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Vitamin D downregulates the IL-23 receptor pathway in human mucosal ILC3

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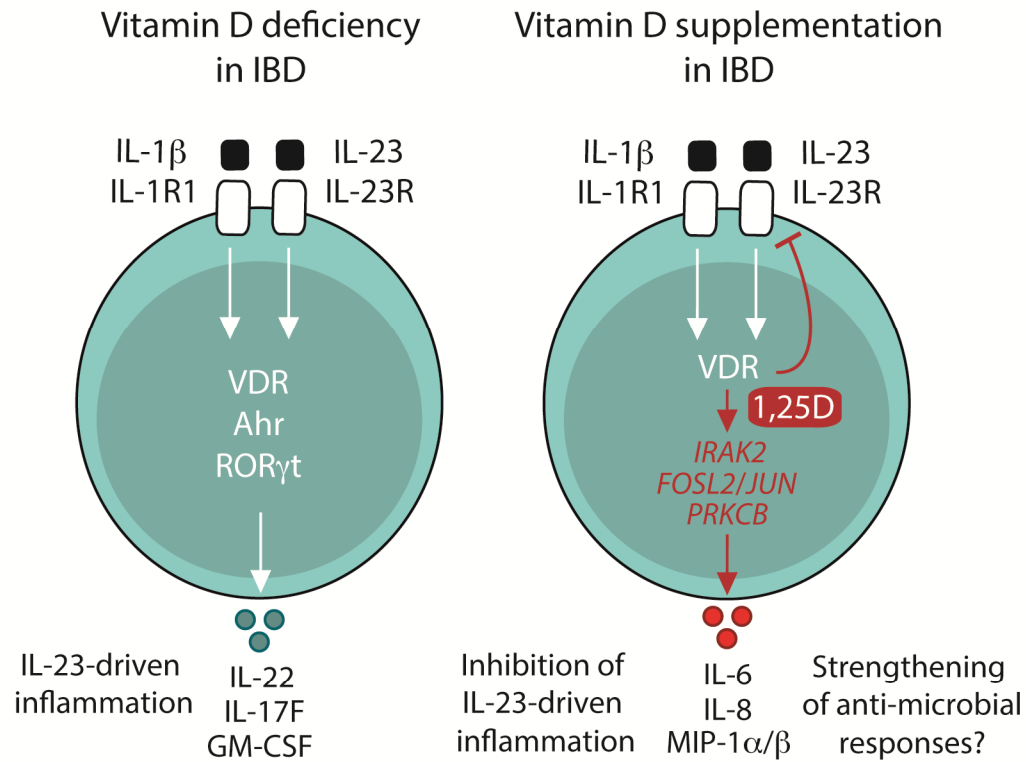
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IBD	inflammatory bowel disease
VDR	Vitamin D receptor
1,25D	1 α ,25-dihydroxy vitamin D3
Ahr	aryl hydrocarbon receptor
ROR γ t	retinoic acid receptor-related orphan receptor γ t

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ABSTRACT

Background: Vitamin D deficiency is a risk factor for inflammatory bowel disease (IBD). The IL-23-driven tissue-resident ILC3 play essential roles in intestinal immunity, and targeting IL-23/12 is a promising approach in IBD therapy.

Objective: We set out to define the role of 1 α ,25-dihydroxy vitamin D3 (1,25D) in regulating functional responses of human mucosal ILC3 to IL-23 plus IL-1 β stimulation.

Methods: Transcriptomes of sorted tonsil ILC3 were assessed by microarray analysis. ILC3 cytokine production, proliferation and differentiation were determined by flow cytometry, ELISA and multiplex immunoassay. Intestinal cell suspensions and ILC3 sorted from gut biopsies of IBD patients were also analyzed along with plasma 25-hydroxy vitamin D3 (25D) detection.

Results: ILC3 stimulated with IL-23 plus IL-1 β upregulated the vitamin D receptor (VDR) and responded to 1,25D with downregulation of the IL-23 receptor (IL-23R) pathway. Consequently, 1,25D suppressed the IL-22, IL-17F and GM-CSF production from tonsil and gut ILC3. In parallel, 1,25D upregulated genes linked to the IL-1 β signaling pathway as well as the IL-1 β -inducible cytokines IL-6, IL-8 and MIP-1 α/β . The 1,25D-triggered skewing in ILC3 function was not accompanied or caused by changes in viability, proliferation or phenotype. Finally, we confirmed low 25D plasma levels in IBD patients with active inflammation.

Conclusion: In the light of the beneficial targeting of IL-23/12 in IBD, 1,25D appears as an interesting therapeutic agent that inhibits the IL-23R pathway, providing a novel mechanism for how ILC3 could be manipulated to regulate intestinal inflammation.

Key messages

- IL-23 plus IL-1 β render human ILC3 responsive to vitamin D by upregulating VDR
- Vitamin D reprograms ILC3 by downregulating the IL-23R pathway while promoting production of IL-1 β -inducible cytokines
- The vitamin D-induced modulation of ILC3 function has therapeutic potential in IBD

Capsule summary

We have unraveled the detailed mechanisms behind how vitamin D efficiently suppresses IL-23 receptor signaling in gut innate lymphoid cells, providing incitement for vitamin D supplementation as a therapeutic approach in IBD.

Keywords: innate lymphoid cells, inflammatory bowel diseases, VDR, IL-22

Abbreviations: 1,25D – 1 α ,25-dihydroxy vitamin D₃, 25D – 25-hydroxy vitamin D₃, Ahr – aryl hydrocarbon receptor, GM-CSF – granulocyte monocyte colony stimulating factor, IBD – inflammatory bowel disease, IFN – interferon, IL – interleukin, ILC – innate lymphoid cell, NKp44 – natural cytotoxicity receptor 44, ROR γ t – retinoic acid receptor (RAR)-related orphan receptor gamma t, VDR – vitamin D receptor

INTRODUCTION

Group 3 innate lymphoid cells (ILC3) are tissue resident innate lymphocytes, functionally resembling Th17/22 cells in the adaptive arm of the immune system. However, ILCs lack rearranged antigen-specific receptors. In humans, expression of the natural cytotoxicity receptor NKp44 distinguishes two subsets of ILC3, the IL-17-producing NKp44⁻ and IL-22-producing NKp44⁺ ILC3^{1, 2}. The human healthy intestine is dominated by the IL-22-expressing NKp44⁺ ILC3^{3, 4}, while IL-17- and IFN- γ -producing NKp44⁻ ILC3 and ILC1 accumulate in the inflamed gut^{3, 4}. Several mouse studies demonstrated the protective role of ILC3-derived IL-22 during intestinal homeostasis and inflammation⁵⁻⁸. However, it is increasingly recognized that dysregulated ILC3 responses can promote intestinal inflammation^{9, 10} and inflammation-induced tumorigenesis¹¹⁻¹⁴. According to mouse studies IL-17 and IFN- γ crucially contribute to the IL-23-dependent innate colitis¹⁵. However, therapeutic targeting of IL-17 (secukinumab) and IFN- γ (fontolizumab) in IBD patients did not provide clear beneficial effects^{16, 17}. In fact, anti-IL-17 treatment worsened inflammation¹⁶. IL-23 is the major driver of human and mouse ILC3 cytokine production, similarly to Th17/22 cells^{5, 18-20}. IL-23 acts on the IL-23 receptor (IL-23R) and induces recruitment of retinoic acid receptor (RAR)-related orphan receptor gamma (ROR γ t) and aryl hydrocarbon receptor (Ahr) to the promoter of ILC3-specific cytokines^{21, 22}. IL-1 β can work in synergy with IL-23 via activating the IL-1R1/MyD88 pathway^{23, 24}. In contrast to anti-IL-17 therapy, initial therapeutic targeting of IL-23/IL-12 (ustekinumab) appears to be very efficient for Crohn's disease (CD) patients unresponsive to anti-TNF treatment²⁵⁻²⁷. We therefore turned our attention to

potential inhibitors of the IL-23R pathway in ILC3 as they would constitute interesting therapeutic agents in IBD.

Recently it was shown that vitamin D receptor (VDR)-deficient mice display increased intestinal accumulation of IL-22-expressing ILCs with accompanying dysbiosis²⁸, suggesting that the vitamin D-VDR signaling might be a negative modulator of ILC3 function. Vitamin D deficiency has been reported as a risk factor for IBD and colorectal cancer in observational studies²⁹⁻³². Similarly, VDR-deficient mice are more susceptible to colitis and develop more severe disease³³⁻³⁵. Vitamin D is a crucial immunomodulator acting via VDR, an intranuclear receptor and transcription factor. Several inflammatory cytokines, like IL-1, IL-6, IL-8 and IL-17 can be positively or negatively regulated by vitamin D, depending on the context and activation status of the immune cells³⁶. Notably, vitamin D reduces Th17-specific cytokine production³⁷⁻³⁹, thus it emerges as a potential regulator of ILC3.

Here, we determined how the active form of vitamin D, 1 α ,25-dihydroxy vitamin D3 (1,25D), modulates the function of IL-23 plus IL-1 β -activated human tonsil and intestinal ILC3. We show that 1,25D represses the IL-23R pathway and consequently the IL-23-regulated production of IL-22, IL-17F and GM-CSF. Simultaneously, 1,25D upregulates key components of the IL-1 β -pathway, and skews ILC3 cytokine secretion towards a more innate cell profile including production of IL-6, IL-8 and MIP-1 α/β . Our study reveals 1,25D as potent modulator of ILC3 function, with important implications for diseases in which ILC3 are involved and 1,25D has beneficial effects, such as IBD and psoriasis.

METHODS

Detailed description of ethical permits, materials and methods is provided in the Online Repository.

Cell isolation from human tonsil and gut

Human tonsil ILC3 and NK cells were isolated by flow cytometric sorting, as previously described ². Gut single cell suspensions and sorted ILC3 were derived from gut biopsies of adult IBD patients in remission (Table E1.).

In vitro stimulation, intracellular staining, and analysis of proliferation

Sort-purified tonsil and gut ILCs and gut single cell suspensions were cultured with 100 nM 1,25D or 25D (25-hydroxy vitamin D3) or vehicle, IL-23 plus IL-1 β (50 ng/mL) and IL-23 plus IL-1 β plus 1,25D for 24h or 72h. When indicated cells were additionally stimulated with 25 ng/mL PMA plus 500 nM ionomycin for 6h. Tonsil NK cells were stimulated with IL-12 plus IL-18 (50 ng/mL). After performing the different staining protocols, cells were immediately acquired on a LSR Fortessa flow cytometer (BD Biosciences).

ELISA, multiplex immunoassay

Cell supernatants were analyzed by Human IL-22 and IL-17F DuoSet ELISA (R&D) and Bio-Plex Pro human cytokine 27-plex kit (Bio-Rad).

RNA isolation and microarray gene expression analysis

Total RNA of ILC3 was isolated by using RNeasy micro kit (Qiagen). Microarray analysis was performed by Human Transcriptome Array 2.0. Data correction and heatmap analysis were performed using R software^{40, 41}.

25D detection in plasma samples of IBD patients

Peripheral blood of IBD patients (Table E2) was collected at the Gastromedical Center, KI. 25D in plasma samples was analyzed by chemiluminescence immunoassay.

Statistical analyses

Data were analyzed by Wilcoxon matched-pairs signed rank test or one-way ANOVA and Tukey's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$) by using GraphPad Prism 6 Software.

RESULTS

Activated ILC3 upregulate VDR and 1,25D induces broad transcriptional changes

In order to assess functional responses of ILC3 to 1,25D, we sort-purified bulk ILC3 containing NKp44⁻ and NKp44⁺ ILC3 from tonsil tissue. Tonsils are mucosa-associated lymphoid tissue (MALT) containing all ILC populations reported so far². We therefore performed our initial experiments using tonsil tissue, to later confirm key findings in the intestinal tissue. ILC3 were sorted by flow cytometry as lineage⁻ cells lacking expression of B, T, myeloid and mast cell markers, and were defined as CD45⁺CD127⁺CD117⁺CD161⁺CRTH2⁻ lymphocytes (Figure 1A). First, we wanted to assess, in an unbiased manner, the effects of vitamin D on human ILC3 by using the active form 1,25D (100 nM). For this purpose, we performed transcriptome analysis of bulk ILC3 after 24h-stimulation with vehicle (0.0002% EtOH), 1,25D, IL-23 plus IL-1 β (each 50 ng/mL) or IL-23 plus IL-1 β plus 1,25D. We analyzed differentially regulated genes among all treatment groups (Figure 1B). Whereas 1,25D alone induced modest transcriptional changes, IL-23 plus IL-1 β stimulation of ILC3 (referred to as activated ILC3 hereafter) led to significant regulation of 321 genes, with VDR being the most significantly upregulated transcript (Figure 1C-D). These data indicated that IL-23 plus IL-1 β -activation rendered ILC3 increasingly responsive to 1,25D (Figure 1C). We used GO-enrichment analysis to assess overall changes in transcriptional patterns, which revealed negative regulation of protein phosphorylation, cytokine-mediated signaling pathways and cytokine production by 1,25D in activated ILC3 (Figure E1). In parallel, 1,25D upregulated genes related to small molecule metabolic processes, innate immune- and stress response and single-organism metabolic processes (Figure E1).

Upon in-depth evaluation of the transcriptional changes we observed that treatment of activated ILC3 with 1,25D triggered significant regulation of several VDR target genes, including *CYP24A1*, a 24-hydroxylase enzyme degrading the active 1,25D compound. Additionally, *CAMP*, (LL-37, anti-microbial peptide)⁴² and *MN1* (meningioma-1, co-activator of VDR-mediated transcription) were significantly upregulated⁴³ (Figure 1C-D). Furthermore, 1,25D upregulated the VDR target genes *IRF8* and *SERPINB1*, factors known as negative modulators of Th17 cells^{44, 45}. Notably, 1,25D skewed the gene expression profile of activated ILC3 towards that of tissue-resident cells as demonstrated by modulation of genes involved in immune cell homing and adhesion as well as in the regulation of actin polymerization and motility (Figure E2A). Additionally, numerous metabolism-related genes were upregulated by 1,25D in activated ILC3, with particular enrichment of lipid metabolism genes (Figure E2B).

These data provide compelling evidence that activated ILC3 respond intensively to 1,25D with transcriptional changes of a wide range of known VDR target genes and with an enhanced transcriptional program aimed at tissue-residency.

1,25D does not affect differentiation, viability or proliferation of activated ILC3

An important function of 1,25D is to modulate differentiation and proliferation of immune cells⁴⁶. Thus, we investigated if 1,25D could influence ILC3 differentiation by affecting the expression of NKp44, which is a marker of IL-22-producing ILC3 in humans¹. Notably, the IL-23 plus IL-1 β -induced increase in ILC3 NKp44 expression was not affected by 1,25D (Figure 2A-B). Additionally, 1,25D had no impact on the expression of other ILC3 markers, such as CD127, CD117 and CD161 (data not shown).

On the transcriptional level, 1,25D modulated the expression of several genes involved in cell survival and proliferation as well as pro- and anti-apoptotic genes (Figure E2C). Thus, we investigated whether 1,25D could influence ILC3 viability and proliferation beyond inducing transcriptional changes. Sort-purified ILC3 were incubated with vehicle, 1,25D, IL-23 plus IL-1 β or IL-23 plus IL-1 β plus 1,25D for 72 hours. Importantly, 1,25D did not affect viability of ILC3 (Figure 2C). Additionally, the IL-23 plus IL-1 β -triggered proliferation of NKp44⁺ and NKp44⁻ ILC3 was also not markedly influenced by 1,25D (Figure 2D-E). These data clearly suggest that the effects of 1,25D on activated ILC3 do not include, and are not mediated by, alterations in viability, proliferation or differentiation status of ILC3.

1,25D downregulates the IL-23R regulatory pathway in activated tonsil ILC3

Transcriptome analysis of highly purified tonsil ILC3 revealed that IL-23 plus IL-1 β treatment caused differential regulation of several genes compared to vehicle treatment. We confirmed here numerous genes including *IL22*, *CSF2*, *IL17F*, *IL26* and *IFNG*, which had been previously shown to be expressed by activated tonsil ILC3 (Figure E2D) ⁴. Co-incubation of activated ILC3 with 1,25D resulted in profound transcriptional changes affecting crucial ILC3 functions, in line with the observed upregulation of VDR in activated ILC3 (Figure 3A). Most importantly, the transcript of *IL23R*, the main driver of ILC3 cellular activity, was significantly downregulated by 1,25D. Consequently, transcripts downstream of the IL-23R including *RORC*, *IL22*, *IL17F*, *IL26*, *IFNG* and *CSF2* were also suppressed. We did not observe significant regulation of *AHR* gene expression at 24 hours of stimulation. We

confirmed the 1,25D-induced downregulation of *IL23R* on NKp44⁺ and NKp44⁻ ILC3 by analyzing the IL-23R cell surface protein by flow cytometry (Figure 3B-C).

Since 1,25D clearly downregulated the IL-23R and cytokine transcripts associated to this pathway, we next assessed the IL-23R-associated and ILC3-specific transcription factors Ahr and ROR γ t. IL-23 plus IL-1 β treatment led to upregulated protein expression of these transcription factors, which was reduced by 1,25D (Figure 3D-E). Interestingly, in response to 1,25D, NKp44⁺ ILC3 showed the more prominent downregulation of Ahr and ROR γ t expression as compared to NKp44⁻ ILC3 (Figure 3D-E).

These results provide the first evidence that 1,25D acts as a silencer of the IL-23R regulatory pathway on human ILC3.

1,25D selectively prevents ILC3 responses to IL-23 cytokine stimulation

We next sought to dissect the relative contribution of the ILC3-activating cytokines IL-23 and IL-1 β in mediating responsiveness to, and the effects of, 1,25D.

Sort-purified ILC3 secreted IL-22 and IL-17F upon IL-23 stimulation and secretion was synergistically enhanced by combined IL-23 plus IL-1 β stimulation (Figure 4A; Figure E3A-B). ILC3-derived GM-CSF can also contribute to the pathogenesis of gut inflammation^{5, 47, 48}. However, in contrast to IL-22, GM-CSF production was dependent on IL-1 β stimulation that was further enhanced by IL-23 after 24 hours of stimulation (Figure E3C). After 72 hours, GM-CSF production might have reached its maximum upon IL-1 β stimulation and was not further inducible by IL-23 (Figure 4B). In line with the 1,25D-induced downregulation of the IL-23R pathway, 1,25D only inhibited the IL-23-stimulated or synergistically co-stimulated secretion of IL-22, IL-17F and GM-CSF but did not modulate IL-1 β -mediated effects (Figure 4A-B).

Similarly, 1,25D reduced the IL-23-dependent intracellular expression of IL-22 and GM-CSF in NKp44⁺ ILC3 (Figure 4C-F), but not the IL-1 β -dependent GM-CSF expression (Figure C-F). Interestingly, although activated ILC3 secreted IL-17F after 24 and 72 hours, which was inhibited by 1,25D (Figure E3B, Figure 4A), intracellular detection of IL-17F revealed no such effects (Figure 4E-F). In contrast to IL-17F, activated ILC3 secreted small amounts of IL-17A, which was not modified by 1,25D (Figure E4A). IL-17A was also not detectable intracellularly in the ILC3 subsets (data not shown). These data are supported by the notion that in our microarray analysis *IL17A* transcripts were not upregulated in activated ILC3. Furthermore, low amounts of secreted and intracellular IFN- γ were detected when ILC3 were stimulated with IL-23 plus IL-1 β , which was inhibited by 1,25D (Figure E4B-D). Notably, tonsil NK cell IFN- γ and GM-CSF production, stimulated by IL-12 plus IL-18, was completely unaffected by 1,25D (Figure E5).

The active form of vitamin D, 1,25D, is synthesized from the inactive proform 25-hydroxyvitamin D3 (25D), by the enzyme 25D-1- α -hydroxylase (*CYP27B1*) that we showed was expressed in ILC3 (Figure 1C)³⁶. Strikingly, 25D mimicked the effects of 1,25D on stimulated ILC3 in reducing IL-22 and GM-CSF cytokine secretion (Figure E6A-B) and also prevented upregulation of ROR γ t and Ahr in both ILC3 subsets (Figure E6C-D). The latter findings suggest that ILC3 can convert 25D to 1,25D intrinsically.

Together, these data demonstrate that 1,25D as well as the inactive proform 25D limit responsiveness of ILC3 to IL-23, as evidenced by reduced IL-22, IL-17F and GM-CSF production.

1,25D upregulates IL-1 β -inducible genes and cytokines in activated tonsil ILC3

Besides the transcriptional changes of genes involved in VDR and IL-23 responses, 1,25D triggered upregulation of a distinct set of genes involved in the IL-1 β signaling pathway (Figure 5A). IL-1 β mediates its effects via the type I IL-1 receptor (IL-1R1) and its signal transduction involves the adaptor molecule IL-1 receptor-activated protein kinase 2 (IRAK2), the downstream mitogen-activated protein (MAP) kinases and c-Jun N-terminal kinases (JNK) and subsequently NF- κ B and members of the activating protein-1 (AP-1) transcription factor family⁴⁹. Canonical IL-1 β target genes are those of IL-6, IL-8, MIP-1 α/β and COX-2. The combination of IL-23 plus IL-1 β plus 1,25D stimulation of ILC3 led to upregulation of *IRAK2*, *JUN* and *FOSL2*, which encode proteins crucially involved in the IL-1 β signaling pathway (Figure 5B). However, expression of IL-1R1 protein was not influenced by 1,25D treatment of activated ILC3 (Figure E7). Notably, we also observed highly significant upregulation of IL-1 β -inducible cytokine/chemokine genes such as *IL6*, *IL8*, *CCL3*, *CCL4* and *CSF1* upon VDR engagement in activated ILC3 (Figure 5A).

In line with the transcriptional changes, we confirmed that secretion of IL-6, IL-8 and MIP-1 α/β was induced by 1,25D treatment in activated ILC3 (Figure 5C). Interestingly, the mechanism behind the enhanced production of IL-6 was different from the latter cytokines. IL-6 was the only cytokine induced by the combination of 1,25D plus IL-1 β , i.e. in the absence of IL-23 (Figure 5C). Notably, IL-23 plus 1,25D-stimulation did not enhance secretion of any of these IL-1 β -inducible cytokine/chemokines. To further validate our findings, and assess the cellular sources of IL-6 within the ILC3 population, we performed intracellular IL-6 detection (Figure 5D-E). Both ILC3 subsets expressed IL-6 following incubation with IL-23 plus IL-1 β plus 1,25D-stimulation, but it was particularly pronounced in NKp44⁺ ILC3 (up to 10%, Figure 5D). Compared to IL-23 plus IL-1 β stimulation, 1,25D shifted NKp44⁺

ILC3 cytokine production to reduce IL-22 but acquire IL-6 expression (Figure 5D). Interestingly, approximately 15% of IL-22-expressing NKp44⁺ ILC3 co-expressed IL-6.

These data show that besides downregulation of the IL-23R regulatory pathway, 1,25D simultaneously turns on genes which belong to the IL-1 β signaling cascade with a consequent induction of IL-1 β -inducible cytokines like IL-6 in activated ILC3.

1,25D selectively inhibits IL-22 and GM-CSF production of activated intestinal NKp44⁺ ILC3 but not in CD4⁺ T cells.

ILC3 are the most abundant intestinal ILC population. The healthy human gut mucosa is dominated by the NKp44⁺ ILC3 subset^{3-5, 20}. We isolated single cell suspensions from biopsy samples of non-inflamed ileum or colon of adult IBD patients in remission (Table E1). To mimic inflammation, we stimulated total intestinal cell suspensions with IL-23 plus IL-1 β , which significantly increased the IL-22 secretion (Figure 6A). As demonstrated for tonsil ILC3, IL-22 production was markedly reduced by 1,25D (Figure 6A). Additionally, we detected low levels of secreted IL-17A and IL-17F from activated intestinal cell suspensions, which were slightly but not significantly decreased by 1,25D (Figure 6A).

We next set out to uncover which cellular source of IL-22 in the intestine is affected by 1,25D. To that end, NKp44⁺ ILC3 and CD4⁺ T cells were analyzed for intracellular expression of IL-22 and also GM-CSF (Figure 6B). As in the tonsil tissue, the gut NKp44⁺ ILC3 subset was gated as lineage⁻ CD45⁺CD127⁺CD117⁺CD161⁺CRTH2⁻NKp44⁺ lymphocytes. CD4⁺ T cells were identified as CD45⁺CD3⁺CD8⁻CD4⁺ lymphocytes. Intestinal NKp44⁺ ILC3 responded to IL-23 plus IL-1 β stimulation with on average 15% of the cells producing IL-22,

while 1,25D co-incubation reduced it to 3.5% (Figure 6C-D). We detected only weak GM-CSF expression upon activation, which was clearly decreased by 1,25D. Notably, we were not able to detect GM-CSF in the intestinal cell suspensions supernatant (data not shown). Upon augmentation of cytokine production by PMA plus ionomycin, approximately 25% of NKp44⁺ ILC3 became IL-22⁺ after cytokine activation, and 1,25D clearly disabled the IL-22 production (Figure 6C-D). In line with our findings in tonsil ILC3, 1,25D reduced IL-22, but did not affect GM-CSF production in PMA plus ionomycin-exposed intestinal NKp44⁺ ILC3 (Figure E8; Figure 6C-D). Additionally, NKp44⁺ ILC3 slightly increased IL-22 expression upon IL-23 plus IL-1 β stimulation, which was inhibited by 1,25D (Figure E9A-B). In contrast, CD4⁺ T cells did not respond to IL-23 plus IL-1 β stimulation with increased IL-22 or GM-CSF expression (Figure 6E). However, upon PMA plus ionomycin exposure, on average 7% of CD4⁺ T cells were IL-22⁺ and 30% GM-CSF⁺ (Figure 6E-F). Strikingly, 1,25D did not influence IL-22 and GM-CSF production in CD4⁺ T cells. CD8⁺ T cells only scarcely displayed IL-22 expression upon activation and it was not affected by 1,25D (Figure E9C-D). These data clearly indicate that 1,25D exclusively controls IL-22 and GM-CSF production by intestinal NKp44⁺ ILC3, but not that of CD4⁺ T cells stimulated with IL-23 plus IL-1 β .

1,25D reprograms sorted intestinal ILC3 by suppressing IL-22 and GM-CSF production while inducing IL-6 secretion.

ILC3 were sort-purified from non-inflamed ileum biopsy samples of IBD patients in remission by using the same gating strategy as for tonsil ILC3 sorting (Figure 1A). Due to low ILC3 sorting yield from human gut biopsies, in some experiments ILC3

were pooled from 2 patients (Table E1). After 72 hours culturing, gut ILC3 retained their phenotype, with high surface expression of CD117 and CD161 (Figure 7A). Activated gut ILC3 showed on average 10% higher frequency of NKp44⁺ ILC3 compared to vehicle, and similarly to tonsil-derived ILC3, the composition of ILC3 subsets was not affected by 1,25D (Figure 7A-B). With regard to cytokine secretion we found augmented IL-22, GM-CSF and IFN- γ levels upon activation, which were almost completely prevented by 1,25D (Figure 7C). Paralleling the findings with tonsil ILC3, activated gut ILC3 also displayed induced IL-6 production upon 1,25D treatment (Figure 7C). These data demonstrate that 1,25D reprograms gut ILC3 cytokine production, which provides important pathophysiological insights. These experiments highlight that ILC3 are functionally very plastic lymphocytes, thus 1,25D is able to suppress IL-23-driven IL-22 and IL-17F expression and enhance IL-1 β -triggered production of IL-6, IL-8 and MIP-1 α/β (displayed in the Graphical Abstract).

IBD patients with active inflammation are vitamin D-deficient

IBD patients have been shown to have lower plasma levels of 25D than healthy controls, but this association is not clear in all cohorts⁵⁰. Therefore, we determined 25D plasma levels in a cohort of IBD patients collected at our gastromedical center (Table E1 and E2, Figure 8). The 25D plasma level of patients with active inflammation was on average lower than 50 nmol/L, which is defined as insufficient according to the IOM guidelines⁵¹. In contrast, subjects with Lynch syndrome and IBD patients in remission exhibited higher 25D levels. These observations, along with our detailed mechanistic description of how 1,25D reprograms intestinal ILC3,

381 strongly argue for the importance of vitamin D supplementation studies of IBD
382 patients.

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DISCUSSION

The IL-23R pathway is one of the most extensively studied risk pathways for IBD, and variants of the *IL23R* gene may be protective or cause an inappropriate immune response to the commensal flora of the gut⁵². Downstream genes of the IL-23R pathway, including *IL22*, *IL17A* and *IL17F* have also been identified as IBD risk loci and found to be overexpressed in colonic mucosa both in CD and ulcerative colitis^{52, 53}. Indeed, targeting IL-23/IL-12 by ustekinumab is beneficial for patients with CD who fail to respond to anti-TNF therapy²⁵⁻²⁷. ILC3 and Th17/22 cells share similar transcriptional programs and cytokine production profiles in response to IL-23⁵³. Strikingly, the IL-23-driven IL-22 secretion of ILC3 can be protective in intestinal homeostasis and inflammation⁵⁻⁸, but it can also exert deleterious effects in gut inflammation and tumorigenesis⁹⁻¹⁴. Similarly, the IL-23-induced production of IL-17 contributes to the development of colitis^{15, 20}, while IL-23-independent IL-17 production was recently shown to promote intestinal barrier function⁵⁴. Consequently, therapeutic targeting of IL-17 was ineffective, even leading to adverse outcomes in CD patients¹⁶. Hence, IL-17 and IL-22 may have both tissue protective and deleterious effects in the intestine. Nevertheless, blocking the IL-23/12 pathway seems to have only beneficial effects in IBD.

Vitamin D deficiency is a known risk factor for IBD⁵⁵, and a recent 5-year longitudinal study revealed that normal 25D level and vitamin D supplementation reduced markers of disease activity in IBD²⁹. We demonstrated in a small cohort of IBD patients that patients with active inflammation are vitamin D-deficient, while patients in remission have normalized 25D levels. However, high-quality randomized and placebo-controlled trials are required to establish vitamin D as therapeutic option for IBD. We demonstrated here that IL-23- plus IL-1 β -activated ILC3 upregulate

VDR expression. In response to the active form of vitamin D, 1,25D, ILC3 downregulated the IL-23R and consequently the IL-23R pathway (Ahr, ROR γ t, IL-22, IL-17F, GM-CSF), which could have dramatic consequences for intestinal health as previous reports identify IL-23 as local driver of intestinal inflammation via inducing production of IL-22, IL-17 and GM-CSF^{15, 20, 47, 56}. Noteworthy, 1,25D only inhibited IL-23R-dependent secretion of GM-CSF, while IL-1 β -induced GM-CSF production was maintained, possibly providing intestinal tolerance as reported by others⁴⁸. Our data therefore suggest that vitamin D supplementation of deficient IBD patients might provide a promising strategy to diminish mucosal inflammation, similarly to what is observed in patients treated with ustekinumab²⁵⁻²⁷.

Vitamin D was previously shown to inhibit Th17 differentiation and cytokine production via inhibiting nuclear factor for activated T cells (NFAT)³⁷. Besides VDR the transcription factor IRF8 and *SERPINB1* gene can also act as silencers of Th17 function^{44, 45}. Notably, both *IRF8* and *SERPINB1* are VDR target genes^{57, 58}, and were significantly upregulated by 1,25D in activated ILC3 in the microarray analysis, suggesting the involvement of multiple factors in downregulating the IL-23R pathway in 1,25D-treated activated ILC3.

Apart from IL-23R, patients with CD commonly display polymorphisms in genes related to pathogen recognition and autophagy relevant to bacterial killing in macrophages (*NOD2/CARD15*, *IRGM*, and *ATG16L1*)⁵². Accordingly, CD is associated with defective acute inflammatory response failing to fight the accumulated bacteria, which then persists and evokes a secondary chronic inflammation⁵⁹. At the onset of IBD there is an inappropriate innate response of macrophages, the major source of IL-6 and IL-8, to the accumulated bacteria. This impaired defense line results in insufficient infiltration of neutrophils to the site of

infection⁵⁹. Notably, 1,25D was shown to enhance bacterial killing of macrophages isolated from CD patients⁶⁰. In addition, 1,25D induced production of IL-1 β , IL-6 and IL-8, but not GM-CSF, in *Mycobacterium tuberculosis*-infected macrophages, which was essential in host defense⁶¹. ILC3, as intestinal mucosa-resident immune cells, are crucially involved in acute inflammation mainly via secreting IL-22⁶². Although vitamin D has shown protective effects in patients with IBD, it can obviously promote production of innate-like cytokines. Yet, the role of ILC3 in producing such cytokines is poorly characterized. We revealed that 1,25D induced upregulation of genes linked to IL-1 β signaling (*IRAK2*, *FOSL2*, *JUN*, *PRKCB*) in activated tonsil ILC3, without affecting IL-1R1 expression. Consequently, IL-1 β -inducible cytokines IL-6, IL-8 and MIP-1 α/β were secreted by activated ILC3 in response to 1,25D. Intestinal ILC3 also shifted the cytokine production profile from IL-22 and GM-CSF to IL-6. In contrast to IL-6, IL-8 and MIP-1 α/β , the IL-1 β -induced GM-CSF production was not affected by 1,25D, which suggests a distinct regulation of this cytokine, as previously shown in macrophages^{61, 63}. Our findings, hence, suggest that 1,25D enables ILC3 to acquire an innate-like cytokine expression profile. Thus, ILC3 might essentially contribute to infiltration of phagocytic monocytes and neutrophils to the site of bacterial infiltration at the onset of IBD. This so far unappreciated involvement of ILC3 in regulating immune cell trafficking during acute inflammation might further enhance the robustness of the intestinal immune system. The role of IL-6 in inflammation is very complex due its soluble and membrane-bound receptors⁶⁴. Interestingly, IL-6, similarly to IL-22, activates Stat3 phosphorylation in intestinal epithelial cells, which triggers wound healing and mucosal repair⁶⁵⁻⁶⁷. Consistently, anti-IL-6R therapy, although it displayed beneficial effects for patients with CD, failed to improve mucosal healing

⁶⁸. This notion supports that the 1,25D-induced modulation of ILC3 function might be of relevance in intestinal inflammation.

Notably, the inactive vitamin D proform 25D mimicked 1,25D in modulating the ILC3 cytokine production profile indicating that ILC3 are able to synthesize 1,25D. Of note, 1,25D did not modulate activated tonsil NK cells. Intestinal CD4⁺ T cells only produced IL-22 and GM-CSF after combined stimulation of IL-23 plus IL-1 β and PMA plus ionomycin. However, 1,25D did not affect CD4⁺ T cells cytokine production, which can be explained by previous reports showing that VDR expression in T cells is TCR activation-dependent ³⁸. Thus, α -CD3/CD28 stimulation would be required for making T cells responsive to 1,25D. Nevertheless, our results suggest that 1,25D exclusively suppresses the innate responsiveness of ILCs and not of T cells.

Taken together, our data clearly demonstrate that activated human ILC3 are rendered responsive to vitamin D, which causes downregulation of the IL-23R pathway and simultaneously shifts ILC3 cytokine production to innate-type cytokines including IL-6, IL-8 and MIPs. We show evidence that IBD patients are vitamin D-deficient, hence, targeting VDR in IBD is highly relevant and would directly affect ILC3 functions crucial in orchestrating innate immune responses.

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AUTHOR CONTRIBUTION

V.K. designed the study, performed experiments, interpreted data and wrote the manuscript. P.C. performed experiments and transcriptional analysis. M.F. performed experiments. A.R performed experiments and contributed to data interpretation. E.K. performed experiments. E.J.V. contributed to study design. S.A., U.L., D.F., C.H. provided clinical samples. P.B. contributed to study design and data interpretation. J.M. designed the study, interpreted data and co-wrote the manuscript. All authors contributed to manuscript writing.

ADDITIONAL INFORMATION

Competing financial interests: The authors declare no competing financial interests.

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715 discussion 47.
- 716

FIGURE LEGENDS

Figure 1. IL-23 plus IL-1 β activation makes ILC3 responsive to 1,25D, which causes regulation of VDR target genes in tonsil ILC3. **A.** Gating strategy for sort-purifying human tonsil ILC3. Lin⁺ stands for lineage⁺ cells and DCM means dead cell marker. **B.** Venn-diagram of differentially expressed genes between the indicated treatments (cut-off criteria: fold change of 1.5 and $p < 0.01$). **C.** Heatmap of VDR target genes regulated in sorted ILC3. ILC3 were stimulated with veh (vehicle, EtOH), 1,25D (100 nM), IL-23 plus IL-1 β (50 ng/mL each) or 1,25D plus IL-23 plus IL-1 β for 24h. **D.** Relative signal intensity $\pm$ SD of VDR and VDR target genes transcripts (* $p < 0.05$, *** $p < 0.005$, **** $p < 0.001$, $n = 3$).

Figure 2. 1,25D does not affect ILC3 phenotype, viability or proliferation. **A.** NKp44 expression of CD117⁺CD161⁺ ILC3 after 72h incubation was analyzed by flow cytometry. **B.** Frequencies of NKp44⁺ and NKp44⁻ ILC3 of total ILC3 ($n = 5$). **C.** Viability of total ILC3 was analyzed by flow cytometry and shown as mean viable ILC3 count $\pm$ SD ($n = 6$). **D.** Proliferation after 72h was analyzed by flow cytometry on gated NKp44⁺ and NKp44⁻ ILC3 (in duplicates, $n = 2$). **E.** Bar graphs show mean percentage of cells in different cell division phases (D0-D5) (in duplicates, $n = 2$).

Figure 3. 1,25D downregulates the IL-23R pathway in activated ILC3. **A.** Heatmap of IL-23R pathway genes regulated by 1,25D in activated ILC3. **B.** IL-23R expression after 24h incubation was analyzed by flow cytometry on NKp44⁺ and NKp44⁻ ILC3. **C.** Normalized geometric MFI of IL-23R expression in ILC3. Dot plots show mean $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, $n = 5$). **D.** Representative plots of Ahr and

ROR γ t expression in NKp44⁺ and NKp44⁻ ILC3 after 72h incubation analyzed by flow cytometry (n=5). **E.** Normalized geometric MFI of Ahr and ROR γ t. Dot plots show mean $\pm$ SD (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001, n=6).

Figure 4. IL-23-induced ILC3 activation is abrogated by 1,25D. **A.** IL-22, IL-17F and **B.** GM-CSF release of sorted tonsil ILC3 after 72h was analyzed by ELISA. Mean concentration +SD is shown (****p<0.001, n=3). **C.** Intracellular IL-22, GM-CSF expression of NKp44⁺ ILC3 was analyzed after 24h by flow cytometry. **D.** Bar graphs show the mean percentage of positive cells +SD (*p<0.05, ****p<0.001, n=3). **E.** Intracellular IL-17F, GM-CSF expression of NKp44⁻ ILC3 was analyzed after 24h by flow cytometry. **F.** Bar graphs show the mean percentage of positive cells +SD (*p<0.05, **p<0.01, n=3).

Figure 5. 1,25D upregulates genes involved in IL-1 β signaling and IL-1 β -inducible cytokines. **A.** Heatmap of IL-1 β -related genes regulated by 1,25D in activated ILC3. **B.** Relative signal intensity of selected transcripts. Bars represent mean $\pm$ SD (**p<0.01, ***p<0.005, ****p<0.001, n=3). **C.** IL-6, IL-8, MIP-1 α / β release of tonsil ILC3 after 72h was analyzed by multiplex ELISA (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001, n=4). **D.** Intracellular expression of IL-22 and IL-6 of NKp44⁺ ILC3 after 38h in the presence of PMA (25 ng/mL) plus ionomycin (500 nM) in the last 6h was analyzed by flow cytometry. **E.** Bar graphs show the mean percentage of positive cells +SD (****p<0.001, n=3). **F.** Intracellular expression of IL-17F and IL-6 of NKp44⁻ ILC3 after 38h in the presence of PMA plus ionomycin in the last 6h was analyzed by flow cytometry. **G.** Bar graphs show the mean percentage of positive cells +SD (*p<0.05, n=3).

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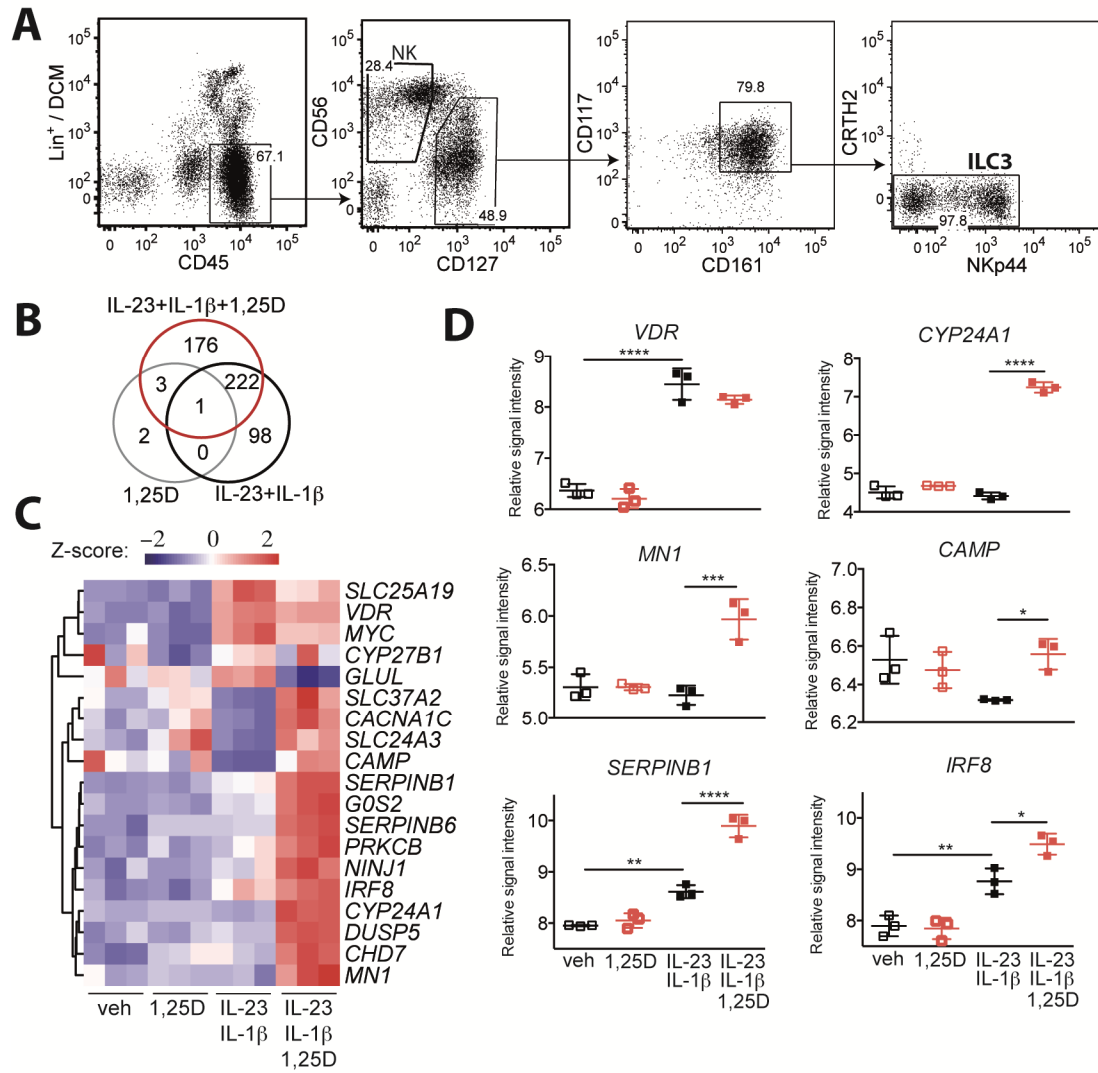
768 **Figure 6. 1,25D selectively inhibits IL-22 and GM-CSF production in intestinal**
 769 **activated NKp44⁺ ILC3 but not in CD4⁺ T cells. A.** Secreted IL-22, IL-17A and IL-
 770 17F non-inflamed gut biopsy cell suspensions was analyzed after 24h incubation by
 771 ELISA. Bars represent mean±SD (*p<0.05, ****p<0.001, n=7). **B.** Gating strategy
 772 for identifying ILC3 and T cell subsets in non-inflamed gut biopsy cell suspensions.
 773 **C, E.** Intracellular expression of IL-22 and GM-CSF was analyzed on gated NKp44⁺
 774 ILC3 (**C**) and on CD4⁺ T cells (**E**) after 24h. In some cases cell suspensions were
 775 incubated in the presence of PMA (25 ng/mL) plus ionomycin (500 nM) in the last 6h
 776 (n=7). **D.** Individual percentages of positive cells are shown (*p<0.05, **p<0.01,
 777 ***p<0.005, n=7). **F.** Individual percentages of positive cells are shown (n=7).

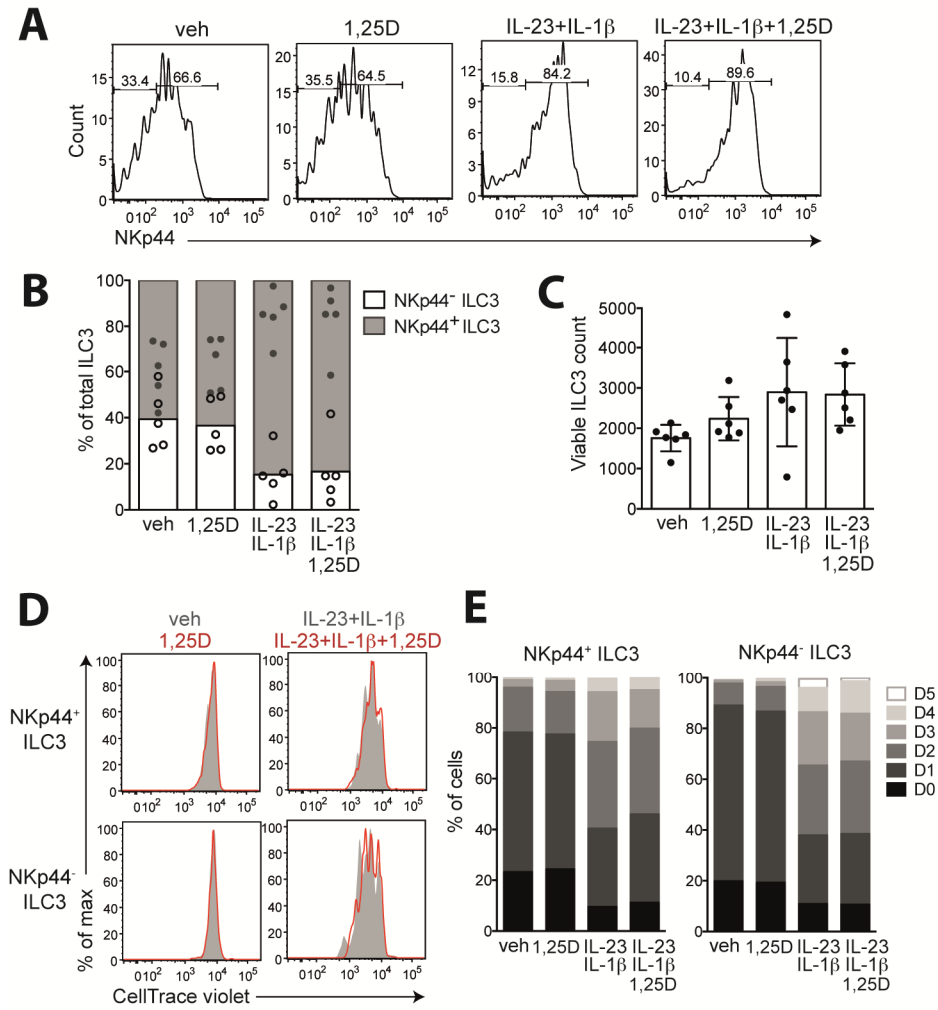
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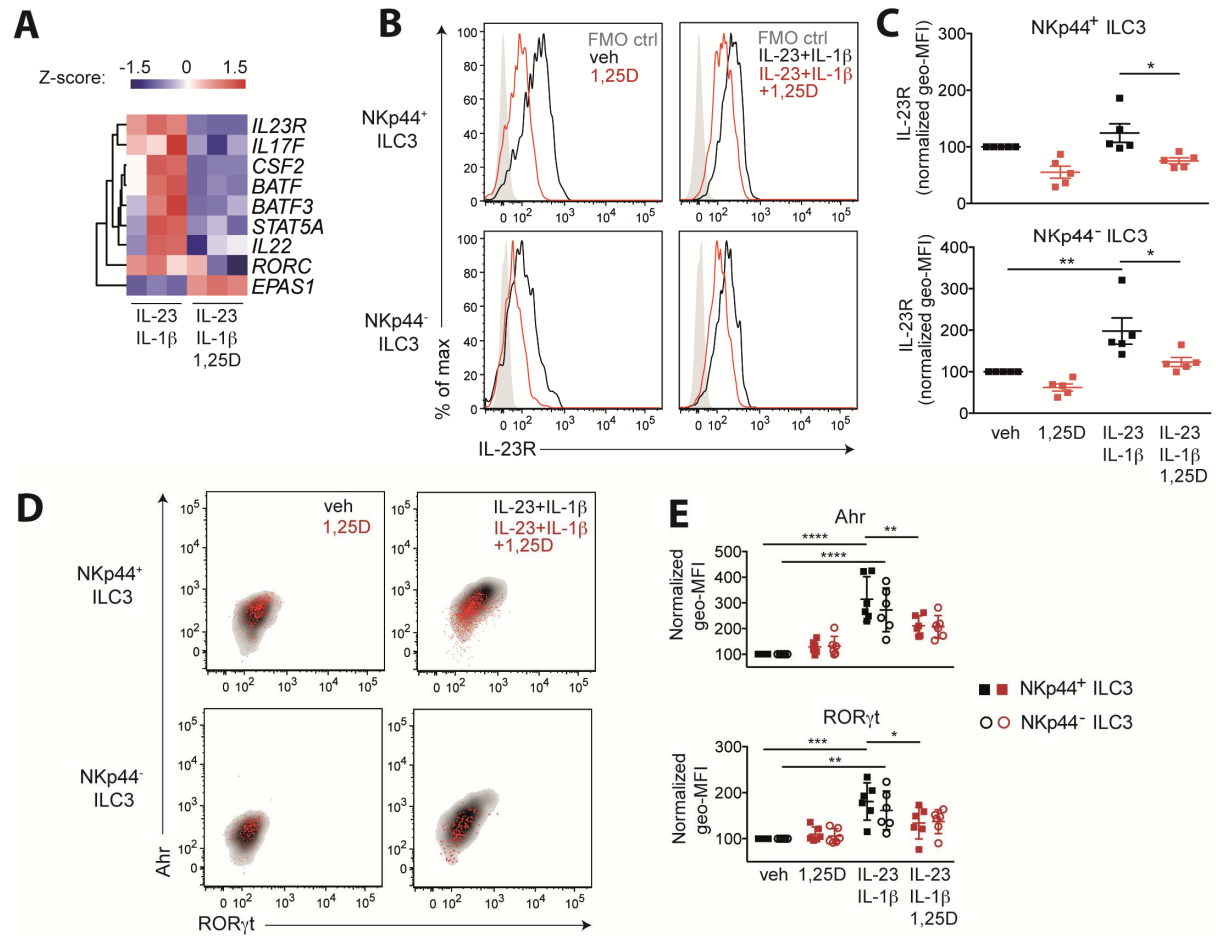
779 **Figure 7. 1,25D reprograms gut ILC3 cytokine production by suppressing IL-22**
 780 **and GM-CSF production and inducing IL-6 secretion. A.** ILC3 were sort-purified
 781 from non-inflamed gut biopsies. Expression of CD117 and NKp44 was analyzed by
 782 flow cytometry after 72h. **B.** Mean frequency of NKp44⁺ and NKp44⁻ ILC3 of total
 783 gut ILC3 (n=3). **C.** IL-22, GM-CSF, IFN-γ and IL-6 release of sort-purified gut ILC3
 784 was analyzed by multiplex ELISA after 72h. Bars represent mean±SD (*p<0.05,
 785 **p<0.01, n=3).

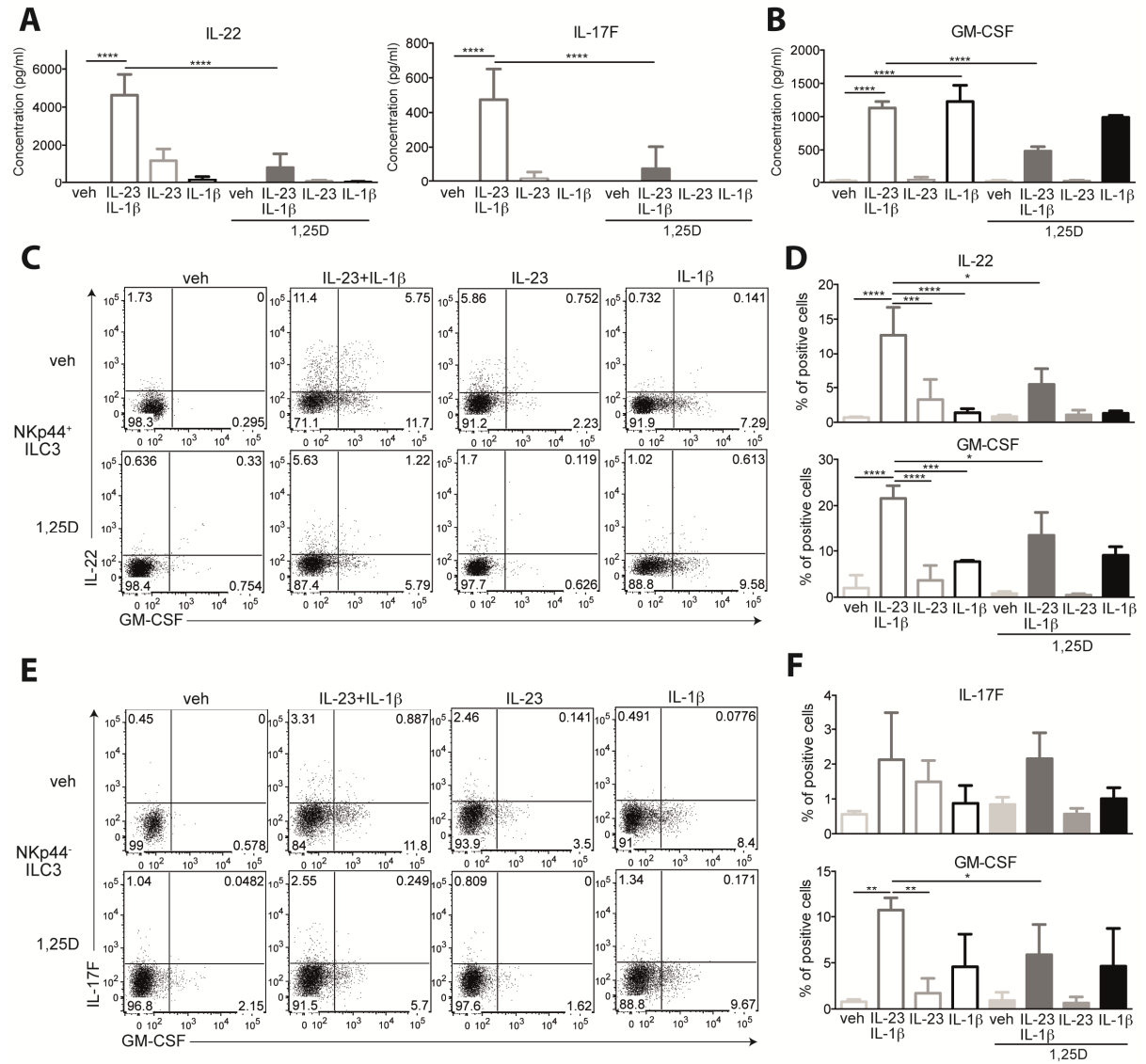
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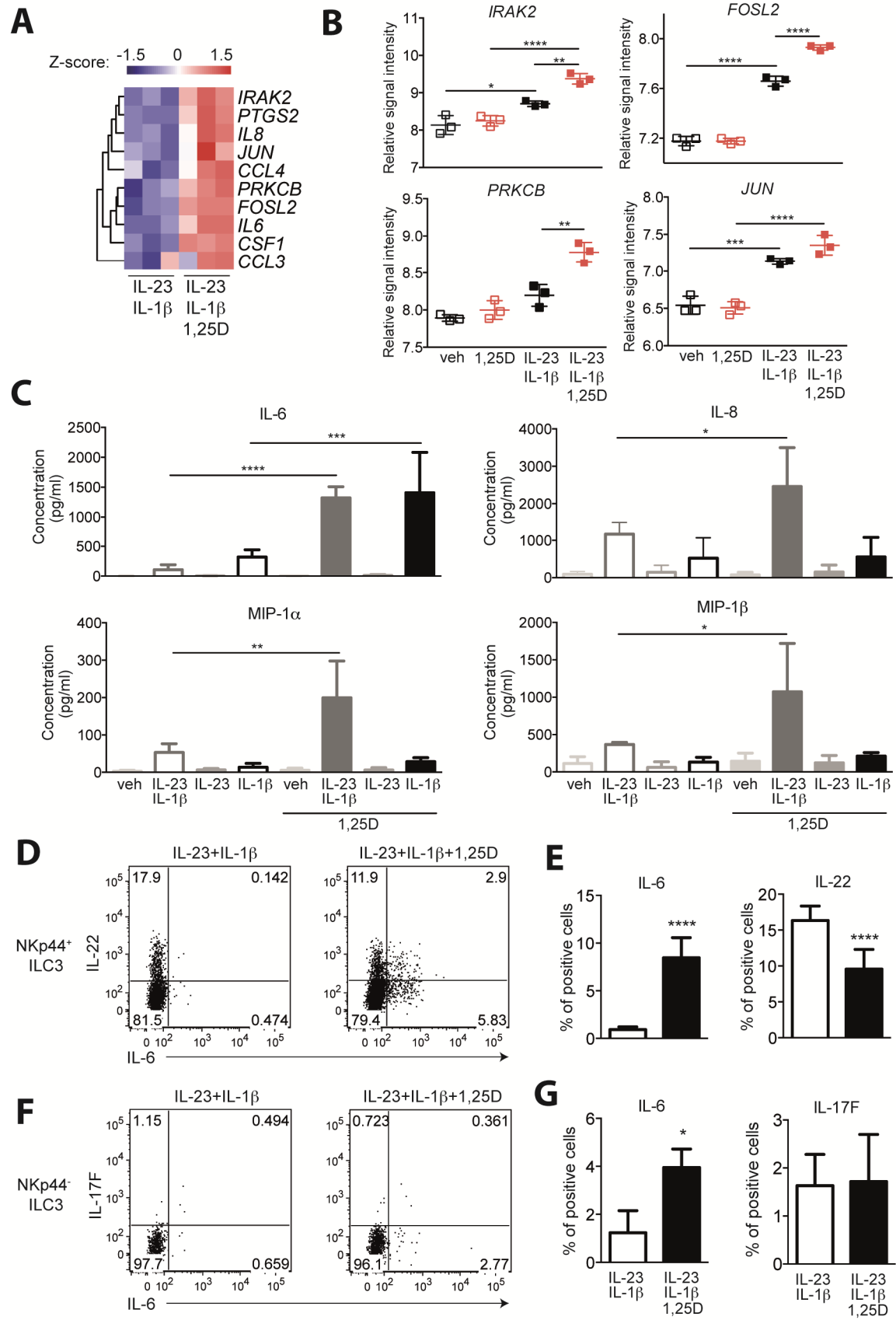
787 **Figure 8. IBD patients with active inflammation are vitamin D-deficient. 25D was**
 788 detected in plasma samples of the control group with Lynch syndrome (an inherited
 789 disorder that increases the risk of colorectal cancer), IBD patients with new diagnosis,
 790 chronic disease (diagnosed for longer than one year) or in remission. Mean±SD of
 791 25D concentration is shown (*p<0.05, **p<0.01).

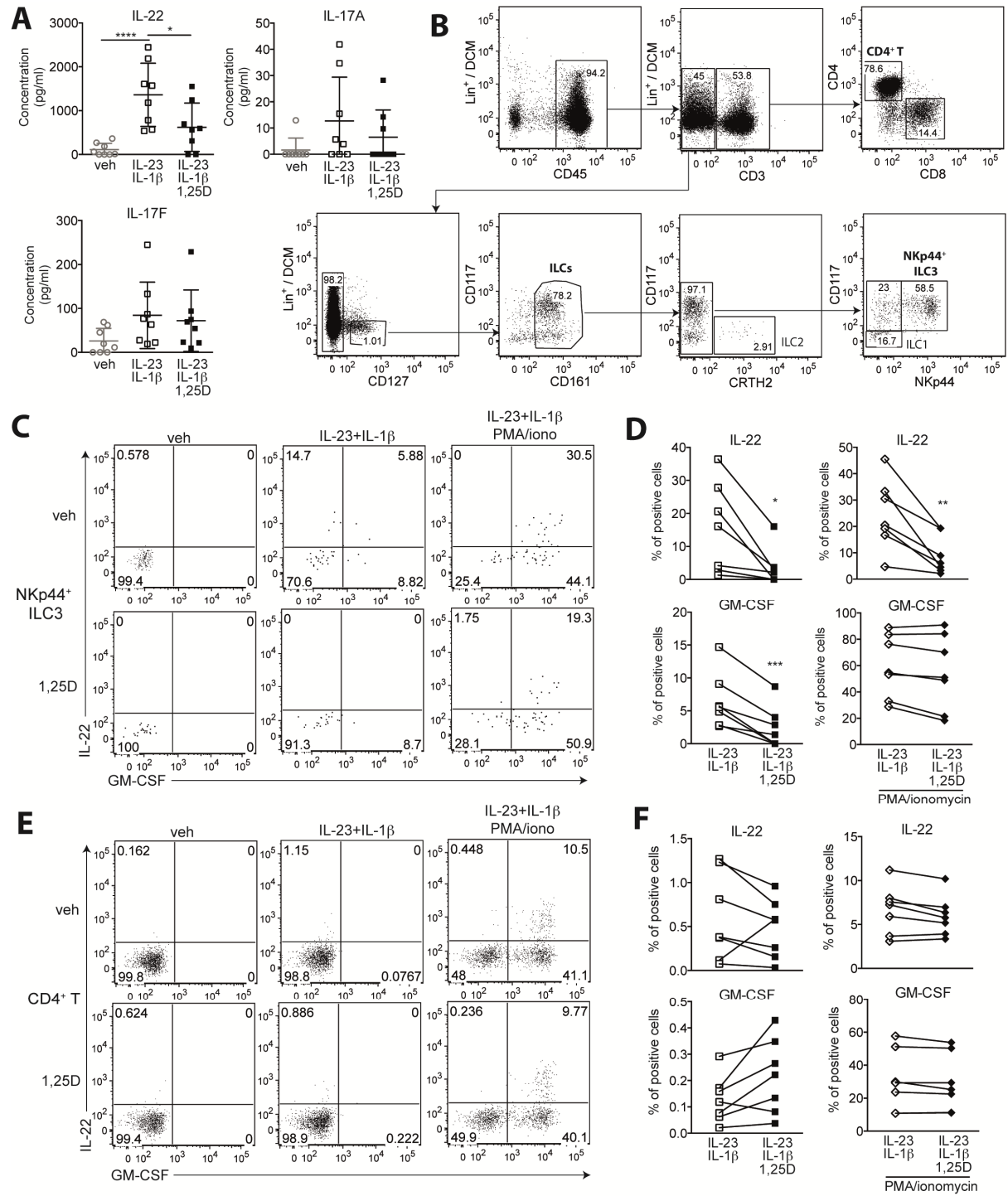


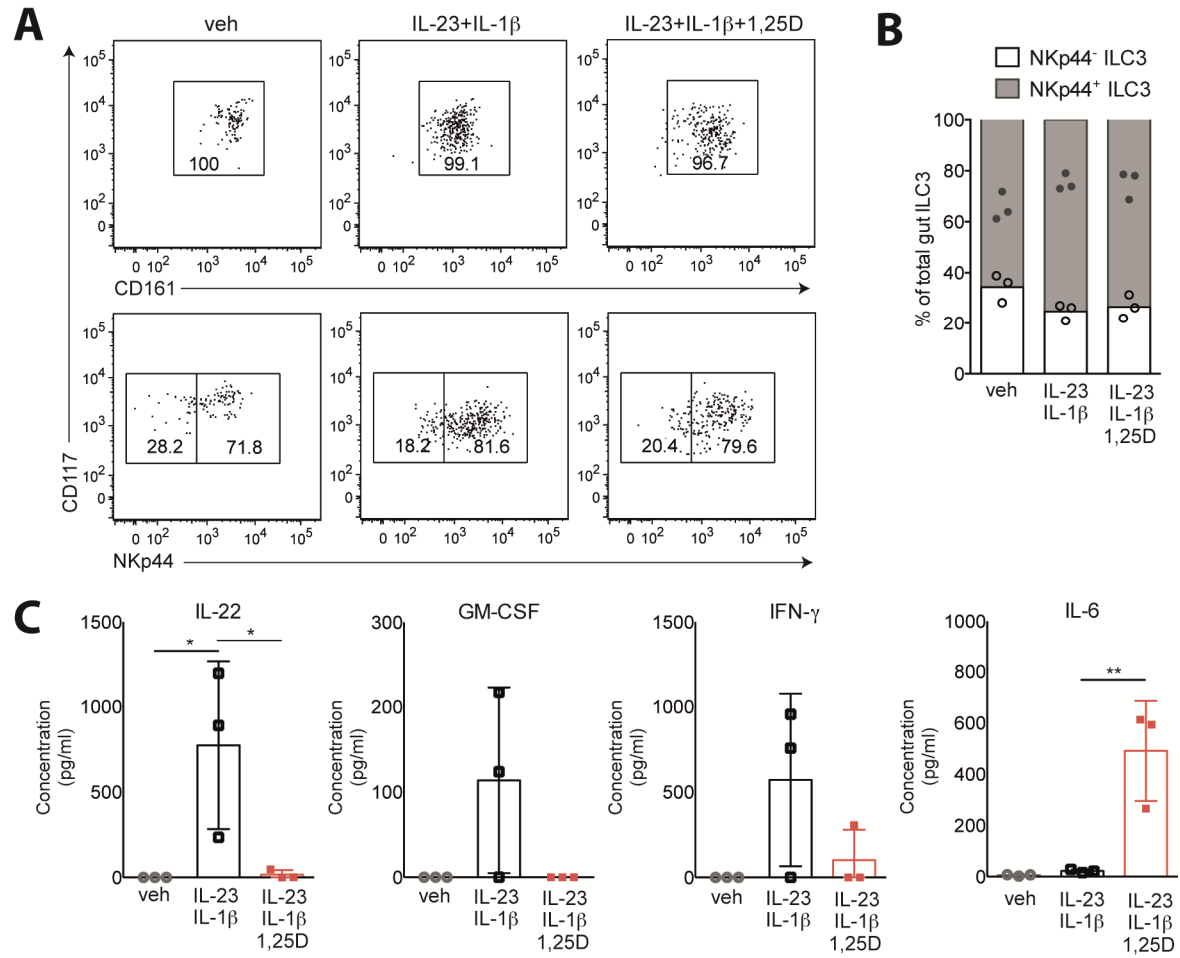


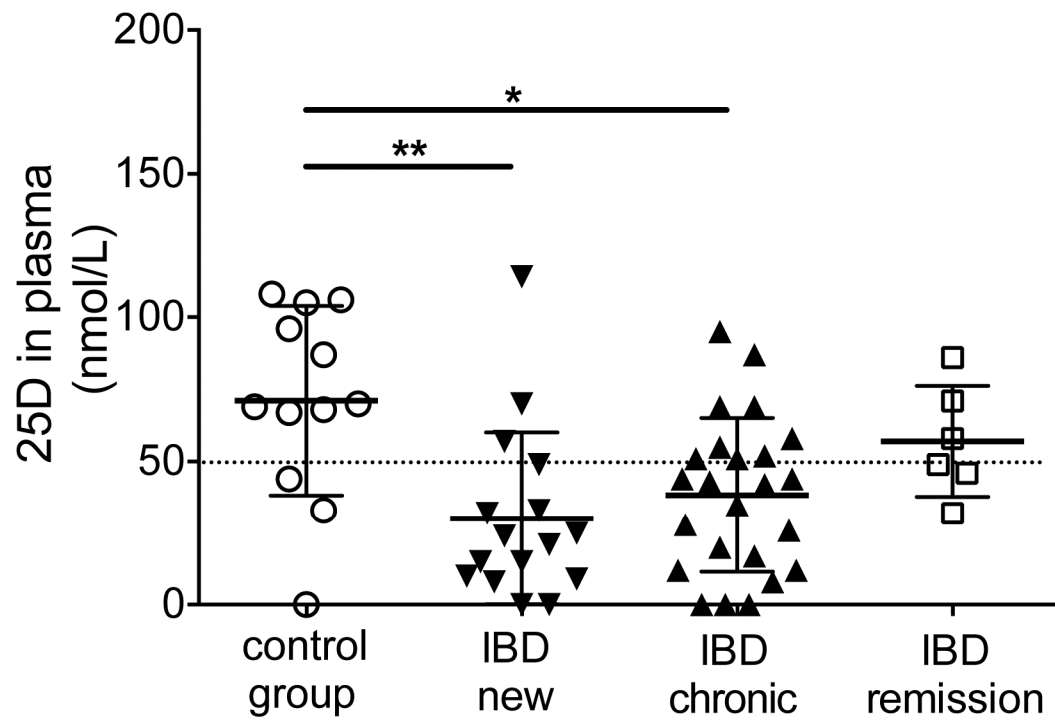












1 ONLINE REPOSITORY MATERIAL**2 Vitamin D downregulates the IL-23 receptor pathway in human**
3 mucosal ILC3

4

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28 **METHODS**

29 **Cell isolation from human tonsil and gut**

30 ILC3 and NK cells were isolated from fresh human tonsils from tonsillectomies of
31 adult or pediatric patients with obstructive sleep apnea syndrome, as previously
32 described ¹. Collection and experimental use of tonsil tissue was approved by the
33 regional ethical board at Karolinska Institutet. All patients, or parents if they
34 were younger than 18 years gave their informed consent. Shortly, tonsils were
35 mechanically disrupted and single cells were isolated followed by separation of
36 mononuclear cells (MNCs) by using lymphoprep and centrifugation. MNCs were
37 incubated with directly-labeled anti-CD3, anti-CD14 and anti-CD19 microbeads
38 (lineage markers) for 20 minutes according to manufacturer's instruction.
39 Lineage⁺ cells were depleted by using LD MACS columns (Miltenyi Biotech) and
40 Lineage⁻ MNCs were directly stained for flow cytometric sorting.

41 Gut ILC3 were isolated from biopsies obtained from non-inflamed ileum or colon
42 of adult IBD patients in remission (Table E1.). Written informed consents were
43 obtained from the patients. The study was approved by a regional ethical board
44 at Karolinska Institutet. In detail, biopsies were cut into small pieces and
45 enzymatically digested by incubation with DNase and collagenase II (each 250
46 ug/mL) in a 37°C water bath with magnetic stirring at 450 rpm for 30 minutes.
47 Single cells were filtered through 40 um cell strainer. In some experiments gut
48 single cell suspensions were directly used for *in vitro* stimulation. Otherwise,
49 isolated cells were immediately stained for flow cytometric sorting.

50

51 **Flow cytometric sorting of tonsil and gut ILC populations**

Lineage⁻ tonsil MNCs and gut single cell suspensions were resuspended in Yssels-supplemented IMDM (Gibco) with 1% NHS (Invitrogen), penicillin (100 U/mL, HyClone) and streptavidin (0.1 mg/mL, HyClone). Cells were incubated with the following antibodies for 30 minutes at 4°C. Lineage marker positive cells were defined by FITC-labeled antibodies: anti-CD14 (TÜK4, Dako); anti-CD1a (HI149), anti-CD3 (17A2), anti-CD34 (581), anti-CD123 (6H6), anti-FcεRIα (AER-37 CRA-1), anti-TCRα/β (IP26) and anti-TCRγ/δ (B1) (all Biolegend); anti-BDCA2 (AC144, Miltenyi Biotech) and anti-CD19 (4G7, BD Biosciences). Additionally we used BV605-conjugated anti-CD161 (HP-3G10), BV711-labeled anti-CD56 (HCD56) (both Biolegend); V450-labeled anti-CRTH2 (BM16), V500-labeled anti-CD45 (30-F11) (both BD Biosciences); PC5-labeled anti-NKp44 (Z231), PC5.5-labeled anti-CD117 (104D2D1), PC7-labeled anti-CD127 (R34.34) (all Beckman Coulter). In addition, cells were incubated with the LIVE/DEAD® Fixable Green Dead Cell Stain Kit (Life Technologies). Tonsil and gut ILC3 were sort-purified as lineage⁻CD45⁺CD127⁺CD117⁺CD161⁺CRTH2⁻ lymphocytes. Tonsil NK cells were sort-purified as lineage⁻CD45⁺CD127⁻CD117⁻CRTH2⁻CD161⁺CD56⁺ lymphocytes. All ILC sorts were performed using BD FACS Aria III or BD FACS Aria Fusion cell sorters and FACSDiva version 7. FACS data were analyzed by FlowJo version 9.9.

In vitro stimulation, intracellular staining, and analysis of proliferation

Sort-purified tonsil (10,000 – 20,000 cells per 96-well plate well in 200 uL) and gut (500 cells per 96-well plate well in 200 uL) ILCs and gut single cell suspensions (1,000,000 cells per 24-well plate well in 300 uL) were cultured in Yssels-supplemented IMDM with 1% NHS, penicillin, streptavidin and 1 U/mL IL-2 for 24h or 72h. Tonsil and gut ILC3 and gut single cell suspensions were

stimulated with 100 nM 1,25D (the active form of vitamin D, Sigma) or its vehicle (EtOH), 50 ng/mL IL-23 (R&D) plus 50 ng/mL IL-1 β (Peprotech) and 50 ng/mL IL-23 plus 50 ng/mL IL-1 β plus 100 nM 1,25D. In some cases we used 100 nM 25D (the inactive preform of 1,25D, Sigma). When indicated cells were additionally incubated with 25 ng/mL PMA plus 500 nM ionomycin for 6h. Tonsil NK cells were stimulated with 50 ng/mL IL-12 (Peprotech) plus 50 ng/mL IL-18 (R&D) for 24h or 72h.

Cell surface staining of sort-purified ILCs was performed by using V450-labeled anti-CRTH2 (BM16 BD Biosciences); BV605-conjugated anti-CD161 (HP-3G10), BV711-labeled anti-CD56 (HCD56) (both Biolegend); PC5-labeled anti-NKp44 (Z231), PC5.5-labeled anti-CD117 (104D2D1), PC7-labeled anti-CD127 (R34.34) (all Beckman Coulter). Additionally, cells were incubated with the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies). In some experiments cell surface expression of IL-23R was detected by using PE-labeled anti-IL-23R antibody (218213, R&D). IL-23R expression is shown as geometric MFI normalized to the vehicle treatment that represents the basal expression, i.e. corresponding to 100% expression.

Cell surface staining of gut single cell suspensions was performed by using FITC-labeled antibodies: anti-CD14 (TÜK4, Dako); anti-CD1a (HI149), anti-CD34 (581), anti-CD123 (6H6), anti-Fc ϵ R1 α (AER-37 CRA-1), anti-TCR α / β (IP26) and anti-TCR γ / δ (B1) (all Biolegend); anti-BDCA2 (AC144, Miltenyi Biotech) and anti-CD19 (4G7, BD Biosciences). Additionally, we used AF700-labeled anti-CD45 (30-F11), BV785-labeled anti-CD3 (OKT3), BV711-labeled anti-CD8 α (53-6.7), BV605-labeled anti-CD161 (HP-3G10), BV650-labeled anti-CD56 (HCD56) (all Biolegend); V450-labeled anti-CRTH2 (BM16), APC-Cy7-labeled anti-CD4 (RPA-

T4) (both BD Biosciences); PC5-labeled anti-NKp44 (Z231), PC5.5-labeled anti-CD117 (104D2D1), PC7-labeled anti-CD127 (R34.34) (all Beckman Coulter). Cells were also incubated with the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies).

Intracellular transcription factor staining was performed after 72h incubation with the above-mentioned stimuli. Cells were fixed and permeabilized by using the Foxp3/transcription factor staining buffer set (eBioscience) and were incubated with eFlour660-labeled anti-AHR (FF3399, eBioscience) and PE-labeled anti-RORγt (Q21-559 BD Biosciences) for 30 minutes at room temperature. Data are shown as geometric MFI normalized to the vehicle treatment that represents the basal expression, i.e. corresponding to 100% expression.

Intracellular cytokine staining was performed after 24h or 38h incubation with the above-mentioned stimuli, with Golgi plug and Golgi stop (both BD Biosciences) present in the last 5h. Cells were fixed by Cell fixation/permeabilization kit (BD Biosciences) followed by incubation with FITC-labeled anti-IL-6 (AS12), BV650-labeled anti-IL-17F (O33-782), PE-CF594-labeled anti-GM-CSF (BVD2-21C11), AF700-labeled anti-IFN-γ (B27) (all BD Biosciences); PE- or eFlour450-labeled anti-IL-22 (22URTI eBioscience) and APC-Cy7-labeled anti-IL-17A (BL168 BioLegend) for 30 minutes at room temperature.

Proliferation of ILC3 was assessed by using the CellTrace™ violet cell proliferation kit (Molecular Probes) according to manufacturers instruction. After loading ILC3 with the dye, cells were incubated with the above-mentioned stimuli for 72h. Cell surface and cell viability staining was performed as

described above for sort-purified tonsil ILC3. Cells were then fixed in 2% paraformaldehyde.

After performing the different staining protocols, cells were immediately acquired on a LSR Fortessa flow cytometer (BD Biosciences) by using FACSDiva version 7 and data were analyzed by FlowJo version 9.9.

ELISA, multi-Plex ELISA

Cell supernatants were collected after 24h or 72h of incubation with the above-mentioned stimuli. IL-22 was detected by Human IL-22 DuoSet ELISA (R&D), while IL-6, IL-8, IL-17A, IFN- γ , GM-CSF, MIP-1 α and MIP-1 β were detected by using Bio-Plex Pro human cytokine 27-plex kit or customized multi-Bio-Plex kit according to manufacturer's instruction (Bio-Rad). Samples were analyzed by a MagPix instrument (BioRad).

RNA isolation

Sort-purified ILC3 (100,000) were cultured in Ysels-supplemented IMDM with 1%NHS, penicillin, streptavidin and 1 U/mL IL-2 for 24h. Cells were treated in triplicates with vehicle (ethanol), 100 nM 1,25D, 50 ng/ml IL-23 plus IL-1 β or 100 nM 1,25D plus 50 ng/mL IL-23 plus IL-1 β . Total RNA was isolated by using RNeasy micro kit (Qiagen).

Microarray gene expression analysis

100 ng RNA was used for microarray analysis by Human Transcriptome Array 2.0 applying standard Affymetrix platform. All data correction and analysis described below were performed using R software. Log2-transformed

expression values were quantile-normalized to correct data distribution across samples. By PCA analysis, we observed samples clustered according to donors and not to experimental conditions that was corrected using SVA ComBat function ² for batch effect removal. Unknown sources of variation were removed using SVA package ² and differential expression was computed using Limma package ³. Transcript probes with fold change cut-off of 1.5 and $p < 0.01$ were considered differentially expressed. Heatmaps were displayed by using CRAN pheatmap package. All microarray gene expression data will be made publically available upon final acceptance of this paper.

Plasma sample collection from IBD patients

In this study, plasma from four different patient groups was collected and analyzed at the Gastromedical Center, Unit for Endoscopy at Karolinska University Hospital, Stockholm. All patients gave their informed consent to participate in this study, which was approved by a regional ethical board at Karolinska Institutet. The control group is represented by subjects below 50 years with Lynch syndrome, an inherited disorder that increases the risk of colorectal cancer (n=12). Two patient groups consisted of patients with active intestinal inflammation that were either newly diagnosed with IBD (n=16) or diagnosed with IBD for longer than one year (n=23). The fourth group of patients included IBD patients in remission without ongoing intestinal inflammation (n=7). For every patient the exact diagnosis of CD or UC, regular medication intake, age and gender were documented. Exclusion criteria for participation in this study were tumors or other immune disease of the intestine and partial colectomy. IBD patients with active inflammation were only included if they did

not take any substances besides 5-AZA within the last 3 months, i.e. no treatment with anti-TNF or thiopurines (including AZA treatment). In addition patients did not receive systemic corticosteroids within the last month.

Peripheral blood was collected in heparin tubes, and was centrifuged at 1500 g for 10 min to separate the plasma. Plasma samples were then aliquoted and stored frozen at -80 °C until analysis.

25D detection in plasma

Levels of 25D in plasma samples were analyzed by chemiluminescence immunoassay (CLIA) on a LIAISON-instrument (DiaSorin Inc, Stillwater, MN, USA) detectable range 7.5-175 nmol/L, CV 2-5% at the Department of Clinical Chemistry, Karolinska University Hospital.

Statistical analyses

Differences between two groups were analyzed by using Wilcoxon matched-pairs signed rank test, three or more groups were compared by one-way ANOVA and Tukey's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$) by using GraphPad Prism 6 Software. Experimental repeats are specified in the respective figure legends.

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SUPPLEMENTAL METHODS

IL-1R1 surface staining of CD127-enriched tonsil lymphocytes

Mononuclear cells (MNCs) were isolated from tonsils as described in the “Cell isolation from tonsil” methods description. MNCs were incubated with directly-labeled anti-CD3, anti-CD14 and anti-CD19 microbeads (lineage markers) for 20 minutes according to manufacturer’s instruction. Lineage⁺ cells were depleted by using LD MACS columns (Miltenyi Biotech) and Lineage⁻ MNCs were directly used for enriching CD127-expressing cells using the CD127 microbead kit and LS columns (Miltenyi Biotech) according to manufacturer’s instructions. For experiments 200,000-400,000 CD127-enriched cells were cultured in 96-well plate well in 200 uL Yssels-supplemented IMDM with 1% NHS, penicillin, streptavidin and 1 U/mL IL-2. CD127-enriched cells were stimulated with 100 nM 1,25D (Sigma) or its vehicle (EtOH), 50 ng/mL IL-23 (R&D) plus 50 ng/mL IL-1 β (Peprotech) and 50 ng/mL IL-23 plus 50 ng/mL IL-1 β plus 100 nM 1,25D for 24h.

For cell surface staining of CD127-enriched tonsil cells the following antibodies were used. PE-CF594-labeled anti-CRTH2 (BM16) (BD Biosciences); AF700-labeled anti-CD45 (HI30), BV421-labeled anti-CD14 (HCD14) and anti-CD19 (HIB19), BV605-labeled anti-CD161 (HP-3G10), BV711-labeled anti-CD56 (HCD56), BV785-labeled anti-CD3 (OKT3) (all Biolegend); PC5-labeled anti-NKp44 (Z231), PC5.5-labeled anti-CD117 (104D2D1), PC7-labeled anti-CD127 (R34.34) (all Beckman Coulter); IL-1R1 expression was detected by using the FITC-labeled anti-human IL-1R1 polyclonal goat antibody (Catalog#FAB269F, R&D). In addition, cells were stained with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies). Staining was performed for 30 min at room

234 temperature, followed by fixation with 2% PFA for 20 min and cells were
235 immediately acquired on an LSR Fortessa flow cytometer (BD Biosciences) by
236 using FACSDiva version 7 and data were analyzed by FlowJo version 9.9.

ACCEPTED MANUSCRIPT

SUPPLEMENTAL FIGURE LEGENDS

Figure E1. GO enrichment analysis of tonsil ILC3. **A.** Heatmap of transcripts regulated by 1,25D, IL-23 plus IL-1 β and IL-23 plus IL-1 β plus 1,25D stimulation compared to veh (vehicle)-treated ILC3. Microarray analysis was performed by Human Transcriptome Array in sorted ILC3 stimulated for 24h with the indicated stimuli (n=3, cut-off criteria: fold change of 1.5 and p<0.01). Z-score was calculated using normalized expression values.

Figure E2. 1,25D induces several transcriptional changes in IL-23 plus IL-1 β -activated tonsil ILC3. **A.** Heatmap of transcripts related to homing markers and motility regulated by 1,25D. **B.** Heatmap of transcripts associated to lipid metabolism regulated by 1,25D. **C.** Heatmap of apoptosis- and proliferation-related transcripts regulated by 1,25D. **D.** Heatmap of IL-23 plus IL-1 β treatment-induced differentially expressed genes. Z-score was calculated using normalized expression values. Microarray analysis was performed by Human Transcriptome Array in sorted ILC3 stimulated for 24h with the indicated stimuli (n=3).

Figure E3. 1,25D reduces IL-22, IL-17F and GM-CSF secretion of activated tonsil ILC3 after 24h incubation. **A.** IL-22, **B.** IL-17F and **C.** GM-CSF release of sorted tonsil ILC3 after 24h was analyzed by ELISA. Mean concentration +SD is shown ((*p<0.05, ***p<0.005, ****p<0.001, n=3).

Figure E4. 1,25D reduces IFN- γ secretion and intracellular expression in activated tonsil ILC3. **A.** IL-17A and **B.** IFN- γ release of sorted tonsil ILC3 after 72h incubation was analyzed by multiplex ELISA. Mean concentration $\pm$ SD is shown (** p <0.01, **** p <0.001, n =3). **C.** Intracellular expression of IFN- γ in NKp44⁺ and NKp44⁻ ILC3 was analyzed after 24h incubation with the indicated stimuli by flow cytometry. In some cases ILC3 were incubated in the presence of PMA (25 ng/mL) plus ionomycin (500 nM) in the last 6h. **D.** Bar graphs show the mean percentage of IFN- γ positive cells $\pm$ SD (n =3).

Figure E5. 1,25D does not affect IFN- γ and GM-CSF intracellular expression in activated tonsil NK cells. **A.** Intracellular expression of IFN- γ and GM-CSF in NK cells was analyzed after 24h incubation with the indicated stimuli by flow cytometry. In some cases NK cells were incubated in the presence of PMA (25 ng/mL) plus ionomycin (500 nM) in the last 6h. **B.** Bar graphs show the mean percentage of positive cells $\pm$ SD (n =5).

Figure E6. The inactive form of vitamin D 25D inhibits the IL-23 plus IL-1 β -activated ILC3 function. **A.** IL-22 and **B.** GM-CSF release of sorted tonsil ILC3 after 72h incubation was analyzed by ELISA. Mean concentration $\pm$ SD is shown (** p <0.01, *** p <0.005, **** p <0.001, n =3). **C.** Representative plots of Ahr and ROR γ t expression in NKp44⁺ and NKp44⁻ ILC3 after 72h incubation with the indicated stimuli analyzed by flow cytometry (n =5). **E.** Bar graphs show normalized geometric MFI. Bars represent mean $\pm$ SD (* p <0.05, n =3).

Figure E7. 1,25D does not influence the IL-1R1 expression in activated ILC3.

A. IL-1R1 expression after 24h incubation was analyzed by flow cytometry on gated NKp44⁺ and NKp44⁻ ILC3 in CD3/CD14/CD19-depleted and CD127-enriched tonsil mononuclear cell suspensions. **B.** Normalized geometric MFI of IL-1R1 expression in ILC3. Dot plots show mean $\pm$ SD (**p<0.01, ***p<0.005 n=3).

Figure E8. 1,25D inhibits IL-22 and GM-CSF expression in IL-23 plus IL-1 β -activated tonsil ILC3, however 1,25D does not affect GM-CSF production after PMA plus ionomycin treatment.

A. Intracellular expression of IL-22 and GM-CSF in NKp44⁺ ILC3 cells was analyzed after 24h incubation with the indicated stimuli by flow cytometry. In some cases cells were incubated in the presence of PMA (25 ng/mL) plus ionomycin (500 nM) (shown in blue) in the last 6h. **B.** Bar graphs show the mean percentage of positive cells $\pm$ SD (**p<0.01, ****p<0.001, n=5).

Figure E9. 1,25D inhibits IL-22 in intestinal activated NKp44⁻ ILC3 but not in CD8⁺ T cells.

Intracellular expression of IL-22 and GM-CSF was analyzed on gated NKp44⁻ ILC3 (**A**) and on CD8⁺ T cells (**C**) after 24h incubation with the indicated stimuli. In some cases cell suspensions were incubated in the presence of PMA (25 ng/mL) plus ionomycin (500 nM) in the last 6h (n=7). **B.** Individual percentages of positive NKp44⁻ ILC3 cells are shown (*p<0.05, n=7). **D.** Individual percentages of positive CD8⁺ T cells are shown (n=7).

Table E1. Biopsies were collected from IBD patients in remission.

UC indicates ulcerative colitis, CD indicates Crohn's disease. 25D concentration is

309 displayed as nmol/L, NA – not applicable. 25D plasma levels are displayed in
310 Figure 8.

311

312 **Table E2. Information of patients recruited for plasma 25D level detection.**

313 CD indicates Crohn's disease, UC indicates ulcerative colitis. 25D concentration is

314 displayed as nmol/L, ND – not detected. 25D plasma levels are displayed in

315 Figure 8.

Patient	Diagnosis	Gender	Age	25D	Medication
IBD remission - <i>Figure 6, 8 and Figure E6</i>					
1	UC	female	35	71	prednisolon, imurel, prograf
2	CD	male	41	32	-
3	CD	female	48	58	-
4	UC	male	66	46	colazid
5	CD	male	37	86	asacol
6	UC	male	70	49	salazopyrin
7	UC	male	38	60	dipentum, asacol, colifoam
<i>Figure 7</i>					
8	UC	male	27	NA	-
9	UC	female	39	NA	sulfasalazin
10	CD	male	31	NA	-
11	CD	male	51	NA	puri-nethol
12	CD	male	49	NA	asacol

Patient	Diagnosis	Gender	Age	25D	Medication
Control group with Lynch syndrome					
1	-	male	33	ND	-
2	-	male	53	44	-
3	-	female	46	105	-
4	-	female	35	67	-
5	-	female	44	96	-
6	-	female	50	70	-
7	-	female	39	106	-
8	-	female	48	68	-
9	-	female	41	108	-
10	-	male	29	87	-
11	-	male	43	69	-
12	-	female	32	33	-

Patient	Diagnosis	Gender	Age	25D	Medication
Newly diagnosed IBD patients					
1	CD	female	21	8	-
2	CD	female	49	10	-
3	CD	female	38	21	-
4	CD	male	32	57	-
5	CD	female	33	15	-
6	CD	female	30	49	-
7	UC	male	30	ND	-
8	UC	male	23	ND	-
9	UC	male	56	24	asacol
10	UC	female	20	114	mesasal
11	UC	male	34	32	-
12	UC	female	21	70	-
13	UC	female	62	15	-
14	UC	female	38	33	-
15	UC	male	49	25	asacol
16	UC	male	50	9	asacol

Patient	Diagnosis	Gender	Age	25D	Medication
Chronic IBD patients					
1	CD	female	30	8	colazid
2	CD	male	36	ND	asacol
3	CD	female	53	17	azathioprim, salazopyrin
4	CD	male	78	12	salazopyrin
5	CD	female	34	87	-
6	CD	female	44	44	-
7	CD	male	20	55	salazopyrin
8	CD	male	48	35	asacol
9	CD	female	30	20	-
10	CD	male	52	42	asacol
11	CD	male	29	44	-
12	CD	female	23	95	pentasa
13	UC	male	62	ND	-
14	UC	male	39	12	pentasa, asacol
15	UC	male	23	52	asacol
16	UC	male	22	69	-
17	UC	female	57	51	-
18	UC	female	39	43	-
19	UC	male	40	ND	asacol
20	UC	male	34	58	colazid
21	UC	female	46	28	-
22	UC	male	59	69	salazopyrin
23	UC	female	40	51	asacol

