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POSTER ABSTRACTS

201.GRANULOCYTES, MONOCYTES, AND MACROPHAGES

Peptidyl Arginine Deiminase (PAD) 4 Modulates the GI Microbiome and Baseline Inflammation in Mice: Implications for Sepsis

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Introduction: As part of the innate immune response, neutrophils release neutrophil extracellular traps (NETs), webs of decondensed chromatin coated with histones that capture bacteria but cause collateral tissue damage when produced in a dysregulated manner. Deficiency of PAD4, a protein that converts histone arginine to citrulline to promote chromatin decondensation essential to NET release (NETosis), has been associated with improved outcomes in murine sepsis models, leading some to propose that inhibiting NETosis may be protective. This conclusion has primarily been drawn from studies using the murine cecal-ligation and puncture (CLP) model of polymicrobial sepsis, in which the cecum is perforated to disseminate GI microbiota. However, NET release is an evolutionarily conserved function, present in protozoa and retained in all studied animal species, suggesting that it plays a crucial role in the immune response. In this study, we seek to characterize the baseline immunophenotype of NET-deficient, PAD4 knockout (PAD4 $^{-/-}$) mice, to determine how PAD4 influences the GI microbiome to impact outcomes in GI models of polymicrobial sepsis.

Methods: Whole blood samples were collected from wild type (WT) C57BL/6 mice, PAD4 $^{-/-}$ mice generated by our group through deletion of exon II in the PAD4 gene (PAD4D $^{\Delta II}$), and PAD4 $^{-/-}$ mice with deletion of exon IX and X (PAD4D $^{\Delta IX+X}$) obtained from Jackson laboratory. Complete blood counts (CBCs) and weights were obtained from PAD4 $^{-/-}$ and healthy WT mice at 9-12 weeks. The Olink target 96 mouse exploratory panel was used to quantify variation in levels of 96 plasma proteins. Next generation sequencing was used to assess the abundance of different bacterial species in stool samples from WT, PAD4D $^{\Delta II}$, and PAD4D $^{\Delta IX+X}$ animals. Polymicrobial sepsis was then induced through peritoneal injections of cecal slurry (CS), using cecal samples from WT, PAD4D $^{\Delta II}$, and PAD4D $^{\Delta IX+X}$ donor mice. 24 hours post injection, mean sepsis score (MSS) was assessed, and blood was obtained for CBC measurement, plasma proteomic analysis, and quantification of bacterial colony forming units (CFUs). Relative abundance of bacterial species was quantified in blood and liver homogenates. Survival outcome was performed on parallel subsets of WT and PAD4 $^{-/-}$ mice.

Results: At comparable ages, both strains of PAD4 $^{-/-}$ animals had lower weights and significantly higher baseline neutrophil counts than WT controls bred in the same colony. Proteomic analysis revealed that PAD4D $^{\Delta II}$ mice had baseline elevation in the inflammatory markers IL6 and Plin1, while PAD4D $^{\Delta IX+X}$ mice, the strain with more pronounced neutrophilia, had higher GM-CSF and decreased IL23r, proteins crucial to neutrophil mobilization. In CS sepsis studies, WT animals that received WT CS were sicker with higher MSS, lower platelet counts, higher inflammatory markers, and a 30% mortality rate. In contrast, PAD4D $^{\Delta II}$ mice that received PAD4D $^{\Delta II}$ CS had a milder clinical course, lower bacterial dissemination, and 100% survival (Fig2). To assess the basis for this unexpected outcome, we compared bacterial colony-forming units (CFU) in WT to PAD4 $^{-/-}$ CS and found lower levels of bacteria in PAD4D $^{\Delta II}$ CS. Deep sequencing of stool from WT, PAD4D $^{\Delta II}$, and PAD4D $^{\Delta IX+X}$ animals revealed marked differences in the relative abundance of bacterial species. Sequencing of blood and liver samples obtained 24 hrs after CS injection showed that the predominant proliferating organism in WT mice was *Enterococcaceae* species while there was an abundance of *Lactobacillaceae* species in the blood and liver of PAD4D $^{\Delta II}$ mice (Fig1). Sepsis studies repeated with genotype switched CS showed that WT mice treated with PAD4D $^{\Delta IX+X}$ CS had a milder disease course with 100% survival, whereas PAD4D $^{\Delta IX+X}$ mice injected with WT CS had elevated MSS, a trend towards higher plasma inflammatory markers, and a 50% mortality rate, similar to that observed in WT mice injected with WT CS (Fig2).

Conclusions: These studies show that NETosis influences baseline inflammation and modulates the GI microbiome in mice. Differences in disease severity and survival between PAD4 $^{-/-}$ and WT mice observed in the CS model of polymicrobial sepsis is

primarily due to greater pathogenicity of the WT CS inoculum rather than collateral tissue damage secondary to dysregulated NETosis. The basis for differences in inflammation and microbiome composition in PAD4 $^{-/-}$ mice needs to be examined.

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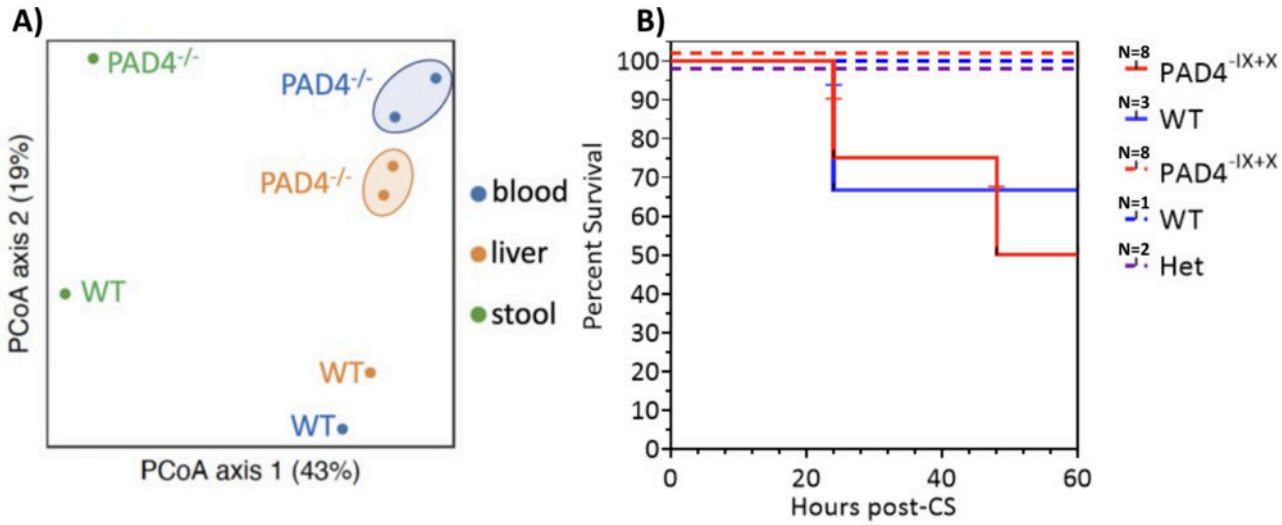

Figure 1. PAD4 expression influences gut microbiota in mice. WT and PAD4$^{-/-}$ mice were injected with 100 µg/g cecal slurry (CS) from genotype-matched donors. Unweighted UniFrac distance was used to compare bacterial species composition in blood and liver 24H post treatment, as well as in the injected stool. Results are displayed using Principle Component Analysis with percent of total variance captured displayed on each axis. Next generation sequencing showed a marked difference in the relative abundance of bacterial species in CS from WT and PAD4 deficient animals (green). 24 hours following intraperitoneal injection of CS, blood and livers were collected and also subjected to microbial sequencing revealing differences in bacterial species proliferation in the blood (blue) and livers (orange) of WT vs. PAD4$^{-/-}$ mice.

Figure 2. Decreased mortality in recipients of cecal slurry from PAD4 deficient mice. WT, PAD4$^{\Delta IX+X}$/WT heterozygous (Het), and PAD4$^{\Delta IX+X}$ mice were injected with CS obtained from PAD4$^{\Delta IX+X}$/(dotted line) or WT (solid line) donor mice. Deaths were recorded over a 60-hour time span. All WT, Het, and PAD4$^{\Delta IX+X}$ mice that received CS from PAD4$^{\Delta IX+X}$ donors survived. 50% of PAD4$^{\Delta IX+X}$ mice that received WT stool survived (red line), significantly less than PAD4$^{\Delta IX+X}$ CS receiving counterparts (Mantel Cox, p=0.0254). The mortality rate for WT mice that received WT stool was similar at 30% (blue line).

Figure 1

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