

## APPENDIX Hecker et al.

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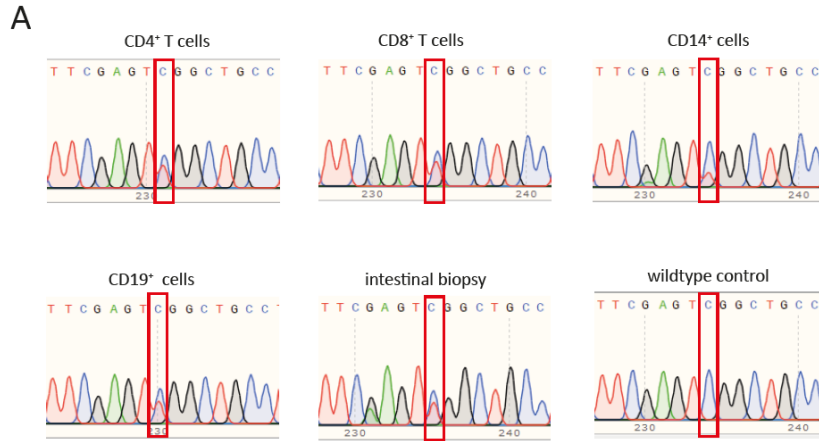
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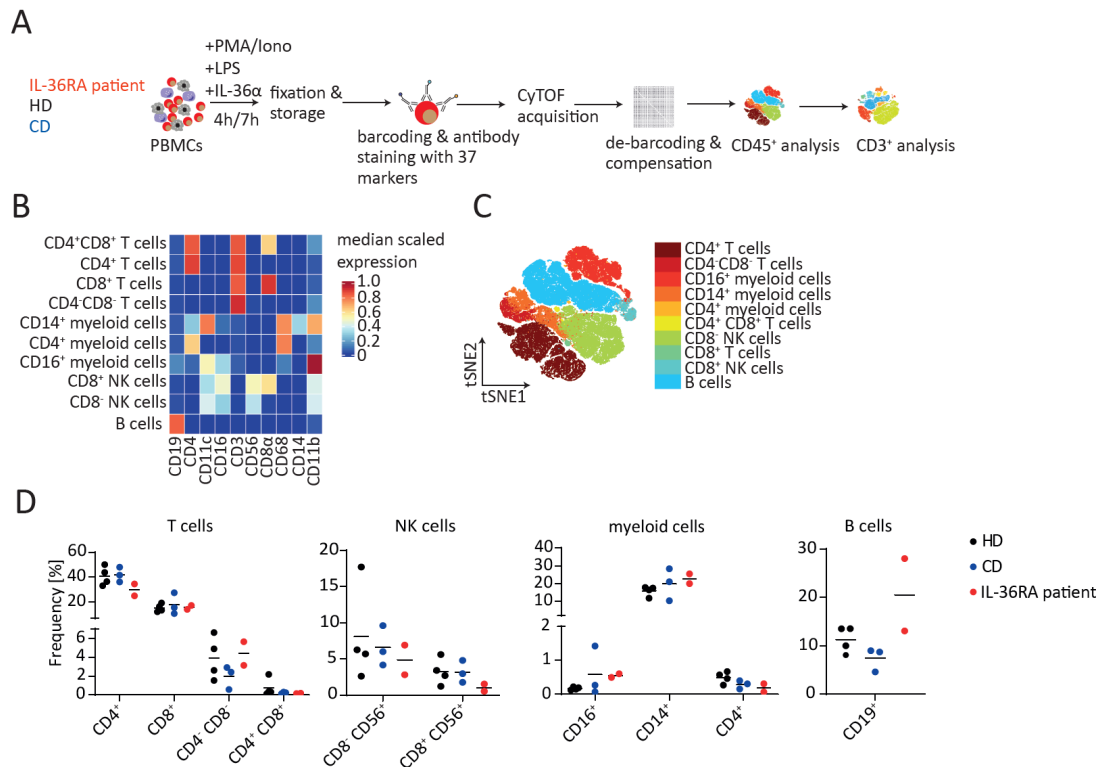
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**Appendix Figure S1: Sanger Sequencing of sorted cells of the IL-36RA patient**

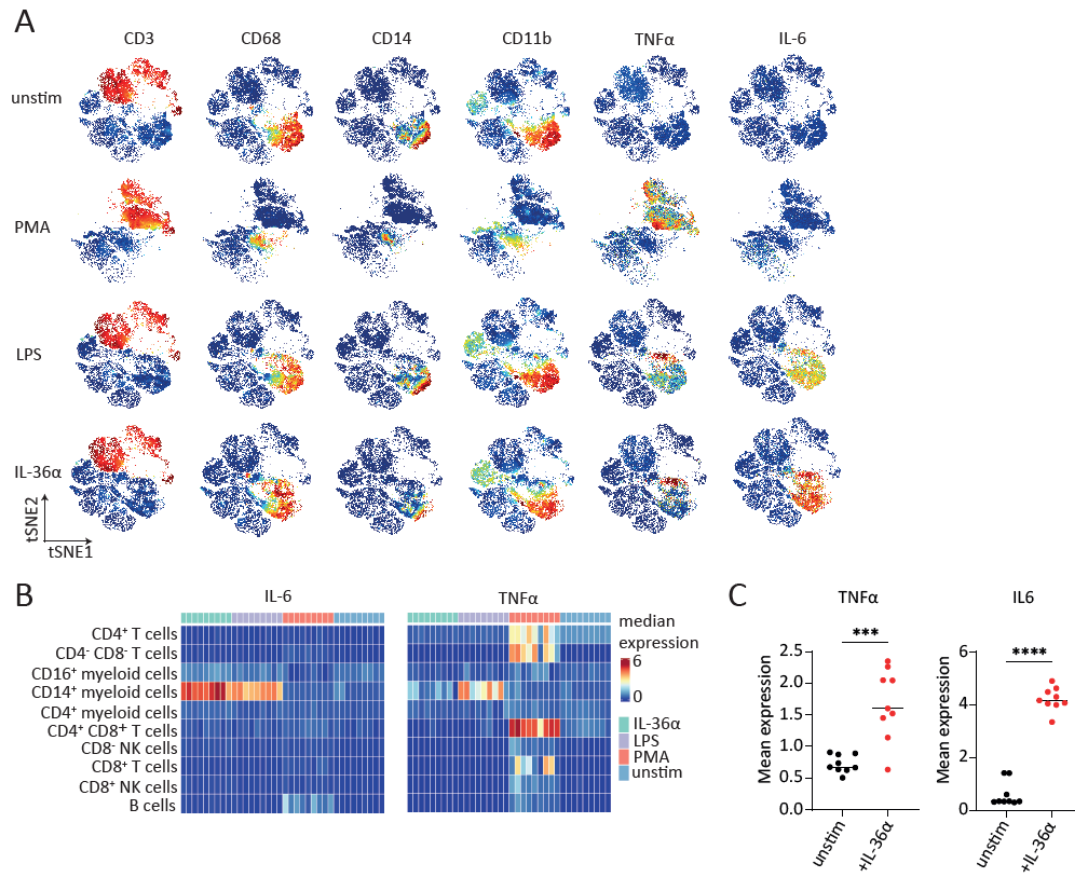
**(A)** Sanger sequencing of sorted CD4<sup>+</sup>, CD8<sup>+</sup>, CD14<sup>+</sup> and CD19<sup>+</sup> cells and of an intestinal biopsy of the *IL36RN*-mutated patient as well as of PBMCs of an unrelated control (wildtype control). The red square indicates the position of the mutation (c.338C) in the *IL36RN* gene.



**Appendix Figure S2: Deep immune cell profiling of the IL-36RA patient by mass cytometry**

**(A)** Schematic summary of the immune cell characterization of the *IL36RN*-mutated patient (IL-36RA patient) by mass cytometry. PBMCs of the IL-36RA patient at two different time points, Crohn's disease patients (CD) and healthy donors (HD) were *in vitro* stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Iono) or lipopolysaccharide (LPS) for 4 h or with IL-36 $\alpha$  for 7 h and fixed. Subsequently, PBMCs were stained with 37 metal-conjugated antibodies and acquired by mass cytometry (CyTOF). Resulting FCS files were de-barcoded, compensated and gated on CD45<sup>+</sup> cells. Following, reduced-dimensional (2D) t-SNE maps were generated, clusters were identified and differential expression and abundance was analyzed. **(B)** Heat map showing the expression of 10 selected markers used for clustering of CD45<sup>+</sup> PBMCs. **(C)** t-SNE plot colored by the 10 identified clusters. **(D)** Frequency of the 10 different clusters in unstimulated PBMCs. Line in the plots indicates the median. HD: n = 4, CD: n = 3, IL-36RA patient: n = 2 (same patient, different time points).



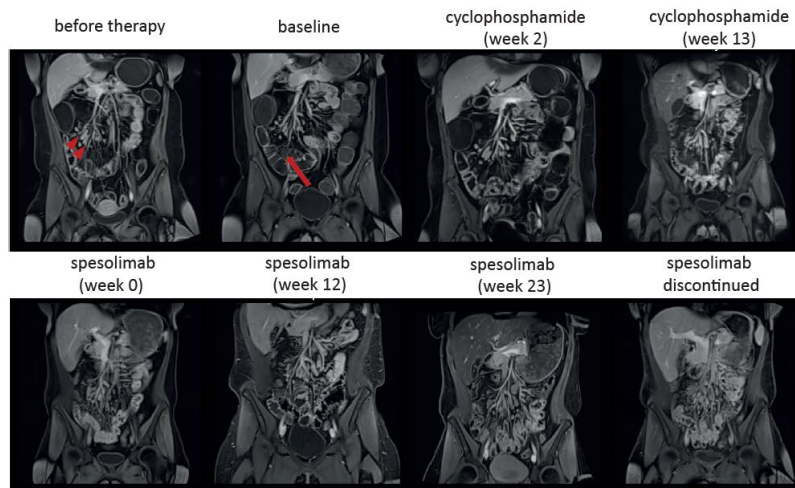


**Appendix Figure S5: Myeloid cells are the main responders to IL-36α stimulation in the blood**

**(A-C)** Peripheral blood mononuclear cells (PBMCs) of Crohn's disease patients and healthy donors (n= 9) were stimulated *in vitro* with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Iono) or lipopolysaccharide (LPS) for 4 h or with IL-36α for 7 h. Subsequently, the samples were analyzed by mass cytometry. **(A)** t-SNE plots colored by the expression of selected markers.

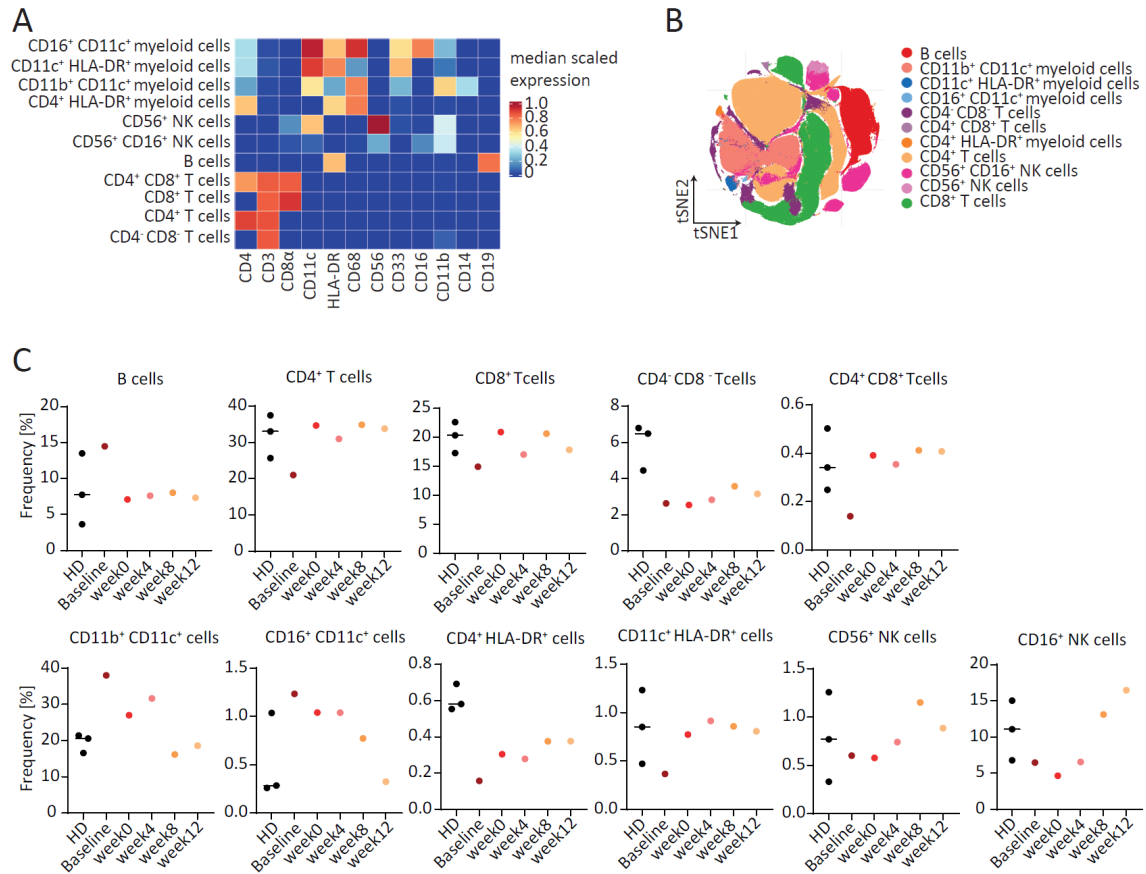
**(B)** Heatmaps showing the expression of IL-6 and TNFα in different subsets identified in PBMCs. **(C)** Mean expression of TNFα and IL-6 in CD14<sup>+</sup> myeloid cells. Statistical significance was determined by paired t test. \*\*\*\*p<0.0001, \*\*\*p<0.001, \*\*p<0.01, \*p<0.05.

A



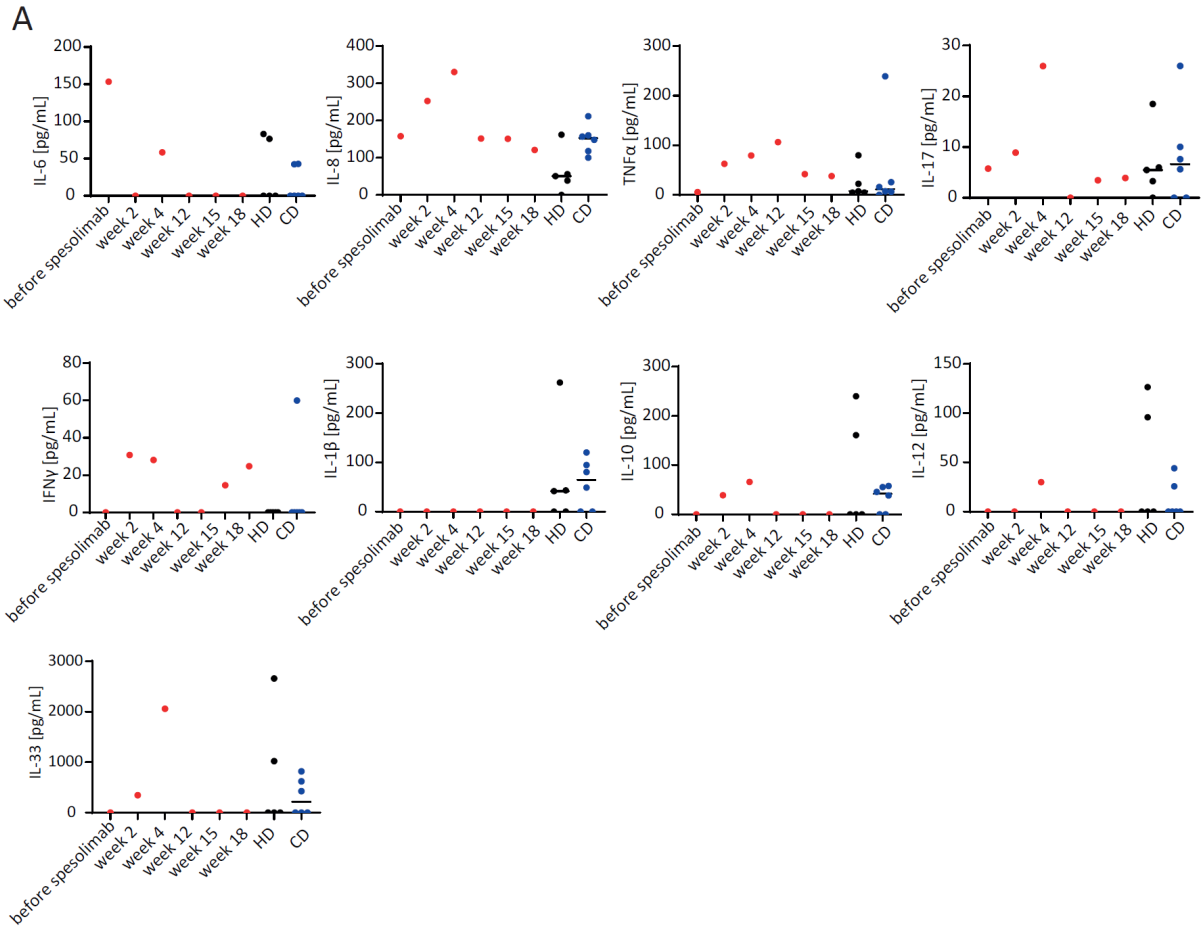
**Appendix Figure S6: MRI scans of the IL-36RA patient before and during spesolimab therapy**

**(A)** T1 weighted, fat-saturated MRI after i.v. contrast administration in coronal plane. There is a moderately pronounced mesenteric lymphadenopathy (arrowheads) and a Comb-sign in the mid-abdomen (arrows) with otherwise unremarkable visualization of the mesenteric vascular tree over the course of 12 months. Segmentally inflamed bowel loops were not seen at any time.



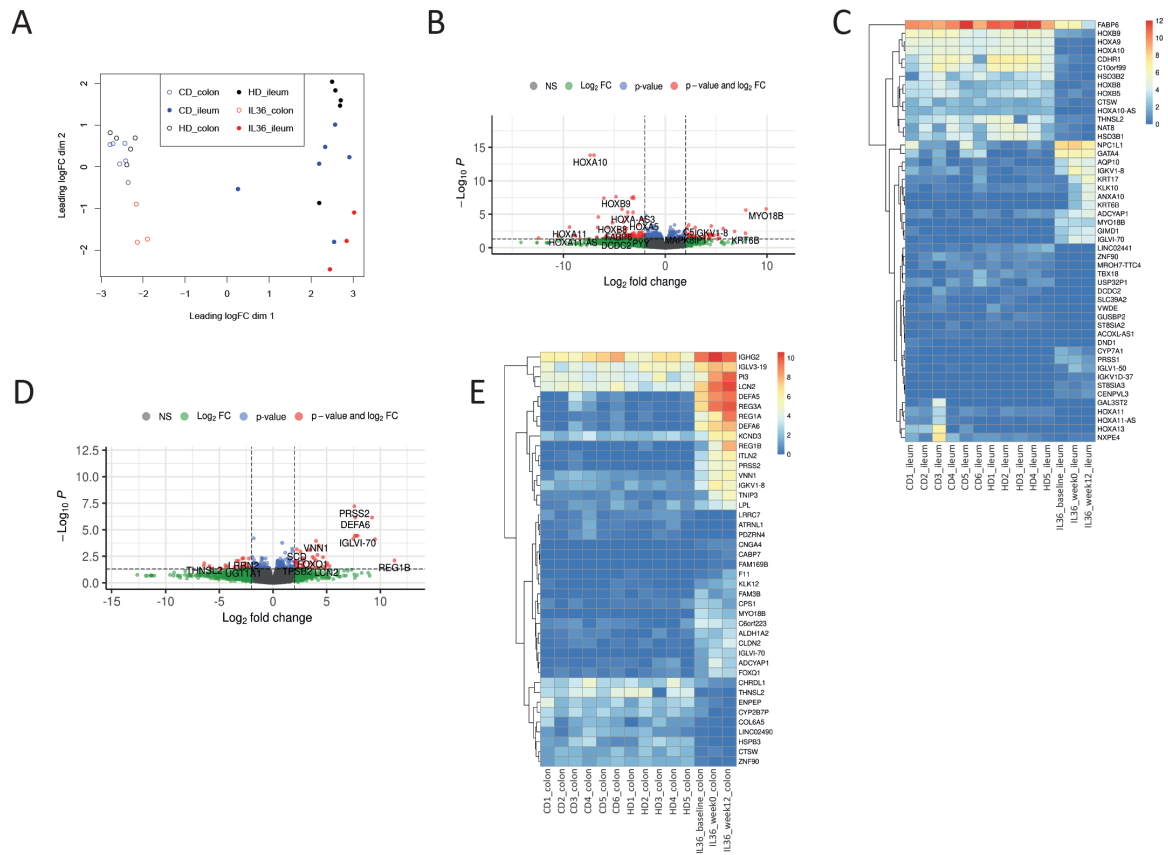
**Appendix Figure S7: Changes in the immune cell composition of the IL-36RA patient during spesolimab therapy**

**(A-C)** Peripheral blood mononuclear cells (PBMCs) of the *IL36RN*-mutated patient (IL-36RA patient) at different time points before and during spesolimab therapy and of healthy donors (HD) were *in vitro* stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Iono) or lipopolysaccharide (LPS) for 4 h or with IL-36α for 7 h and subsequently analyzed by mass cytometry **(A)** Heat map showing the expression of 12 selected markers used for clustering of CD45<sup>+</sup> PBMCs. **(B)** t-SNE plot colored by the 11 identified clusters. **(C)** Frequency of the 13 different clusters in unstimulated PBMCs. Line in the plots indicates the median. HD: n = 3, IL-36RA patient: n = 1.



**Appendix Figure S8: Changes in serum cytokine levels of the IL-36RA patient during spesolimab therapy**

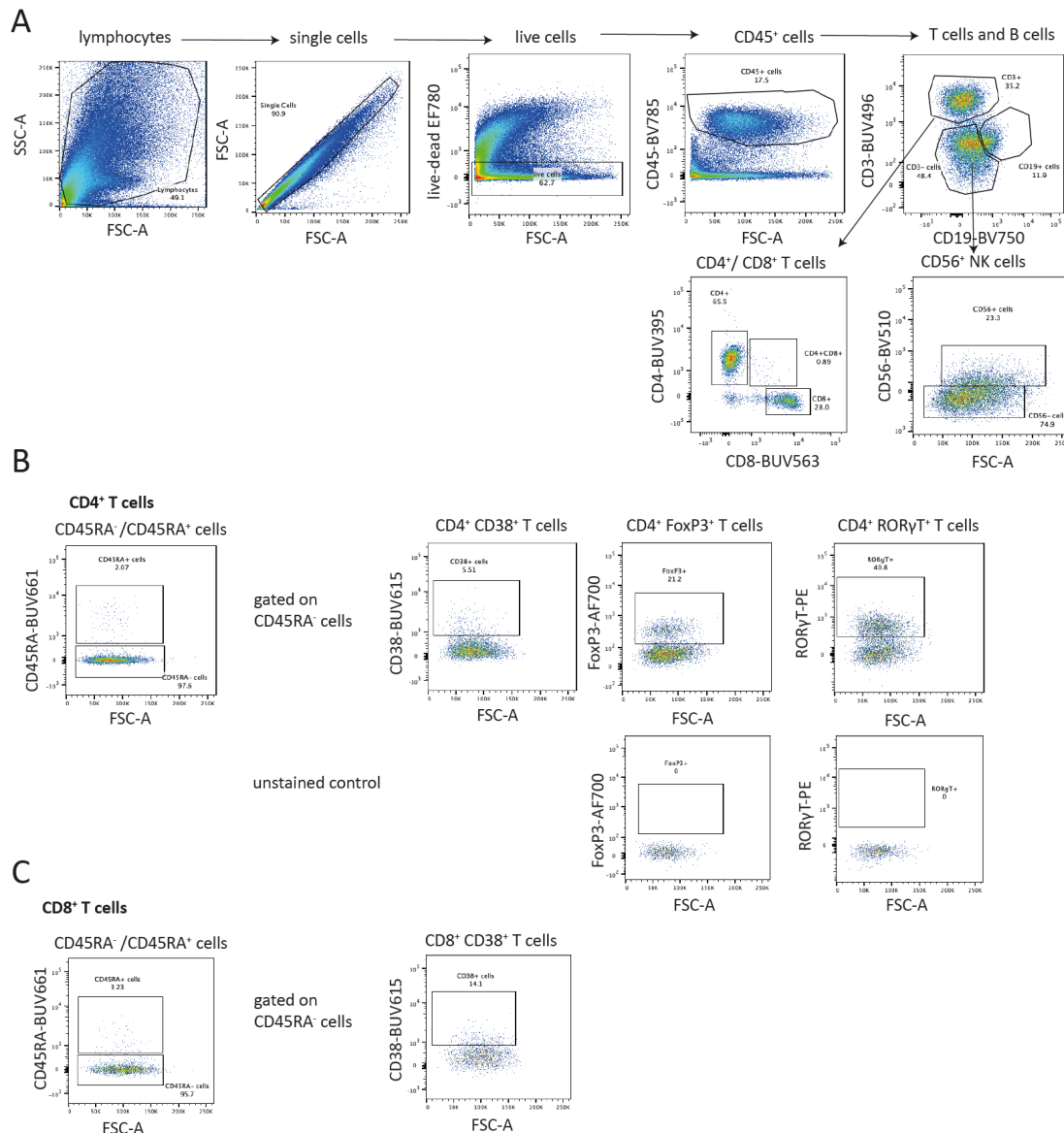
**(A)** The concentration of pro-inflammatory cytokines in the serum of healthy donors (HD), Crohn's disease patients (CD) and the *IL36RN*-mutated patient (IL-36RA patient) at different time points before and during spesolimab therapy. HD: n = 5, CD: n= 6, IL-36RA patient: n= 1.



**Appendix Figure S9: RNA sequencing of intestinal biopsies of the IL-36RA patient**

**(A)** MDS plot of RNA sequencing data from intestinal biopsies of the *IL36RN*-mutated patient (IL-36RA patient), Crohn's disease patients (CD) and healthy donors (HD). **(B)** Volcano plot of RNA sequencing analysis of ileal biopsies of the IL-36RA patient compared to CD patients (padjusted<0.05, log<sub>2</sub>FC cutoff = 2) **(C)** Heatmap showing the normalized expression of selected genes in ileal biopsies (padjusted<0.05, log<sub>2</sub>FC cutoff = 4). **(D)** Volcano plot of RNA sequencing analysis of colonic biopsies of the IL-36RA patient compared to CD patients (padjusted<0.05, log<sub>2</sub>FC cutoff = 2). **(E)** Heatmap showing the normalized expression of selected genes in colonic biopsies (padjusted<0.05, log<sub>2</sub>FC cutoff = 3). HD: n = 5, CD: n = 6, IL-36RA patient: n = 3 (same patient, different time points).





**Appendix Figure S10: Gating strategy for the analysis of different immune cell subsets in LPMCs by flow cytometry**

**(A)** Gating strategy for the identification of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, CD19<sup>+</sup> B cells and CD56<sup>+</sup> NK cells in lamina propria mononuclear cells (LPMCs) isolated from biopsies of the ileum and colon of the *IL36RN*-mutated patient (IL-36RA patient) and other Crohn's disease (CD) patients. **(B)** Identification of different subsets among CD4<sup>+</sup> T cells in LPMCs of the IL-36RA patient and other CD patients. **(C)** Identification of different subsets among CD8<sup>+</sup> T cells in LPMCs of the IL-36RA patient and other CD patients.

**Appendix Table S1: Treatment history of the IL-36RA patient**

Age [years]	Treatment	Reason for discontinuation
19	Azathioprine	Intolerance: vomiting
20	6-mercaptopurine	Intolerance: pancreatitis
20	Adalimumab	Secondary treatment failure
20	Infliximab	Secondary treatment failure
20	Andante (anti-IL-6)	Treatment failure
20	Methotrexate	Primary treatment failure
20	Protective ileostomy	
21	Ustekinumab	Secondary treatment failure
21	Vedolizumab	Secondary treatment failure
21-22	Certolizumab	Secondary treatment failure
22-23	Methotrexate	Treatment failure
22	Ileal segment resection	
23-24	Golimumab	Treatment failure
25	Autologous stem cell transplantation	
25	Ileocecal resection	
25-27	Adalimumab	Tertiary loss of response
27	Infliximab	Fulminant allergic reaction
27	Golimumab	Therapy failure
27-28	Upadacitinib	Secondary treatment failure
28	Methotrexate	
28	Protective ileostomy	
29	Spesolimab therapy	Discontinuation of therapy due to small perianal abscess
29	Risankizumab	

**Appendix Table S2: SES-CD score and weight of the IL-36RA patient during spesolimab therapy**

Table showing the weight and the Simple Endoscopic Score for Crohn's Disease (SES-CD) of the *IL36RN*-mutated patient before and during treatment with spesolimab.

Timepoint	Weight [kg]	SES-CD
Baseline	Not assessed	12
Week 0 (after cyclophosphamide)	56,4	9
Week 2 of spesolimab treatment	56,4	Not assessed
Week 4 of spesolimab treatment	54,7	Not assessed
Week 8 of spesolimab treatment	56,0	Not assessed
Week 12 of spesolimab treatment	55,0	6
Week 15 of spesolimab treatment	54,0	Not assessed
Week 18 of spesolimab treatment	53,9	Not assessed

**Appendix Table S3: p-values from the statistical analysis conducted in this study**

Figure panel and comparison	Statistical test and p-value
Figure 1C	Ordinary One-way ANOVA, Tukey's multiple comparisons test
WT vs. S113L	0.0002
WT vs. untrans.	<0.0001
WT vs. H <sub>2</sub> O	<0.0001
S113L vs. untrans.	0.0161
S113L vs. H <sub>2</sub> O	0.0125
untrans. vs. H <sub>2</sub> O	0.9995
Figure 1D	Ordinary One-way ANOVA. Tukey's multiple comparisons test
WT vs. S113L	<0.0001
WT vs. untrans.	<0.0001
S113L vs. untrans.	0.7577
Figure 3B	Ordinary One-way ANOVA. Tukey's multiple comparisons test
WT vs. S113L	0.0011
WT vs. P76L	0.008
WT vs. L133I	0.5912
WT vs. H <sub>2</sub> O	<0.0001
WT vs. untrans.	<0.0001
S113L vs. P76L	0.9715
S113L vs. L133I	0.0637
S113L vs. H <sub>2</sub> O	0.0648
S113L vs. untrans.	0.052
P76L vs. L133I	0.2811
P76L vs. H <sub>2</sub> O	0.0105
P76L vs. untrans.	0.0081
L133I vs. H <sub>2</sub> O	<0.0001
L133I vs. untrans.	<0.0001
H <sub>2</sub> O vs. untrans.	>0.9999
Figure 3C	Ordinary One-way ANOVA. Tukey's multiple comparisons test
WT vs. S113L	<0.0001
WT vs. P76L	<0.0001
WT vs. L133I	<0.0001
WT vs. untrans.	<0.0001
S113L vs. P76L	>0.9999
S113L vs. L133I	<0.0001
S113L vs. untrans.	0.9994
P76L vs. L133I	<0.0001
P76L vs. untrans.	0.9993
L133I vs. untrans.	<0.0001
Figure EV1C	Ordinary One-way ANOVA. Tukey's multiple comparisons test
TNF $\alpha$ unstim. vs. spesolimab	>0.9999
TNF $\alpha$ unstim. vs. IL-36 $\alpha$	<0.0001

TNF $\alpha$ unstim. vs. IL-36 $\alpha$ + spesolimab	<0.0001
TNF $\alpha$ spesolimab vs. IL-36 $\alpha$	<0.0001
TNF $\alpha$ spesolimab vs. IL-36 $\alpha$ + spesolimab	<0.0001
TNF $\alpha$ IL-36 $\alpha$ vs. IL-36 $\alpha$ + spesolimab	<0.0001
IL-6 unstim. vs. spesolimab	>0.9999
IL-6 unstim. vs. IL-36 $\alpha$	<0.0001
IL-6 unstim. vs. IL-36 $\alpha$ + spesolimab	<0.0001
IL-6 spesolimab vs. IL-36 $\alpha$	<0.0001
IL-6 spesolimab vs. IL-36 $\alpha$ + spesolimab	<0.0001
IL-6 IL-36 $\alpha$ vs. IL-36 $\alpha$ + spesolimab	<0.0001
IL-23 unstim. vs. spesolimab	>0.9999
IL-23 unstim. vs. IL-36 $\alpha$	<0.0001
IL-23 unstim. vs. IL-36 $\alpha$ + spesolimab	0.0300
IL-23 spesolimab vs. IL-36 $\alpha$	<0.0001
IL-23 spesolimab vs. IL-36 $\alpha$ + spesolimab	0.0300
IL-23 IL-36 $\alpha$ vs. IL-36 $\alpha$ + spesolimab	<0.0001

**Appendix Table S4: Sample IDs of control samples for bulk RNA sequencing**

<b>Sample ID used in this paper</b>	<b>Sample ID in IBDome database</b>
HD1_ileum	ibdome_berlin:20008a500
HD1_colon	ibdome_berlin:20008a501
HD2_ileum	ibdome_berlin:20008a502
HD2_colon	ibdome_berlin:20008a503
HD3_ileum	ibdome_berlin:20008a505
HD3_colon	ibdome_berlin:20008a506
HD4_ileum	ibdome_berlin:20008a507
HD4_colon	ibdome_berlin:20008a508
HD5_ileum	ibdome_berlin:20008a511
HD5_colon	ibdome_berlin:20008a512
CD1_ileum	ibdome_berlin:20008a409
CD1_colon	ibdome_berlin:20008a410
CD2_ileum	ibdome_berlin:20008a416
CD2_colon	ibdome_berlin:20008a417
CD3_ileum	ibdome_berlin:20008a420
CD3_colon	ibdome_berlin:20008a421
CD4_ileum	ibdome_berlin:20008a430
CD4_colon	ibdome_berlin:20008a431
CD5_ileum	ibdome_berlin:20008a453
CD5_colon	ibdome_berlin:20008a454
CD6_ileum	ibdome_berlin:20008a457
CD6_colon	ibdome_berlin:20008a458

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