal, etomoxir-, and oligomycin-dependent respiration essentially identical OCR values have occurred in *Egr2*$^{+/+}$ and *Egr2*$^{fl/fl}$ cells. In the case of ECAR, we detected a low range of reduction in *Egr2*$^{fl/fl}$ cells.

Finally, we concluded the myeloid-specific deficiency of EGR2 leads to a metabolically intact macrophage, which locates in the alveolar space in higher ration than normal AMs and represents a unique profile of cell surface markers.

EGR2 as a likely direct DNA bound transcription factor is a dominant modulator of alveolar macrophage epigenome

Next, we sought to understand the molecular events mediated by EGR2 in the chromatin of AMs, so we performed ATAC-seq and described the differences in chromatin openness of EGR2 sufficient and deficient AMs. Then we analysed the differences in chromatin accessibility between *Egr2*$^{+/+}$ and *Egr2*$^{fl/fl}$ AMs. We identified 1906 closing and 4792 opening significantly changed genomic regions in the chromatin in *Egr2*$^{fl/fl}$ AMs compared to *Egr2*$^{+/+}$ cells. We mapped the genomic distribution profile of differentially accessible regions (DARs). Both classes were localized in larger proportion at enhancer region than promoter region.

To identify sequence motifs for transcription factors that directly bind to DNA within DARs, we conducted a *de novo* motif enrichment analysis. For closed DARs, there was a significant enrichment of EGR motifs (50.53%). Analyzing the specificity of EGR motifs revealed that closing regions predominantly featured strongly. Notably, the absence of EGR motif enrichment in the opening sites suggests that EGR2 is unlikely to function as a direct repressor through direct DNA binding.

To further investigate EGR2's molecular function, we decided to integrate chromatin accessibility studies with transcriptomic analyses.

EGR2 directly regulates transcription and plays a crucial role in controlling the gene network associated with pathogen elimination

To uncover the molecular specifics of these likely direct activating transcriptional events, we examined the connection between potential EGR2-bound enhancers and their

associated regulated genes. Consequently, we conducted bulk RNA-seq experiments on control and *Egr2*$^{fl/fl}$ AMs in their steady state. This led to the identification of 364 significantly repressed and 621 significantly induced genes in AMs lacking EGR2.

By linking the closing DARs to the repressed DEGs, we discovered that a large number of closed DARs were located within a 100 kilobase pair (kbp) distance (n=373). However, over 1000 DARs were positioned more than 1000 kbp away from a repressed gene. We integrated the DARs and DEGs by employing a stricter criterion of ±100 kbp distance from the gene's TSSs. We identified 158 repressed DEGs as potential direct targets of EGR2. The closing DARs connected to repressed DEGs suggest that EGR2 is likely to function as a transcriptional activator.

Then we conducted CUT&RUN experiments focusing on the H3K4me3 histone mark to identify active gene promoters and on BRD4 as a marker of active enhancers and promoters. By analyzing the distribution of the H3K4me3 signal in the 500 bp downstream region relative to the TSS of EGR2-dependent genes, we observed that H3K4me3 levels corresponded with differences in gene expression, reinforcing the idea that EGR2 primarily activates chromatin structure and gene regulation. BRD4 showed a similar pattern at promoters and enhancers, further supporting EGR2's significant role as an epigenetic regulator.

Encouraged by these findings, we performed gain-of-function experiments on ESC-derived myeloid progenitors with a DOX-inducible EGR2 genetic construct. In this system, RT-qPCR analysis confirmed *Egr2* mRNA overexpression upon DOX treatment, resulting in significantly elevated levels of mRNA and protein for the CD11c and SIGLECF AM marker. We also examined transcriptional changes in a selection of potential direct target genes (*Kazald1, Clec7a, Atp6v0d2, Rhoc*) and their associated enhancers, which showed an upregulation trend following EGR2 overexpression. These experiments led us to conclude that the identified DARs serve as enhancers, and their activity depends on EGR2.

Next, we analyzed both the EGR2-dependent DEGs from a functional perspective. In the absence of EGR2, the mRNA levels of core macrophage marker genes and AM signature genes were partially altered. However, nearly half of the altered AM signature genes, including *Epcam, Krt79, Cldn1*, and *Kazald1*, appeared to be proximal EGR2 targets.

Next, we used the KEGG database to predict the biological pathways affected by the list of DEGs. The transcriptional changes associated with the absence of EGR2 are related to pathogen recognition, ROS formation, phagolysosome acidification, and maturation. Among these genes, several are part of a transcriptional network involved in regulating pathogen elimination.

The absence of EGR2 results in impaired zymosan phagocytosis

Our findings led us to investigate some of the critical pathways in detail. In EGR2-deficient AMs, the BRD4 marks and eRNA levels of an enhancer linked to *Clec7a* were significantly reduced, as was the H3K4me3 signal at the gene's promoter, reinforcing this observation. *Clec7a* encodes the Dectin-1 receptor, a well-known PRR for fungal cell wall components like zymosan, which is essential in the antifungal response of AMs. We assessed Dectin-1 protein expression on the surface of both control and EGR2-null AMs using flow cytometry on cells isolated from BAL. Reflecting its mRNA levels, the protein levels of Dectin-1 were considerably lower in $Egr2^{fl/fl}$ AMs.

To functionally assess the activity of this protein, we examined the zymosan uptake in $Egr2^{+/+}$ and $Egr2^{fl/fl}$ AMs *ex vivo* using a pHrodo-conjugated zymosan bioparticle assay. After 3 hours, we observed a significant reduction in the proportion of phagocytosing EGR2-null AMs compared to WT AMs, with their phagocytic capacity reduced to approximately 70%.

Additionally, we used confocal microscopy to monitor the progression of uptake by AMs during *ex vivo* treatment with Texas Red-conjugated zymosan. We counted the number of

internalized bioparticles at 0-, 20-, 60-, and 120-minutes post-treatment. The results indicated a decrease in the average number of internalized zymosan in *Egr2*$^{fl/fl}$ AMs compared to the control at 20 minutes and even at 120 minutes. The phagocytic capacity of *Egr2*$^{fl/fl}$ AMs did not reach that of WT AMs, the KO cells internalized fewer zymosan particles.

EGR2 affects the initial transcriptional response to zymosan *in situ*

Zymosan, which is not only phagocytosed but also triggers a strong inflammatory response, prompted us to further investigate the early phase of the zymosan-induced inflammatory response *in vivo* in *Egr2*$^{+/+}$ and *Egr2*$^{fl/fl}$ mice. We administered 300 µg of zymosan intranasally and analyzed the induced changes at 6 and 24 hours post-administration. We sorted the CD45+ and F4/80+ AM populations from control and zymosan-treated groups. Using bulk RNA-seq, we identified 1755 genes in *Egr2*$^{fl/fl}$ AMs that responded to zymosan compared to controls. Notably, the majority of zymosan-responsive genes (n=6047) which showed dynamic expression changes unaffected by the absence of EGR2. This indicates that EGR2 is not essential for inflammatory gene expression in general but plays a distinct and selective role in the early regulation of *in situ* inflammatory genes.

The 1755 EGR2-dependent and zymosan-responsive genes were organized into ten clusters according to their similar time-dependent expression patterns. These were then reintegrated with the previously identified closed DARs across the ten EGR2-dependent and zymosan-responsive gene clusters. We identified zymosan-responsive and EGR2-dependent repressed DEGs and linked these genes to closed DARs located more than 100 kbp from the TSS of DEGs under control conditions between *Egr2*$^{+/+}$ and *Egr2*$^{fl/fl}$ AMs. The expression of these genes was significantly affected by EGR2 during zymosan-induced inflammation, but not under unstimulated, physiological conditions. This observation indicates that EGR2 plays a dual role in shaping the AM epigenome and modifies various signal-dependent transcriptional

responses without affecting the gene's basal expression. Thus, it functions as an epigenomic bookmarker.

We analyzed gene sets for TLR, TGF-β, Jak-Stat, phosphatidyl-inositol (PI), and arachidonic acid (ARA) signaling pathways to pinpoint exact EGR2-dependent changes linked to the canonical inflammatory response. While all pathways included DEGs, the impacted genes exhibited varying ratios and expression patterns across the groups. Genes primarily upregulated were associated with the TGF-β, TLR, and Jak-Stat pathways. The DEGs tied to the PI signaling pathway were notably repressed, including genes coding for components of phosphorylation cascades. Additionally, we identified *Lta4h* as a proximal, likely direct target, which was consistently repressed following zymosan treatment in EGR2-null AMs.

Given that transcriptional changes significantly impacted genes encoding cytokines and lipid mediator-synthesizing enzymes, we measured the cytokine profile using an ELISA-based array from BAL fluid and ARA metabolism-related lipid mediators via mass spectrometry from total lung homogenate. These methods revealed a notable decrease in IL-4 protein levels 6 hours after zymosan treatment and an increase in the levels of CCL17, CXCL11, and CSF1 after 24 hours of treatment. While IL-4 is secreted by Th-cells, the upregulated cytokines were specifically derived from macrophages. The induction of CXCL11 is particularly noteworthy as this cytokine is not induced when EGR2 is present, indicating a novel and highly specific response. Among ARA-derived lipid mediators, RvE1 was undetectable in the lungs of EGR2-deficient mice. This lipid mediator, produced by the Leukotriene A4 Hydrolase (LTA4H) enzyme, plays a crucial role in resolving inflammation. Although *Lta4h* expression is reduced in zymosan-treated EGR2-null AMs.

We further examined the inflammatory process by analyzing the histology of paraffin-embedded, H&E-stained lung samples after 24 and 72 hours of zymosan treatment. Comparable infiltration of PMNs was observed in both WT and *Egr2*$^{fl/fl}$ mice 24 hours after

zymosan exposure, although *Egr2*$^{fl/fl}$ lungs showed a greater presence of macrophage-like mononuclear cells with large, irregular nuclei. Additionally, after 72 hours, the alveoli in the lungs of *Egr2*$^{fl/fl}$ mice remained unresolved, and there was progression towards tissue remodeling and incipient fibrosis.

Inadequate response to *Aspergillus fumigatus* in AMs lacking EGR2

Finally, we expanded our research to include pathophysiologically relevant *ex vivo* and *in vivo Aspergillus fumigatus* infection models. Initially, we treated *Egr2*$^{+/+}$ and *Egr2*$^{fl/fl}$ AMs, with *AF* conidia for 1 hour, allowing the AMs to internalize the particles. We washed out the spores. At that point, and after an additional 6 hours of incubation, we lysed the cell cultures and measured the samples' colony forming capacity. We observed a significant decrease in CFUs after 1 hour and a significant increase at 7 hours in EGR2-null AMs. Furthermore, the ratio of internalized and inactivated *AF* conidia was lower in *Egr2*$^{fl/fl}$ AMs. Using time-lapse microscopy, we monitored the morphological changes following the internalization of *AF* conidia. We found that EGR2 deficiency in AMs resulted in a greater proportion of hyphae-containing AMs and an earlier onset of hyphae growth compared to WT cells.

We also investigated the *in vivo* effects of *AF* conidia through intranasal infection. Clinically, no significant differences were observed in WT mice under the test conditions following infection. However, *Egr2*$^{fl/fl}$ mice quickly began to lose body weight, suggesting severe inflammation. Histological analyses indicated a trend of unresolved, prolonged inflammation with early onset of progressive pulmonary fibrosis in EGR2 KO lungs. In contrast, the control lungs demonstrated regressing inflammation without fibrosis.

AF is an opportunistic pathogen, and severe or fatal *AF* infections occur only in immunocompromised patients. Thus, cyclophosphamide (CP)-induced immunosuppression serves as an appropriate model, leading to rapid *aspergilloma* formation in the lungs. We

employed this agent in combination with gentamycin bacterial prophylaxis to evaluate the effects of myeloid-specific EGR2 loss under immunosuppressive conditions. Initially, we used flow cytometry to assess changes in the number of various immune cells in the lung after 1 day of infection. CP treatment resulted in a significant decrease in the number of B and CD4+ T cells and a slight, though not significant, reduction in AMs and CD8+ T cells in the lungs of both control and EGR2-deficient mice compared to non-immunocompromised infections. The reduction in monocyte and neutrophil numbers was noticeable only in control animals.

Next examined the BALF isolated from the lungs one day after *AF* infection. We did not observe any significant changes in total protein levels across different conditions. However, under immunosuppression, we found significantly higher LDH activity and a tendency for increased concentrations of inflammatory cytokines (TNFα, IL-6) and profibrotic markers (CXCL9, CXCL11, CXCL13) in *Egr2*$^{fl/fl}$ mice compared to controls.

Histological analysis of lung sections revealed significantly larger *aspergillomas* containing *AF* hyphae and necrotizing inflammation characterized predominantly by large macrophage-like mononuclear cells with pleomorphic large nuclei or apoptotic cells in *Egr2*$^{fl/fl}$ mice. The lungs of *Egr2*$^{fl/fl}$ mice harbored persistent inflammatory cells which induced large spindle-shaped cells, indicative of activated fibroblast morphologies. Masson's staining of these areas revealed coarse collagen deposits, indicating significant fibrosis.

These results collectively offer further *in vivo* evidence of the crucial role EGR2 plays in the antifungal activity of AMs, although it is important to note that the observed findings reflect a complex multicellular phenotype.

DISCUSSION

EGR2 is a late-stage regulator of alternative macrophage polarization

Understanding the hierarchical transcriptional program governing macrophage polarization is crucial for advancing knowledge of cell type specification and developing targeted therapies. In our study, we identified EGR2 as a key component of the IL-4-mediated and STAT6-dependent alternative polarization program. EGR2 connects early, transient events to later, more stable ones, establishing critical hierarchical relationships among various transcription factors. Acting as a molecular linchpin, EGR2 links the transient STAT6 signal to enduring epigenomic changes, supporting stable gene expression. We leveraged the mapping of genome activity, using active enhancer markers P300 and H3K27ac, over a time course of macrophage polarization to study both the immediate and long-term effects of IL-4-activated STAT6. This strategy led to the discovery of EGR2, a powerful transcriptional regulator with previously unrecognized functions in myeloid cells. While *Egr2* had been reported as an IL-4-induced gene in macrophages in transcriptome-wide analyses, its specific roles had remained unclear. In our model, IL-4 selectively induces *Egr2* from the family members.

The reanalysis of mouse tissue-resident macrophage RNA-seq data revealed that *Egr2* exhibits a lung-restricted expression pattern in steady-state. Combined with findings about basophil-derived IL-13's role in AM function, this supports EGR2's potential *in vivo* role.

The function of EGR2 as an auxiliary or secondary LDTF

AM identity is established by general macrophage LDTFs such as PU.1 and C/EBPβ which is essential for AM differentiation. However, EGR2 stands out as a distinctive component due to its specialized function: it directly activates only a limited subset of mature AM marker genes, such as SiglecF, while leaving others unchanged. Our findings support that EGR2 deficiency caused only a partial reduction in differentiation-dependent gene expression.

Thus, EGR2 acts more as an accessory or secondary LDTF, collaborating with other factors to specify and enhance certain aspects of AM identity and function.

The dual mechanism of EGR2 action

EGR2, however, plays a critical role in the terminal differentiation stage of human monocytes to macrophages, as identified in numerous studies associating it with AMs. Recent research using genetic models similar to ours has confirmed the necessity of EGR2, which we further explored, showing its likely integration into the AM epigenome as a direct DNA-bound transcriptional activator and epigenomic marker. This is suggested by the enrichment of the EGR motif in chromatin regions that close in the absence of EGR2, while those that open do not show such enrichment. This indicates EGR2 is unlikely to act as a repressor through direct DNA-binding but may have repressive effects through protein interactions or indirect pathways.

EGR2 directly regulates Dectin-1, a key player in antifungal response and phagocytosis, thereby positioning EGR2 upstream of clinically important pathways involved in fungal recognition, clearance and it has a role in responses to zymosan.

Moreover, EGR2 links distinct stimuli with silent bookmarking functions revealed upon exposure to specific conditions, explaining the high number of genomic sites closing in EGR2's absence and their connection to non-cognate stimuli like the zymosan response.

The impact of EGR2 deficiency on lung pathophysiology and its clinical significance

The clinical significance of these findings is multifaceted. Firstly, EGR2 is an evolutionarily conserved transcription regulator in AMs. The regulated gene network is particularly important in the context of lung health, as it relates to conditions such as invasive *Aspergillosis* and fungal allergies, highlighting the need for a specialized transcriptional regulator. Single nucleotide polymorphisms (SNPs) in Dectin-1 have been linked to

Aspergillosis and other fungal infections in human populations. Our data support the involvement of EGR2 in selective responses against specific pathogens.

Furthermore, histological analysis of lung tissue in both steady-state and following zymosan and *Aspergillus fumigatus* treatment corresponds with observed transcriptional and phenotypical changes. In a steady-state, myeloid-specific deficiency of EGR2 results in increased cellularity in interalveolar spaces, with some mononuclear cells showing larger size and irregular nuclear structures, possibly indicating poorly differentiated AMs. Morphological changes are more pronounced in pathogen-activated inflammatory cells in EGR2-deficient lungs, particularly large macrophage-like inflammatory cells with irregular nuclei, compared to control lungs with wild-type AMs. Additionally, prolonged inflammation with greater fibrosis was noted in EGR2-deficient lungs during zymosan or *AF*-induced inflammation. These fibrosis patterns are morphologically similar to "*bronchiolitis obliterans* with organizing *pneumonia*" (BOOP) in humans.

Using a cyclophosphamide-based immunosuppression model, which mimics the immunocompromised state of patients with invasive *Aspergillosis,* we provided further evidence for increased *aspergilloma* formation and enhanced fibrosis in the lungs.

Interestingly, we observed a reduction in the inflammation-resolving lipid mediator RvE1 in the lungs of EGR2-deficient mice, which may relate to reduced LTA4H levels in EGR2-deficient AMs. RvE1 plays roles in promoting macrophage phagocytosis during zymosan-initiated inflammation resolution and offers protective benefits in asthma and allergic airway inflammation.

SUMMARY

Our research demonstrates that EGR2 is a crucial epigenomic and transcriptional modulator of alternative polarization and AM gene expression in steady-state and inflammatory conditions. Through distinct mechanisms such as direct transcriptional regulation and silent bookmarking, EGR2 controls cell-type-specific gene expression and function. Given its specific role in AMs, EGR2 presents a potential target for therapeutic intervention using small molecules or through its upstream regulators to modulate AM function and antifungal response.

As summary, in our findings we demonstrated that (1) EGR2 acts as a late-stage regulator of alternative macrophage polarization in BMDMs, and (2) as part of the AM epigenome activating gene expression likely a DNA-bound transcriptional activator. (3) It serves as a proximal nexus regulator for a subset of terminal stage lineage marker genes and numerous phagocytosis-related genes in AMs and (4) regulates Dectin-1 and Dectin-1-mediated phagocytosis of zymosan and fungi. (5) Some zymosan-induced inflammatory events are modulated by myeloid EGR2 as an epigenomic bookmarker, leading to increased and/or newly induced profibrotic cytokines (e.g., CXCL11) and reduced production of resolving lipid mediators (e.g., RvE1) when EGR2 is deficient. (6) Myeloid specific loss of EGR2 in AMs manifests in impaired inflammation resolution and compromised fungal killing during *Aspergillus fumigatus* infection.

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LIST OF PUBLICATIONS

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