Re-shaping the thinking of IBD: Investigating rare genetic variations in the pro- and anti-inflammatory balance between innate immunity, intestinal epithelia and gut microbiota

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Abstract

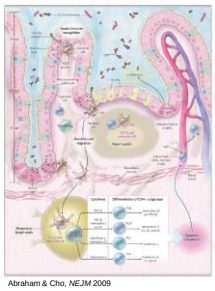
Crohn's disease (CD) has the highest prevalence in Ashkenazi Jewish (AJ) populations. We sought to identify rare, CD-associated frameshift variants of high functional and statistical effects. Exome-sequencing and array-based genotyping was performed in 1477 AJ CD cases and 2614 AJ controls. Replication genotyping of a CSF2RB (colony-stimulating factor 2, receptor beta) frameshift was performed in 1515 CD cases and 6980 controls. Intestinal CSF2RB gene expression and GM-CSF responsive cells were defined by CyTOF. Wild-type and mutant CSF2RB function was compared in cellular transfection and primary monocyte studies. We observed evidence for CD association for the CSF2RB frameshift (*P* value 8.52x10-4) in the discovery cohort, which was significantly replicated (combined P value 3.42x10-6). In the intestine, robust GM-CSF induction of STAT5 phosphorylation is observed, with lesser induction of pERK and pAKT. Co-transfection of wild-type and mutant CSF2RB displayed decreased pSTAT5 with GM-CSF stimulation, consistent with a dominant negative effect. Monocytes from heterozygous frameshift carriers, present in 6% of AJ CD cases, demonstrated diminished GM-CSF responses, with markedly decreased aldehyde dehydrogenase enzyme activity associated with immune tolerance. Our findings define a primary role of diminished GM-CSF signaling and impaired innate immunity in CD pathogenesis. We are currently working on expanding our CSF2RB finding to other immune modulators such as PGE2, IL-3, and IL-5, that demonstrate the similar genetic architecture.

Introduction

A major focus of our research is understanding the prevalence of IBD in the Ashkenazi Jewish population. We are working on further identifying rare disease associated variants in Ashkenazi Jewish population by Exome Chip technology as well as the biological function of those identified genes. Dr. Cho's research has contributed to defining the pathophysiologic mechanisms of IBD by identifying associations to NOD2, IL23R, and 163 loci to IBD. With new findings our research is evolving to now looking at the function of lipid mediators and their related cytokines in innate immune cells, and the full transcriptome of enteroids, the intestinal epithelial stem cells.

Furthermore, we investigate the relationship between environmental factors and IBD by examining how hosts (humans) interact with gut microbes and how the interaction may lead to IBD susceptibility and/or maintain IBD pathogenesis. By dissecting the relationship between IBD genetics, immune response, and microbes, we would have a better chance to develop treatment for blocking pro-inflammatory proteins, inflammatory pathways, or immune cell entry into intestine. The integration of our research with the clinical research for IBD has great potential for the future of IBD treatment.

IBD Genetics



Abraham & Cho, *NEJM* 2009

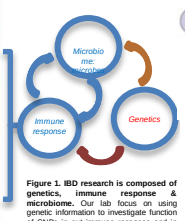


Figure 1. IBD research is composed of genetics, immune response & microbiome. Our lab focus on using genetic information to investigate function of SNPs in gut immune response and in cross-talk between epithelia and gut microbes.

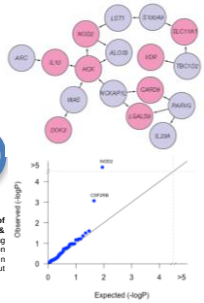


Figure 2. NOD2-focused cluster of the IBD causal sub-network. Pink genes are in IBD associated loci, blue are not. Arrows indicate inferred causal direction of regulation of expression. Jostins *et. al., Nature* 1012

Figure 3. Frameshift mutation in *CSF2RB* associated to CD. Quantile-quantile plot of 88 frameshift mutations in the CD case-control discovery cohort (1477 cases, 2614 controls).

Functional studies- human ileal resection with CyTOF analysis

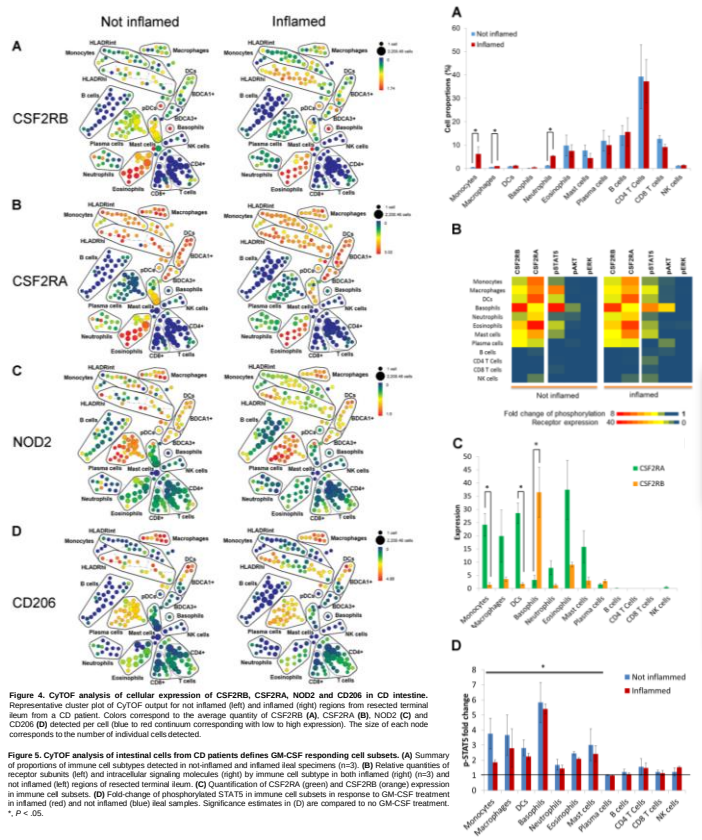


Figure 4. CyTOF analysis of cellular expression of CSF2RB, CSF2RA, NOD2 and CD206 in CD intestine. Representative cluster plot of CyTOF output for not inflamed (left) and inflamed (right) regions from resected terminal ileum from a CD patient. Colors correspond to the average quantity of CSF2RB **(A)**, CSF2RA **(B)**, NOD2 **(C)** and CD206 **(D)** detected per cell (blue to red continuum corresponding with low to high expression). The size of each node corresponds to the number of individual cells detected.

Figure 5. CyTOF analysis of intestinal cells from CD patients defines GM-CSF responding cell subsets. (A) Summary of proportions of immune cell subtypes detected in not-inflamed and inflamed ileal specimens (n=3). **(B)** Relative quantities of receptor subunits (left) and intracellular signaling molecules (right) by immune cell subtype in both inflamed (right) (n=3) and not inflamed (left) regions of resected terminal ileum. **(C)** Quantification of CSF2RA (green) and CSF2RB (orange) expression in immune cell subsets. **(D)** Fold-change of phosphorylated STAT5 in immune cell subsets in response to GM-CSF treatment in inflamed (red) and not inflamed (blue) ileal samples. Significance estimates in (D) are compared to no GM-CSF treatment. *, *P* < .05.

Functional studies- generating mutant cell line & recalling mutation carriers for peripheral blood monocytes signaling analysis

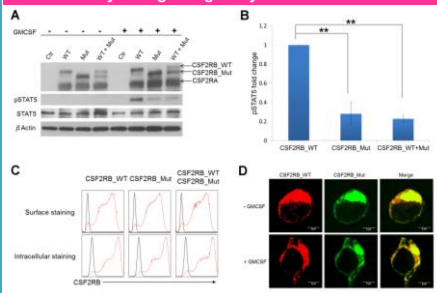


Figure 6. The CD-associated frameshift in *CSF2RB* results in a truncated protein and attenuates GM-CSF-induced STAT5 phosphorylation in a dominant negative manner. (A) HEK293 cells were transfected with CSF2RA + wild-type (WT) and/or frameshift mutation (Mut) CSF2RB, alone or in combination. Cells were then treated with 10ng/ml GM-CSF for 15min. Representative western blot displaying products of transfection and phospho-STAT5. STAT5 and b-actin are shown as loading controls. **(B)** Quantitative levels of pSTAT5 relative to wild-type control (CSF2RB_WT) from three replicated experiments. **, *P* < .01. **(C)** Transfected HEK293 cells were stained with isotype antibodies (black line) or anti-CSF2RB antibodies (red line) on their surface (top row) and intracellularly (bottom row). Representative histograms are shown. **(D)** Confocal fluorescent micrograph illustrating intracellular localization of wild-type (left), mutant (middle) construct products and merge (right) in cells co-transfected with both constructs with 10ng/ml GM-CSF treatment for 15min. Yellow areas indicate the co-localization of CSF2RB_WT and CSF2RB_Mut receptors. Scale bar: 5μm.

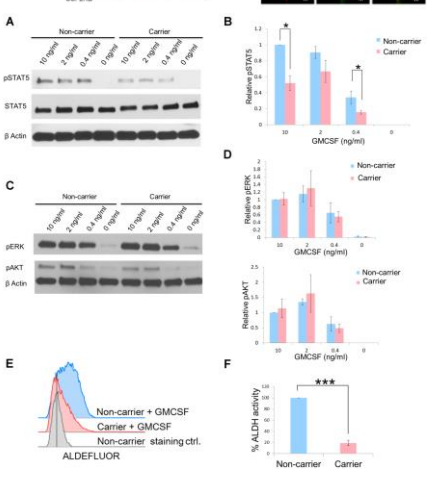


Figure 8. Primary monocytes from *CSF2RB* frameshift carriers demonstrate diminished GM-CSF mediated pSTAT5 and aldehyde dehydrogenase activity with intact ERK and AKT activation. (A) Representative immunoblot of peripheral monocytes from a *CSF2RB* frameshift carrier and matched control displaying pSTAT5 and total STAT5 15 min after stimulation with various concentrations of GM-CSF. **(B)** Quantification of pSTAT5 levels after GM-CSF stimulation in three phenotypically matched pairs with normalizing to loading control. *, *P* < .05. **(C)** Immunoblot of pERK and pAKT in a frameshift carrier and a matched non-carrier after GM-CSF stimulation. **(D)** Quantification of pERK and pAKT in 3 matched pairs of frameshift carriers and non-carriers. **(E)** Activity of aldehyde dehydrogenase (ALDH), an enzymatic catalyst involved in retinoic acid synthesis, as measured by flow cytometry detecting ALDEFLUOR after 10ng/ml of GM-CSF stimulation for 15 minutes, in a *CSF2RB* frameshift non-carrier (top) and a matched carrier (middle). **(F)** Quantification of ALDH activity by mean ALDEFLUOR intensity in 3 pairs of frameshift carriers and non-carriers. ***, *P* < 0.001.

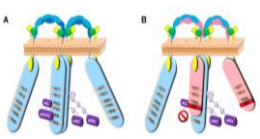


Figure 7. Schematic model of altered GM-CSF signaling with the *CSF2RB* frameshift mutation. GM-CSF signaling includes GM-CSF cytokine (green), CSF2RA (yellow) and CSF2RB (wild-type in blue, mutant in pink) components. With GM-CSF stimulation of wild-type CSF2RB (A, blue), potent induction of pSTAT5, pAKT, and pERK is observed. However, with heterozygous *CSF2RB* frameshift carriage (wild-type in blue, mutant in pink CSF2RB with the frameshift region in red) pAKT and pERK signaling is largely intact, reflecting interaction with more proximal tyrosine residues within the CSF2RB tail.

Functional studies- zebrafish DSS colitis model

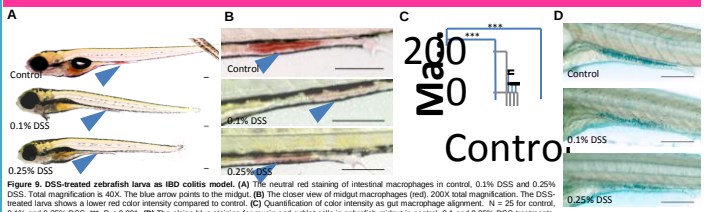
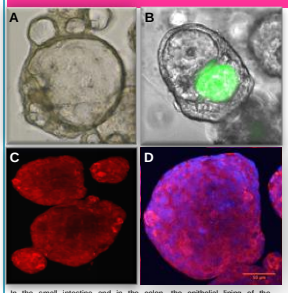


Figure 9. DSS-treated zebrafish larva as IBD colitis model. (A) The neutral red staining of intestinal macrophages in control, 0.1% DSS and 0.25% DSS. Total magnification is 40X. The blue arrow points to the midgut. **(B)** The closer view of midgut macrophages (red). 200X total magnification. The DSS-treated larva shows a lower red color intensity compared to control. **(C)** Quantification of color intensity as gut macrophage alignment. N = 25 for control, 0.1% and 0.25% DSS. ***, P < 0.001. **(D)** The alcian blue staining for mucin and goblet cells in zebrafish midgut in control, 0.1 and 0.25% DSS treatments. Total magnification is 200X. Bar 100 μm.

Functional studies- human enteroids (colonic & intestinal stem cells)



In the small intestine and in the colon, the epithelial lining of the gastrointestinal tract is in constant renewal. This process is triggered by proliferation of intestinal stem cells (ISCs) that continuously produce progeny to rapidly replace the surface intestinal lining during epithelial turnover. Commonly termed enteroids and colonoids, these ISCs are derived from the small and large intestines respectively. Both these enteroids and colonoids continuously differentiate into all cell types found normally within the intestinal epithelium. This *in vitro* organ-like culture system provides a novel platform for studying the interactions between immune response, microbiome and genetics in IBD.

Figure 10. Human enteroid biobank from intestinal biopsy and tissue resection. (A) The bright-field image of enteroid spheres. **(B)** Staining ROS (green) in live enteroid culture. **(C)** Staining of ISC marker LGR5 (red) in PFA fixed spheres. **(D)** The merged image of LGR5 (red) and nucleus (DAPI; blue) shows the heterogeneity of cells in spheres.

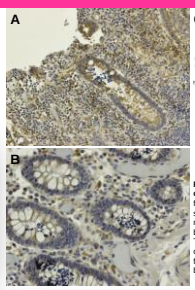


Figure 11. Investigating the interaction between crypts and microbes. (A) Cross section of ileal resection from IBD patient. The nucleus staining (blue) shows the infiltration of mononuclear cells such as neutrophils, monocytes and macrophages. Bacteria could be found within the damaged crypts of the IBD patient. **(B)** The closer view of the bacteria (dark blue) inside the crypts. **(C)** Identifying and profiling the "crypt bacteria" from IBD resection sample by next generation sequencing-based 16s ribosomal RNA.

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