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Title of the project: The role of the transcription factor BACH1 in macrophage function and tissue homeostasis
A project magyar címe: A BACH1 transzkripciós factor szerepe makrofágokban és szöveti homeosztázisban

Background:

Transcriptional master regulators of the MF lineage (like PU.1, Cebpa/b, etc.) are believed to be the main drivers of chromatin reprogramming during differentiation and polarization[(1)]. Such TFs act via their pioneer ability to open chromatin *de novo* and bookmark, protect or safeguard genomic regulatory sites (enhancers and promoters) to later recruit signal-dependent TFs, co-factors, and the basal transcriptional machinery[(2)]. However, recent studies by us and others **challenged this "developmental TF-centric" mode**l. They suggested that signal-dependent TFs, particularly repressors, can also have a pioneer and thus safeguarding role in the MF epigenome at baseline and during acute inflammation[(3-5)]. This observation highlights **a gap in our understanding of inflammation control** and remains unexploited from a therapeutic point of view. Such an expanded model predicts that *active transcriptional repression* (and not just a passive placeholder road-blocking repression) and chromatin bookmarking by signal-dependent master regulators are key mechanisms for safeguarding the enhancer repertoire and proper priming, deployment, and resolution of an inflammatory response. Such active repression mechanisms are mediated via repression 'domains' recruiting co-repressor complexes and interacting with RNA polymerase II basic transcription machinery to inhibit the formation of an active transcription complex at a promoter. *This realization led to our hypothesis* that negative transcriptional regulators (repressors) are developmentally hardwired and in place before MF activation to bookmark genomic regulatory sites. Importantly, they also set basal gene expression levels, shape the kinetics and amplitude of the inflammatory gene expression response – in combination with other known mechanisms down-regulating gene expression during/after inflammation[(6)].

We seeked to address three key questions concerning BACH1's function in macrophages: What are the common and dinstict molecular mechanims on **how BACH1 mediates its repressor function** in specific regulatory sites genome-wide and what is **BACH1's impact on transcriptional activity and chromatin dynamics**? **How BACH1 regulates blood monocyte differentiation** to safeguard the proper deployment of homestatic functions in steady state and during disease?**How BACH1 shapes the cellular specification and heterogeneity of monocyte/macrophage populations *in situ*** during tissue inflammation? To this end, we proposed *three interrelated and integrated but independent Aims* in which we combined new genetic mouse models with cutting edge molecular biology approaches to establish BACH1 as a critical modulator of inflammatory gene expression. **Our approach aimed to gain deep mechanistic insights on BACH1 function** and experimental approaches were designed to answer our outlined questions above and establish **BACH1 as a novel signal-dependent master regulator** and as part of the core hardwired transcriptional circuit of macrophages safeguarding proper inflammation dynamics. Moreover, we think our results are expected to push the field forward by **consolidating the notion that signal-dependent TFs can also have a pioneer role in the macrophage epigenome** at baseline and during acute inflammation.

Results:

1. **TRE/MARE DNA motif binding TFs are the main drivers of the transient inflammatory transcriptional programs *in vivo*.** We have used a murine acute muscle injury model to assess global chromatin accessibility and gene expression dynamics in infiltrating monocytes during tissue regeneration upon injury. More than 9,000 genomic regulatory elements become *de novo* accessible during monocyte infiltration[(7)]. Importantly, motif analysis showed that these sites were highly enriched (>60%) for the TRE (AP-1)/MARE (extended TRE) motif, compared to other common MF-specific motifs (PU.1, C/EBP)[(7)]. These findings support a novel role for signal-dependent TRE/MARE motif-binding TFs as master regulators in early chromatin priming and enhancer

safeguarding and *led to the **unbiased identification of BACH1** as a broad regulator of the inflammatory response in vivo during muscle injury*.

2. **BACH1 as a repressor.** BACH1, a heme-regulated TF, emerged as a candidate likely to regulate the inflammatory transcriptional response of MFs. **BACH1** belongs to the CNC-bZip TF family[8], and it forms heterodimers with small Maf proteins to TRE/MAREs to act as a **transcription repressor[8, 9].** In addition, it also possesses a BTB/POZ domain at its N-terminal end, which is required for protein-protein interactions (**Fig. 2A**)[8]. BACH1 is expressed at low levels in most cell types, but it is upregulated in myeloid cells [8, 10]. It possesses six **heme-binding regions** with dipeptide cysteine-proline (CP) motifs and thus can be directly bound by labile **heme** released during the degradation of many hemoproteins[8]. The **current model** of its mechanism of action is that upon heme binding, BACH1 is destabilized, inhibiting its DNA binding capacity and promoting its nuclear export and degradation via the proteasome. Subsequently, the TF NF-E2/Nrf2 competes for the same binding sites, heterodimerizes with small Mafs, and activates BACH1 target genes. These genes are believed to be restricted to *Hmox1* and a few others to catabolize heme to biliverdin, ferrous iron, and carbon monoxide[11]. Interestingly, and supporting a broader role, BACH1 has been predicted by transcriptomic analysis to be among the core regulator of MF identity and is highly expressed in blood monocytes[10]. BACH1 is also a highly relevant candidate in the context of inflammation during hemolysis or muscle injury. Extensive hemolysis or myolysis results in increased release of labile-heme from hemoglobin and myoglobin[12] that acts as a critical alarmin molecule. This, in turn, signals to MFs to regulate their polarization/specification. As part of this grant, we reported cardiotoxin-induced injury experiments in WT and Bach1 ex2 -/- mice and observed significantly impaired muscle regeneration. Importantly, comparing in WT vs. Bach1 ex2 -/- mice, we observed altered dynamics of the MF phenotype transition from inflammatory ($Ly6C^{high}$) to resolving ($Ly6C^{low}$) and transcriptional deregulation of inflammatory and repair-related genes [7].
3. **BACH1 is part of the hardwired transcriptional program of unstimulated MFs.** We used the gold standard Bone Marrow-Derived Macrophage (BMDM) differentiation system and performed comprehensive ChIP-seq experiments to map BACH1's cistrome in unstimulated MFs. Using a new custom-made, specific, and mass spectrometry validated antibody against the C-terminal of BACH1 protein, we found BACH1 to bind extensively to unstimulated MF chromatin in more than 45,000 genomic sites, to a level often observed for master regulators or developmental lineage determining factors. Interestingly, these sites were predominantly in enhancer regions, both in open and closed chromatin as defined by ATAC-seq peaks **.** In addition, we found an extensive overlap of BACH1 binding with other known core TFs of MFs (PU.1, RUNX1, IRF8, etc.). Importantly, we found that BACH1 binds to all previously defined classes (active, poised, repressed, latent/cryptic, inactive) of distal elements in MFs[13], suggestive of potential novel roles both in active repression but also in the fine-tuning of activation thresholds of poised and active enhancers. Interestingly, we find that BACH1 binds to ~20% of' cryptic/latent enhancers', a new class of regulatory elements, that are completely inactive (no PU.1 binding or active histone marks) in the unstimulated state but get activated upon stimulation[13]. These results strongly suggest that BACH1 acts as multifaceted chromatin bookmarking and enhancer safeguarding TF. This work is being prepared for publication.
4. **BACH1 controls transcriptional responses to inflammatory stimuli.** We profiled the transcriptomic changes (RNA-seq) of BMDMs in WT and Bach1 KO mice (new full-body exon 3-4 KO) in control and upon stimulation with LPS. We found that 641 genes were deregulated in untreated cells while, upon LPS, 758 genes were upregulated in Bach1 KO in any of the three time-points while 1,214 were downregulated. Clustering the affected genes, we observed that the lack of Bach1 affected gene expression by controlling both the baseline and the amplitude of gene induction. The direct regulation of BACH1 stability, DNA binding, and active repression by extracellular and intracellular labile heme levels is intriguing and suggests a role for **heme as a driver signal-metabolite in MF differentiation, activation, and subtype specification programs that are actively BACH1-repressed**. However, the role of labile heme in MF activation has been controversial, with studies that suggest either a pro-inflammatory or anti-inflammatory role[12, 14, 15]. This discrepancy largely stems from different protocols used[14]. We used PolII ChIP-seq of low- or high- heme concentration treated BMDMs to see if they have differential or overlapping transcriptional responses. Both heme treatments induced transcriptional changes in 24,659

regulatory elements collectively. However, our data revealed a priming effect on inflammatory genes at low heme concentration, while the higher concentration induced a specific set of anti-inflammatory genes. Interestingly, 42% of the affected gene bodies (n=1,553) in any of the heme concentrations have a proximal BACH1 peak close to their promoters, suggesting that a significant fraction of heme-elicited pro- and anti-inflammatory transcriptional responses converge on BACH1-bound sites. Since BACH1 has been found to regulate a significant fraction of heme and LPS responses, we asked whether combining heme and LPS could synergistically over-activate known inflammatory genes. We confirmed that heme and LPS have an additive effect on gene expression (e.g., IL6;), while in Bach1 KO, this could be reached by LPS alone. Collectively, these data suggested that interaction of heme with TLR signaling leads to a premature upregulation, sustained or higher activation of pro-inflammatory genes and that BACH1 acts as a negative regulator of enhancer function setting thresholds for activation. This work is alos being prepared for publication.ß

5. **Evidence for active repression by BACH1**. In addition to the conditional knock-out mouse for Bach1, we have now generated an additional myeloid-specific conditional knock-out mouse for Nrf2, while we also have bred both lines together to get a double KO mouse (Bach1 fl/fl; Nrf2 fl/fl; LysM Cre+). As mentioned before, the final step in the classic mechanism for BACH1 repression by heme binding NRF2 replaces BACH1 at target gene enhancers. We tested these lines in gene expression experiments in BMDMs in untreated and in heme-treated conditions. We found constitutive upregulation of several Bach1 target genes in Bach1 KO BMDMs, which seems to be recapitulated to a great extent in the Double KO too, suggesting that even in the absence of is coupled an activator (Nrf2) the release of Bach1 active repression is a key regulatory step for gene activation. These data strongly support our hypothesis that the BACH1 is not a passive placeholder but an active repressor in MFs. Heme-binding to BACH1 has been shown to destabilize it, thus inhibiting its DNA binding capacity, being exported from the nucleus, and degraded via the proteasome. Our ChIP-seq results suggest that BACH1 binding to chromatin is indeed severely decreased upon short heme treatment (100 μM, 1h) at 9,152 sites genome-wide. Importantly, these genomic sites correlated with the strongest BACH1 binding at baseline. *In contrast to dogma in the field that heme would only negatively affect BACH1 binding to chromatin, we detected 8,979 genomic sites (with promoters being the most enriched class of sites) to have increased or de novo BACH1 binding* following heme treatment and, very interestingly, most of these "induced or gained" binding events were near the original BACH1-bound genomic sites that were heme-sensitive. Interestingly, heme stimulation also induced BACH1 binding, covering the gene bodies of 305 genes in a similar enrichment pattern as the elongating RNA-Pol II. These observations strongly suggest that BACH1 binding to chromatin is dynamic and, importantly, suggest that nuclear BACH1 is not immediately exported to the cytoplasm, *but rather it is still intact and part of nuclear chromatin complexes that dynamically bind to target promoters and gene bodies*. Furthermore, as these new sites show increased transcriptional activity, we postulate that this striking fast local DNA binding rearrangement (which has not been reported for any specific DNA motif-binding TF) mirrors the dynamic changes in chromatin architecture consistent with the 'transcription hub/ enhancer looping model' [(25)] that facilitates loading TF complexes from distal enhancers to promoters. Consistent with this, HiChlP experiments using anti-RAD21ab show that heme indeed induces chromatin contacts in BACH1-regulated target loci.
6. **BACH1 actively represses critical genes for MF differentiation.** We found that BACH1 regulates gene sets associated with myeloid cell and MF differentiation. This finding prompts us to test if deletion of Bach1 can affect MF differentiation *in vitro*. Indeed, we observed that Bach1 deficient differentiating MFs were significantly upregulating canonical MF markers (e.g., F4/80) compared to WT. These *in vitro* observations further prompted us to study whether macrophage differentiation is also accelerated *in vivo* in Bach1 KO mice. Thus, we tested the new full-body Bach1 KO mouse line (exon 3-4 KO), and we found that these mice accumulated an F4/80+ CD11b+ cell population in peripheral blood. This cell population resembles phenotypically to recently described circulating "inflammatory hemophagocytes" [(29)] as unstimulated steady-state Bach1 KO BMDMs share a significant portion of their unique transcriptional signature, further suggesting that BACH1 actively represses *in vitro* as well as *in vivo* induced circulating MF transcriptional signature. Among the deregulated genes, we found that Csf1 (encoding M-CSF, the master growth factor for MF

differentiation) and SpiC (a TF and master regulator of red pulp MF subset differentiation) are the most prominent examples as they were significantly upregulated in the Bach1 KO BMDMs (14- and 52-fold up respectively). Both genes were also upregulated to similar levels in Bach1 KO muscle infiltrating monocytes/MFs upon CTX injury, suggesting that they can contribute to phenotypes *in vivo*. We also found that Bach1 KO BMDMs were secreting significantly higher amounts of M-CSF in their supernatant as measured by ELISA. Importantly, the F4/80 upregulation in the Bach1 KO was blunted after incubation with a neutralizing antibody against M-CSF at the last three days (Day 3-6) of differentiation, further suggesting that constitutive expression of M-CSF in Bach1 KO MFs contributes, at least partly, to the accelerated differentiation phenotype even *in vitro* likely through an autocrine and paracrine mode of action. We then asked whether these two highly deregulated genes were direct BACH1 targets, and we found that BACH1 binds strongly to several proximal enhancers in the Spic and Csf1 loci**.** These data strongly support our hypothesis that BACH1 is not a passive placeholder repressor but rather has an **active repression role in critical genes for MF differentiation.**

7. **BACH1 is required during inflammatory response upon injury for timely tissue regeneration.** The CTX induced skeletal muscle injury model is a gold standard system to study inflammation dynamics and tissue environment-dependent monocyte-to-effector MF transition processes[(36)]. It is a synchronous and highly reproducible process allowing the isolation and functional characterization of the cellular components implicated in the regeneration and their dynamics. Upon injury, circulating monocytes enter the damaged tissue and differentiate to MFs with an inflammatory phenotype (Ly6C^{high} cells), and that very fast (within 1-2 days), the same cells shift to a Ly6C^{low} repair MF phenotype[(36-39)]. This phenotypic transition from the monocyte progenitors to inflammatory Ly6C ^{high} and then to repair Ly6C^{low} cells is a highly-regulated and not a passive process, dependent both on MF autonomous[(36)] and non-autonomous (e.g., Treg-dependent regulation)[(40)] mechanisms correlated with the tissue regeneration kinetics and is accompanied by dynamic crosstalk between MFs and other muscle tissue components (e.g., muscle satellite cells, FAPs, T-cells). Our recent study[(7)] has revealed a novel role for BACH1 as the main driver to shape the monocyte enhancer landscape during MF development upon tissue injury. In our new myeloid-specific Bach1-deficient mouse, we have established that the delayed muscle regeneration phenotype and the faster shift from an 'inflammatory (Ly6C^{high})' to a 'resolving (Ly6C^{low})' phenotypic shift is indeed a MF-intrinsic phenotype. Bach1 deletion primes MFs to convert faster to a 'resolving' phenotype. However, when we profiled the transcriptional imprint of this skewed phenotypic shift, we found that while Day 1 Ly6C^{high} inflammatory MFs assayed in bulk have a deregulated pro-inflammatory gene expression pattern, Day 4 Ly6C^{low} MFs still expressed many pro-inflammatory molecules (Il6, Tnf, Il1a, and others) (Fig.7C). This finding suggests that although they have down-regulated the "inflammatory monocyte" marker Ly6C, these cells likely represent a mixed population with contrasting functions (pro- and anti-inflammatory). Further characterization of BACH1-deficient MFs showed that both 'inflammatory (Ly6C^{high})' and 'resolving (Ly6C^{low})' MF populations fail to upregulate MHCII in the Bach1-deficient mice. MHCII up-regulation has been recently described as a functional feature of resolving MFs during the regeneration period, and lack of MHCII^{high} MFs in the injured muscle results in decreased numbers of IFN-γ^{+} leucocytes (NK & T-cells)[(40)]. Together, these data suggest that Bach1 genetic perturbations a) affect the functional diversification of infiltrating monocyte populations upon tissue injury and b) reveal the limitations of current markers to describe functional cell states in dynamic tissue environments.
8. **BACH1 is required for blood monocyte and tissue resident macrophage homeostasis.** To test whether Bach1 deficiency results in any defect in blood monocyte development and fitness, we performed bone marrow chimerism experiments with 50:50, 75:25 and 25:75 ratios of WT vs Bach1 KO donor bone marrows (BM). In the these competition/fitness experiments, we found that in all combinations WT donor BM could outcompete Bach1 KO BM suggesting that Bach1 deficiency is causing a fitness disadvantage during BM development. Furthermore, lung and peritoneal resident macrophage populations were also reduced in Bach1 KO animals further suggesting a role for Bach1 in regulating macrophage homeostasis.

Collectively, these results establish BACH1 as a novel, unsuspected, targetable and multifaceted signal-dependent master regulator and transcriptional repressor as part of the core hardwired transcriptional circuit of MFs safeguarding enhancers and ensuring proper MF differentiation and inflammation dynamics. Furthermore, we showed that the heme-BACH1 regulatory axis contributes to MF specification and inflammatory transcriptional response by acting as a signal-integrator and -regulated transcriptional repressor by safeguarding dynamic inflammatory enhancer activation via epigenomic mechanisms in MFs.

Our studies were significant for several reasons: First, they moved the field by providing a deep mechanistic understanding of the active repression mechanisms, which are poorly understood in MFs. This work establishes BACH1 as a prototypic transcriptional repressor of MF activation and differentiation programs. It provided a broad characterization of its genome-wide repression activity and regulating dynamic 3D chromatin structure and promoter-enhancer interactions. Second, these studies provided a systematic characterization of BACH1's contribution to monocyte/MF differentiation and polarization via its novel target genes Csf1/MCSF and SpiC. Third, it provided a mechanistic framework of how heme, a molecule vital for life and cell metabolism but cytotoxic when in excess, can affect the inflammatory potential and response of MFs. The research was innovative because a) It used new mouse lines (full-body and conditional) to characterize BACH1 function in the transcriptional responses of MFs in vitro and in vivo, and this new genetic toolkit will provide a substantially different vantage point. b) It uncovered the mechanistic aspects of BACH1 repression genome-wide using innovative cellular models and cutting-edge genomic (genome tagging and ChIP-seq), proteomic (ChIP-MS), and imaging approaches. c) It showed how repressor binding to chromatin is re-arranged upon activating signaling and will establish the enhancers and the mechanism of BACH1-mediated direct regulation of Csf1/MCSF and Spic1. d) It showed the surprising phenotype of accumulation of differentiated MFs in the circulation in intact full-body Bach1 KO animals. e) It further confirmed the contribution of BACH1 to the cellular heterogeneity of infiltrating cells and their interactions upon tissue injury. We anticipate to publish three major manuscript of these studies in the next year.

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