The gene encoding the interleukin-1 receptor antagonist is differentially expressed in the blood and regulatory T-cells of patients with Crohn's disease and transcriptionally induced in peripheral blood mononuclear cells upon stimulation with muramyl dipeptide.

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Crohn's disease, an inflammatory bowel disease (1), is a significant cause of morbidity and economic burden in the United States (2, 3, 4). We performed differential gene expression analyses and identified the interleukin-1 (IL-1) receptor antagonist, IL1RN (5, 6), as among the genes most differentially expressed in the blood and regulatory T-cells of patients with Crohn's disease using published (7) and public datasets (8). Further analysis of published microarray data (9) revealed that IL1RN was transcriptionally induced upon stimulation of peripheral blood mononuclear cells (PBMC) with muramyl dipeptide, the ligand for the gene product of the Crohn's disease susceptibility locus NOD2. Interestingly, IL-1RN was expressed at significantly lower quantities in the T_{reg} of patients with Crohn's disease than in the T_{reg} of non-affected control subjects. This is the first report of differential expression of IL1RN in patients with Crohn's disease.

Keywords: IL-1 receptor antagonist, IL-1RN, Crohn's disease, systems biology of Crohn's disease.

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2 Crohn's disease is a malady of the developed world (10) and despite this fact is
3 significantly shaped by genetic background (11, 12). Variants in TRIM22 have been identified
4 in Canadian families with severe and very-early onset inflammatory bowel diseases by whole
5 exam sequencing (13), and NOD2 has been identified as the strongest genetic risk factors for
6 the development of Crohn's Disease by genome-wide association (GWAS) (14, 15). Although
7 Crohn's disease is a model for the identification of genetic risk factors by GWAS, there has
8 been little clinical translation from the identification of these risk factors, nor has their been
9 clinical translation of findings from WES in CD. Other systems-level methods of understanding
10 the fundamental biology of Crohn's disease are necessary for basic and translational advances
11 towards these ends.

12
13 Though the gut tissues are primarily affected in IBD, anemia is the most common extra
14 intestinal manifestation of CD patients (16, 17) and allogeneic bone marrow transplantation is
15 effective for CD (18), demonstrating that the hematopoietic compartment is important for the
16 disease process. In this study, we performed transcriptome-level differential gene expression
17 analysis using published and public datasets (7-9) to discover the most significant gene
18 expression differences in the hematopoietic tissues of patients with Crohn's disease. We
19 identified the interleukin-1 receptor antagonist as among the most differentially expressed
20 genes in the blood of patients with Crohn's disease. IL1RN was differentially expressed in the
21 regulatory T-cells of patients with Crohn's disease, and expressed at significantly lower levels
22 than in the regulatory T-cells of non-affected control subjects. The biology of the IL-1 receptor
23 antagonist may be relevant to the pathogenesis of Crohn's disease.

24 25 26 **Methods**

27 We used GSE94648 (7), GSE23996 (8), and GSE68765 (9) for this analysis in
28 conjunction with GEO2R. GSE94648 (7) was generated using Affymetrix Human Genome U133
Plus 2.0 Array technology with n=22 whole blood from controls and n=50 whole blood from
PAGE 2 OF 12

1 patients with Crohn's Disease. GSE23996 (8) was generated using the HCI Human TReg Chip
2 with n=6 CD patients (3 inactive CD patients and 3 active CD patients) and n=29 healthy
3 controls (11 patients; 29 chips). GSE68765 (9) was generated using Illumina HumanHT-12
4 V4.0 expression beadchip technology with n=6 untreated PBMC and n=5 PBMC stimulated
5 with MDP.

6 The Benjamin and Hochberg method of p-value adjustment was used for ranking of
7 differential expression but raw p-values were used for assessment of statistical significance of
8 global differential expression. Log-transformation of data was auto-detected, and the NCBI
9 generated category of platform annotation was used.
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11 A statistical test was performed to evaluate the significance of difference between
12 mRNA expression levels of IL1RN in the regulatory T-cells of patients with Crohn's disease as
13 compared to healthy controls, and between PBMC in the resting states as compared to PBMC
14 treated with muramyl dipeptide using a two-tailed, unpaired t-test with Welch's correction.
15 Only p-values less than 0.05 were considered statistically significant.
16

17 **Results**

18 We mined three separate datasets (5, 6, 7) to assess the most significant differences in
19 the hematopoietic tissues of patients with Crohn's disease. First, we compared the whole
20 transcriptomes of blood from patients with CD and blood from non-affected control subjects.
21 Second, we compared a more limited microarray dataset restricted to genes expressed in the
22 regulatory T-cell, comparing the transcriptomes of CD4+ CD25+ regulatory T-cells from
23 patients with Crohn's disease to regulatory T-cells from non-affected control subjects. Finally,
24 we compared whole transcriptomes of peripheral blood mononuclear cells (PBMC), in the
25 resting state and after stimulation with muramyl dipeptide, the ligand for the gene product for
26 the CD susceptibility gene, NOD2.
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In the whole blood of patients with Crohn's disease, we identified the IL-1 receptor
antagonist as differentially expressed. The expression of IL1RN changed to a greater degree

1 than 73.8% of the rest of the transcriptome. Differential expression of IL1RN in the blood of
2 patients with Crohn's disease was statistically significant (Table 1; $p=4.79E-02$). When ranking
3 all of the genes expressed in whole blood from patients with CD based on change relative to
4 whole blood from control, non-affected subjects, IL1RN ranked 5153 out of 19674 genes.

5 We further identified IL1RN as differentially expressed specifically in the CD4+ CD25+
6 regulatory T-cells of patients with Crohn's disease. When ranking all of the genes expressed in
7 Treg from patients with CD based on change relative to Treg from control, non-affected
8 subjects, IL1RN was ranked 211 out of 397; this was statistically significant (Table 1;
9 $p=3.48E-02$).

10 We further identified IL1RN as transcriptionally induced and differentially expressed in
11 peripheral blood mononuclear cells treated with muramyl dipeptide (MDP). Muramyl dipeptide
12 is the ligand or agonist for the NOD2 pattern recognition receptor, variants in which are the
13 single greatest genetic risk factors for the development of Crohn's disease. The expression of
14 IL1RN, after treatment with MDP changed to a greater degree than 99.5% of the rest of the
15 transcriptome. Differential expression of IL1RN in the PBMC treated with MDP was statistically
16 significant (Table 1; $p=7.72E-05$). When ranking all of the genes expressed in PBMC after
17 stimulation with MDP based on change in expression with and without MDP stimulation, IL1RN
18 ranked 223 out of 47323 genes.

19 Finally, we extracted exact mRNA expression values for IL1RN, from both Treg from
20 patients with Crohn's disease, and from normal PBMC, and compared them to Treg from non-
21 affected control subjects, and to PBMC stimulated with MDP, respectively, in order to compare
22 expression levels of IL1RN between disease and non-diseased groups, or between PBCMs in
23 resting state or after treatment with MDP. We also performed a statistical test to determine if
24 the difference in mRNA expression between groups was statistically significant.

25 The expression of IL1RN was lower in Treg from CD patients than in Treg from non-
26 affected control subjects. This difference was statistically significant (Figure 1; $p=0.0015$).
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1 IL1RN, in PBMC, was transcriptionally induced after stimulation with MDP. The
2 expression of IL1RN in PBMC treated with MDP was significantly higher than in PBMC in
3 resting state (Figure 1; $p=0.0040$).

4 Thus, we found that IL1RN was differentially expressed in the blood and regulatory T-
5 cells of patients with Crohn's disease as compared to control non-affected subjects, and
6 expressed at significantly lower levels in regulatory T-cells from patients with Crohn's disease
7 as compared to Treg from control non-affected subjects. We also found that IL1RN was
8 transcriptionally induced after exposure of normal PBMC with muramyl dipeptide, the ligand for
9 the gene product of the CD susceptibility gene NOD2.
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11 Discussion

12 We found here using whole transcriptome differential gene expression analysis that the
13 IL-1 receptor antagonist IL1RN was differentially expressed in the whole blood of patients with
14 Crohn's disease. Using a microarray dataset measuring a more limited number of genes
15 specifically expressed in the regulatory T-cell, we found that IL1RN was differentially expressed
16 in the CD4⁺ CD25⁺ regulatory T-cells. Moreover, we found that IL1RN was transcriptionally
17 induced and differentially expressed following stimulation of peripheral blood mononuclear
18 cells with muramyl dipeptide.
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20 Mice deficient in IL1rn develop spontaneous autoimmune arthritis (6). Interestingly,
21 BALB/C IL1rn ^{-/-} mice but not C57BL/6 IL1rn ^{-/-} mice develop spontaneous autoimmune
22 arthritis, suggesting that other genetic factors collaborate with IL-1 receptor antagonist
23 deficiency to create this inflammatory arthritis (6). These mice produce large amounts of
24 IL-1beta, a pro-inflammatory cytokine. Interestingly, the arthritic phenotype in these mice is
25 dependent on toll-like receptor (TLR) activation by microbial flora; germ-free mice do not
26 develop joint damage (19). This points to a collaboration between the IL-1R antagonist,
27 microbial flora, TLR2 and TLR4 (19), and most likely other genetic factors that conspire to
28 cause systematic inflammation that leads to arthritis. Inflammatory damage in IL1RN deficient

1 mice is not restricted to arthritis; the cardiovascular system of IL1rn -/- mice is also affected as
2 they develop arterial inflammation (20).

3 Humans genetically deficient in IL1RN develop a disorder similar to the neonatal multi-
4 system inflammatory disorder NOMID (5), caused by mutations in NLRP3 (cryopyrin), the
5 canonical inflammasome component that leads to activation of caspase-1 and subsequent
6 maturation of pro-IL1 β to the active form of IL1 β . Of note, common variants in a regulatory
7 region downstream of the NLRP3 locus have also been reported to be associated with the risk
8 of development of Crohn's disease (21).

10 We found here that the receptor antagonist for interleukin-1, IL1RN, was differentially
11 expressed in the blood and in the regulatory T-cells of patients with Crohn's disease.
12 Moreover, we identified IL1RN as a transcriptional target of muramyl dipeptide in peripheral
13 blood mononuclear cells, as stimulation of normal PBMC with MDP lead to significant
14 transcriptional induction of IL1RN. MDP is the ligand for the CD susceptibility gene, NOD2.
15 Further study should be directed towards understanding the potential significance of IL1RN in
16 the pathogenesis of Crohn's disease.
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References

1. Baumgart, D.C. and Sandborn, W.J., 2012. Crohn's disease. *The Lancet*, 380(9853), pp.1590-1605.
2. Kappelman, M.D., Rifas-Shiman, S.L., Kleinman, K., Ollendorf, D., Bousvaros, A., Grand, R.J. and Finkelstein, J.A., 2007. The prevalence and geographic distribution of Crohn's disease and ulcerative colitis in the United States. *Clinical Gastroenterology and Hepatology*, 5(12), pp.1424-1429.
3. Malarcher, C.A., Wheaton, A.G., Liu, Y., Greenlund, S.F., Greenlund, S.J., Lu, H. and Croft, J.B., 2017. Hospitalizations for Crohn's disease—United States, 2003–2013. *MMWR. Morbidity and mortality weekly report*, 66(14), p.377.
4. Kappelman, M.D., Rifas-Shiman, S.L., Porter, C.Q., Ollendorf, D.A., Sandler, R.S., Galanko, J.A. and Finkelstein, J.A., 2008. Direct health care costs of Crohn's disease and ulcerative colitis in US children and adults. *Gastroenterology*, 135(6), pp.1907-1913.
5. Reddy, S., Jia, S., Geoffrey, R., Lorier, R., Suchi, M., Broeckel, U., Hessner, M.J. and Verbsky, J., 2009. An autoinflammatory disease due to homozygous deletion of the IL1RN locus. *New England Journal of Medicine*, 360(23), pp. 2438-2444.
6. Horai, R., Saijo, S., Tanioka, H., Nakae, S., Sudo, K., Okahara, A., Ikuse, T., Asano, M. and Iwakura, Y., 2000. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. *The Journal of experimental medicine*, 191(2), pp. 313-320.
7. Planell, Núria, M. Carme Masamunt, Raquel Franco Leal, Lorena Rodríguez, Miriam Esteller, Juan J. Lozano, Anna Ramírez et al. "Usefulness of transcriptional blood biomarkers as a non-invasive surrogate marker of mucosal healing and endoscopic response in ulcerative colitis." *Journal of Crohn's and Colitis* 11, no. 11 (2017): 1335-1346.
8. GSE23996. Geffers R, Pfoertner S, Maul J. Robert Geffers, HCI - Helmholtz Centre for Infection Research. Department of Molecular Bacteriology. Genome Analytics Lab.
9. Begun, J., Lassen, K.G., Jijon, H.B., Baxt, L.A., Goel, G., Heath, R.J., Ng, A., Tam, J.M., Kuo, S.Y., Villablanca, E.J. and Fagbami, L., 2015. Integrated

- genomics of Crohn's disease risk variant identifies a role for CLEC12A in antibacterial autophagy. *Cell reports*, 11(12), pp.1905-1918.
10. Economou, M. and Pappas, G., 2008. New global map of Crohn's disease: Genetic, environmental, and socioeconomic correlations. *Inflammatory bowel diseases*, 14(5), pp.709-720
11. Cho, J.H., 2008. The genetics and immunopathogenesis of inflammatory bowel disease. *Nature Reviews Immunology*, 8(6), pp.458-466.
12. Khor, B., Gardet, A. and Xavier, R.J., 2011. Genetics and pathogenesis of inflammatory bowel disease. *Nature*, 474(7351), pp.307-317.
13. Li, Q., Lee, C.H., Peters, L.A., Mastropaolo, L.A., Thoeni, C., Elkadri, A., Schwerd, T., Zhu, J., Zhang, B., Zhao, Y. and Hao, K., 2016. Variants in TRIM22 that affect NOD2 signaling are associated with very-early-onset inflammatory bowel disease. *Gastroenterology*, 150(5), pp.1196-1207.
14. Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H. and Achkar, J.P., 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*, 411(6837), pp.603-606.
15. Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cézard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M. and Binder, V., 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*, 411(6837), pp.599-603.
16. Kaitha, S., Bashir, M. and Ali, T., 2015. Iron deficiency anemia in inflammatory bowel disease. *World journal of gastrointestinal pathophysiology*, 6(3), p.62.
17. Hindryckx, P., Amininejad, L., Van, E.D.V. and Bossuyt, P., 2014. Belgian recommendations for the management of anemia in patients with inflammatory bowel disease. *Acta gastro-enterologica Belgica*, 77(3), pp. 333-344.
18. Lopez-Cubero, S.O., Sullivan, K.M. and McDonald, G.B., 1998. Course of Crohn's disease after allogeneic marrow transplantation. *Gastroenterology*, 114(3), pp.433-440.
19. Abdollahi-Roodsaz, S., Joosten, L.A., Koenders, M.I., Devesa, I., Roelofs, M.F., Radstake, T.R., Heuvelmans-Jacobs, M., Akira, S., Nicklin, M.J.,

1 Ribeiro-Dias, F. and Van den Berg, W.B., 2008. Stimulation of TLR2 and
2 TLR4 differentially skews the balance of T cells in a mouse model of
3 arthritis. *The Journal of clinical investigation*, 118(1), pp.205-216.

4 20. Nicklin MJ, Hughes DE, Barton JL, Ure JM, Duff GW. Arterial inflammation in
5 mice lacking the interleukin 1 receptor antagonist gene. *The Journal of*
6 *experimental medicine*. 2000 Jan 17;191(2):303-12.

7 21. Villani, A.C., Lemire, M., Fortin, G., Louis, E., Silverberg, M.S., Collette, C.,
8 Baba, N., Libioulle, C., Belaiche, J., Bitton, A. and Gaudet, D., 2009.
9 Common variants in the NLRP3 region contribute to Crohn's disease
susceptibility. *Nature genetics*, 41(1), p.71.

Rank	ID	p-value	t	B	gene	gene name
GSE23996						
211	6050654	3.48E-02	-2.1957	-4.3576	IL1RN	interleukin 1 receptor antagonist
GSE68765	ID					
223	ILMN_1774874	7.72E-05	5.8523529	1.89615	IL1RN	interleukin 1 receptor antagonist
GSE94648	ID					
5153	3557_at	4.79E-02	2.0124382	-4.19362	IL1RN	interleukin 1 receptor antagonist

Table 1: The IL1 receptor antagonist (IL1RN) is differentially expressed in the blood and regulatory T-cells (GSE23996) of patients with Crohn's Disease, and transcriptionally induced in peripheral blood mononuclear cells by muramyl dipeptide.

The rank of differential expression by dataset, the probe or transcript ID, the p-value of differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, the gene and gene name are listed on this chart.

Datasets analyzed:

- 1) GSE23996: Regulatory T-cells (CD4+ CD25+) from patients with Crohn's Disease and non-affected control subjects
- 2) GSE68765: Peripheral blood mononuclear cells in the resting state, and with muramyl dipeptide stimulation.
- 3) GSE94648: Whole blood from patients with Crohn's Disease and non-affected control subjects.

Figure 1: IL1RN is expressed at significantly lower levels in the regulatory T-cells of patients with Crohn's Disease than in the regulatory T-cells of control, non-affected subjects.

The mRNA expression value of IL1RN is represented individually by patient with the mean value marked (left) and as a violin plot with the median value marked. The result of a statistical test evaluating the significance of difference between the groups compared, the p-value, is listed on each graph.

Figure 2: IL1RN is transcriptionally induced in peripheral blood mononuclear cells upon stimulation with muramyl dipeptide.

The mRNA expression value of IL1RN in peripheral blood mononuclear cells, in the resting state and with muramyl dipeptide (MDP) stimulation, is represented individually with the mean value marked (left) and as a violin plot with the median value marked (right). The result of a statistical test evaluating the significance of difference between the groups compared, the p-value, is listed on each graph.