Response to therapy evaluation

Response of immune cytopenia to sirolimus therapy is categorized as complete response (CR), partial response (PR) and no response (NR) based on previously published criteria[1].

Flow cytometry

Immunophenotyping of peripheral blood mononuclear cells from patients with **m-IC** and HC was performed using flow cytometry. Fluorochrome conjugated monoclonal antibodies and live dead dye to exclude dead cells are listed in supplemental Table 3. Flow cytometry data was captured on BD FACSymphony™ A5 and analyzed by FlowJo software version 10. Description of cell populations used in this study were listed in supplemental Table 3. Total cTfh cells were defined as $CD4^+$ $CD45RA^-$ $CXCR5^+$ T cells. PD-1^+ cTfh or cTfh cell population was defined as $CD4^+$ $CD45RA^-$ $CXCR5^+$ PD-1^+.

Cytokine/chemokine profiling

Plasma concentrations of soluble CD40 ligand (sCD40L), Interferon-gamma (IFN-γ), C-X-C motif chemokine ligand 9 (CXCL9), CXCL10, Interleukin-2 (IL-2), IL-7, IL-10, IL-18, IL-21 and IL-12p40 were determined using multi-analyte panel on Luminex platform.

Statistical analysis

Shapiro-Wilk test was used for testing the normality. When comparing more than two groups, one-way ANOVA with Tukey's multiple comparison tests was used for normally distributed data. For non-normal distribution, Kruskal-Wallis test with Dunn's multiple comparison test was used. Paired data was compared using paired t test or Wilcoxon signed rank test. GraphPad Prism v8 was used to perform statistical analysis.

Supplement Figure legends

Supplemental Figure 1. Timeline distribution of follow-up in patients with m-IC on sirolimus therapy. Timeline plots represent the duration of follow-up from the start of sirolimus therapy. # represents a patient with poor adherence to sirolimus (<3 ng/mL).

Supplemental Figure 2. Comparison of different immune parameters in patients with m-IC on sirolimus therapy. (A-C) Percentage of HLA-DR$^+$ CD38$^+$, PD-1$^+$ Tim3$^+$ and CD57$^+$ expression on CD8$^+$ EM T cells showing CD8$^+$T cell activation, exhaustion, and senescence in HC and **m-IC** patients. (D-F) Data represent frequencies of Th1, Th2 and Th17 populations gated on CD4$^+$ T cells in HC and **m-IC** patients. (G-I) Dot plots showing frequencies of cTfh1, cTfh2 and cTfh17 gated on cTfh population in HC and **m-IC** patients. Dot plots represent ratios between proportion of Th1 and Th2 (J) and between cTfh1 and cTfh2 populations (K).

Supplemental Figure 3. Comparison of immunoglobulin profiles and absolute number of lymphocytes in patients with m-IC on sirolimus therapy. (A-C) Plots showing levels of immunoglobulins (IgA, IgG and IgM) in patients with **m-IC (n = 10)** before and after sirolimus therapy. (D-H) Plots showing absolute number of B cells, CD4$^+$ T, CD8$^+$ T, lymphocytes (ALC) and NK cells in patients with **m-IC (n = 10)** before and after sirolimus therapy.

Supplemental Figure 4. Evaluation of sirolimus response on different immune subsets in patients with m-IC based on absence or presence of genetic mutations. (A-E) Patients with m-IC were segregated based on presence (gene+) or absence (gene-) of pathogenic or likely pathogenic genetic variants. Plots showing percentage of cTfh, total cTfh, cTfh activation, exhaustion and senescence gated on CD45RA$^-$CD4$^+$ T cells in m-IC sub-groups. (F-G) Plots showing percentage of CD4$^+$ and CD8$^+$ T cell activation gated on CD4$^+$ and CD8$^+$ effector memory T cells in m-IC sub-groups.

Supplemental Figure 5. Longitudinal assessment of immune parameters in patients with m-IC on sirolimus therapy. Data represents the longitudinal follow up of 2 patients with **m-IC** at two different time points based on frequencies of cTfh, CD4$^+$ T cell activation, CD$^+$ T naïve and CSMB populations.

Supplemental Figure 6. Visual abstract showing response to sirolimus therapy in patients with m-IC in terms of cytopenia, lymphoproliferation and immune-phenotypes.

Reference

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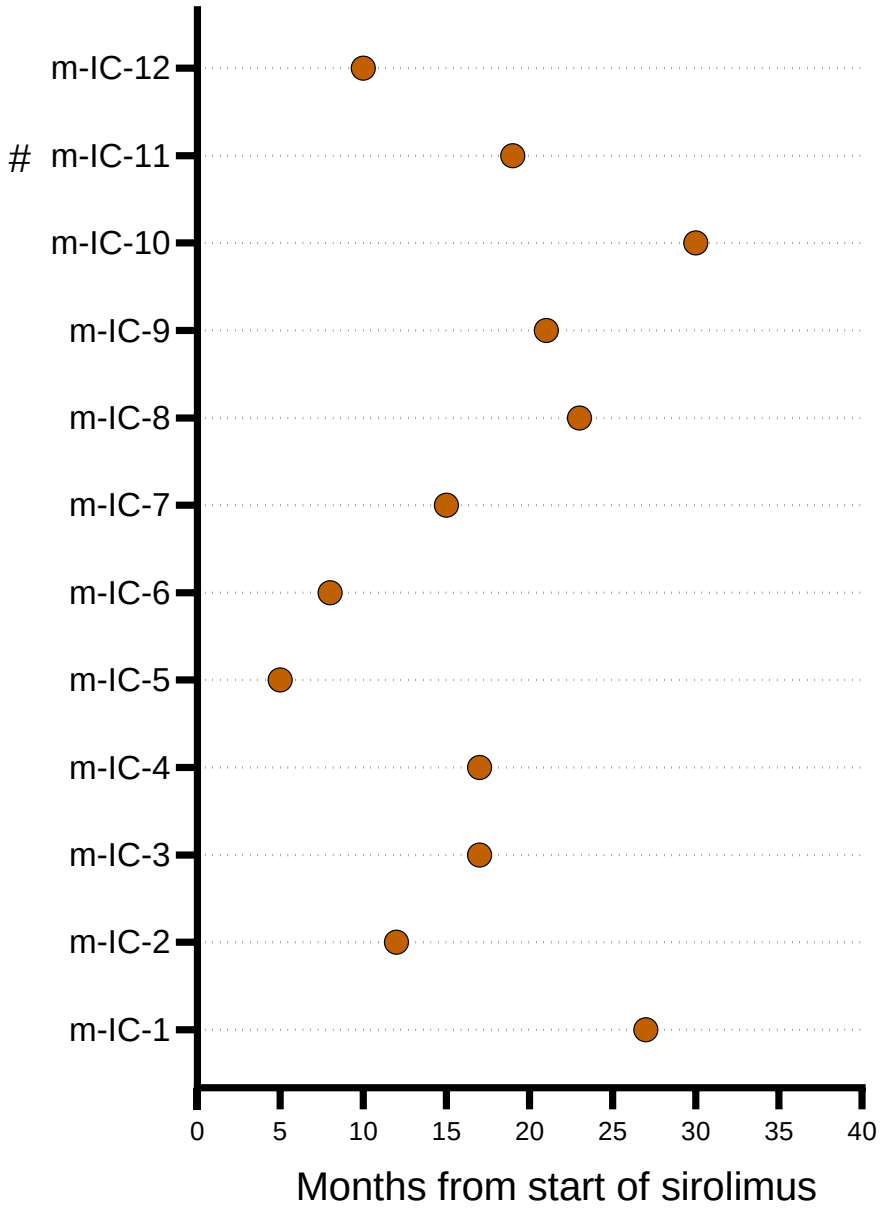
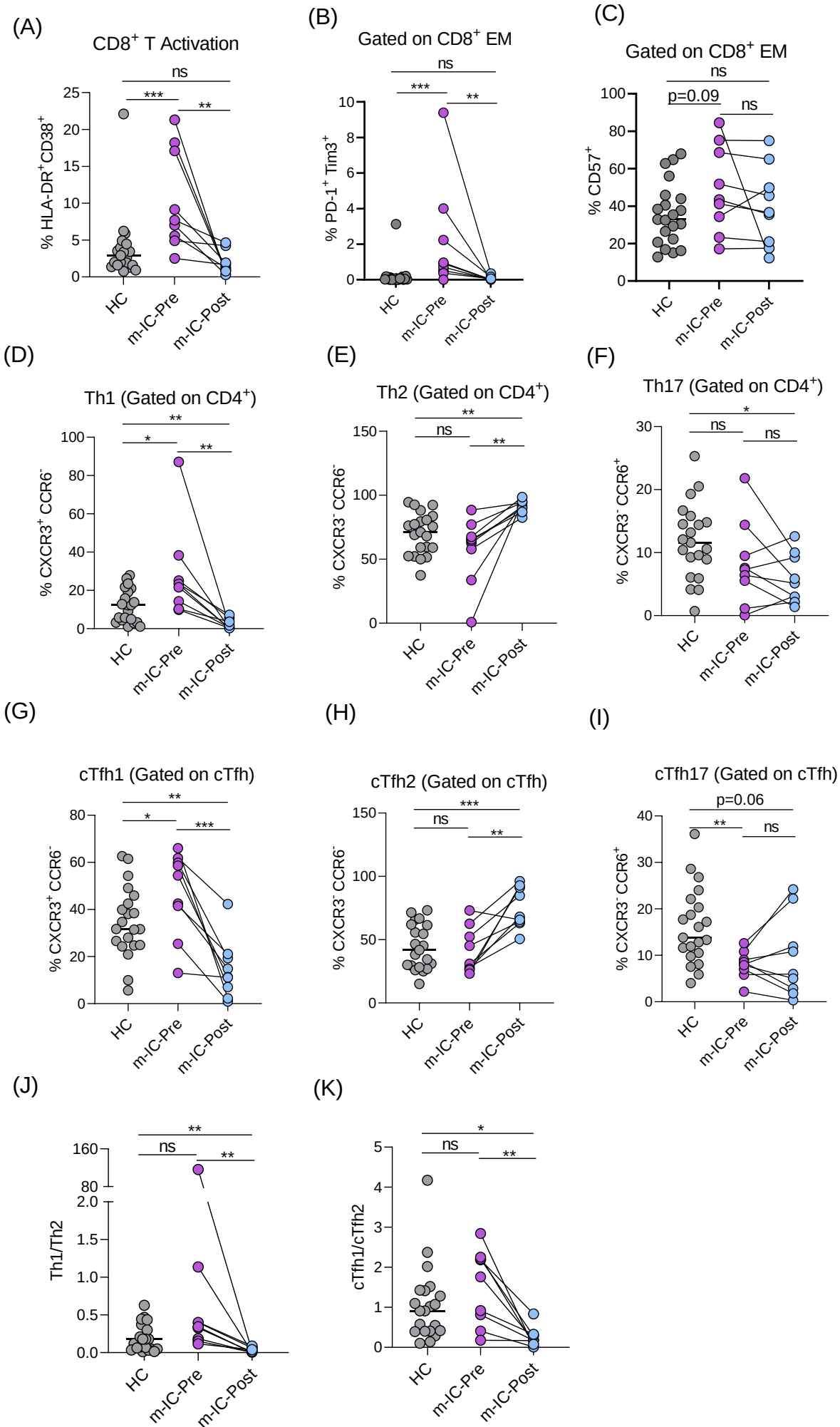
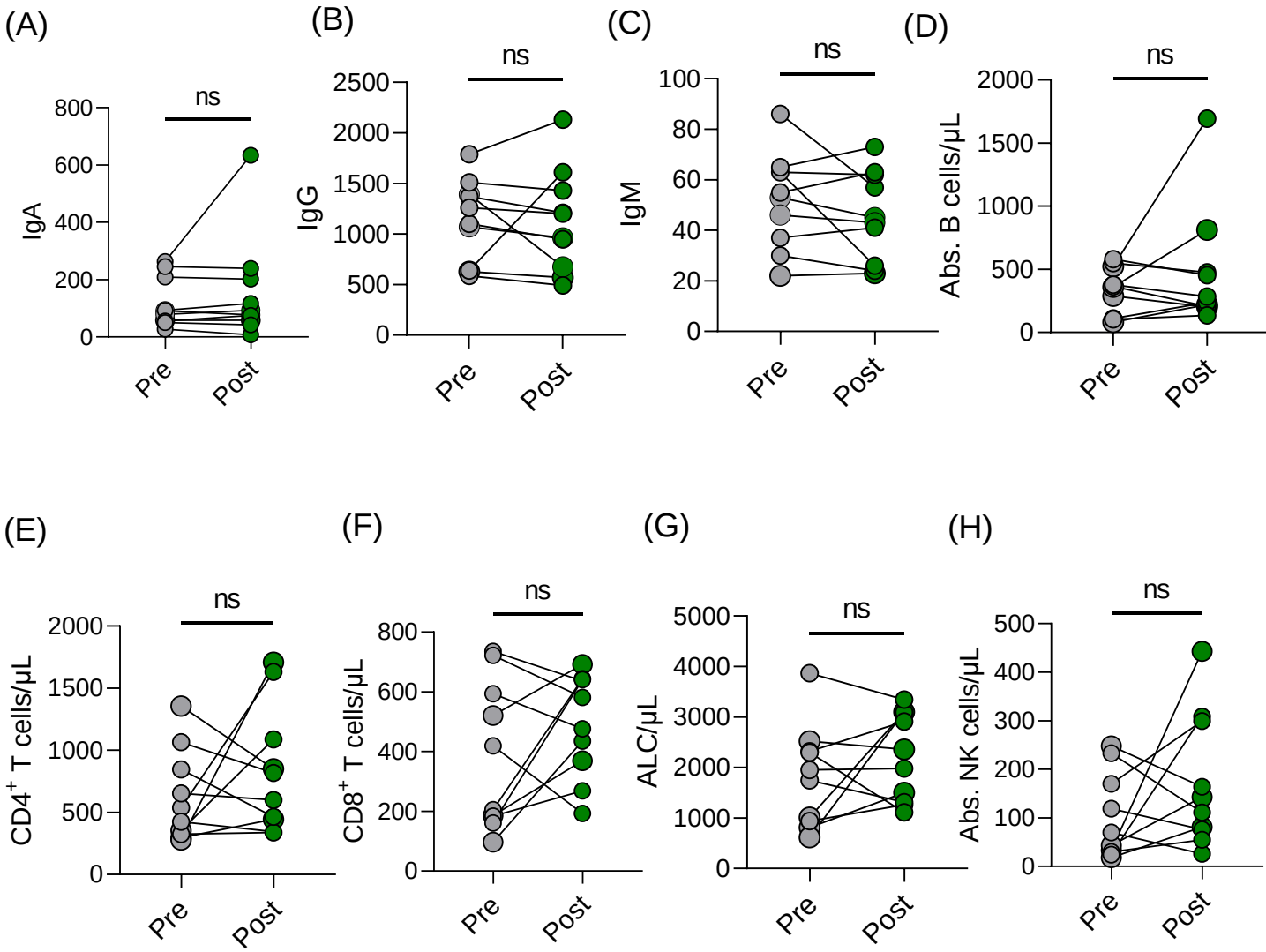
Supplemental Figure 1.



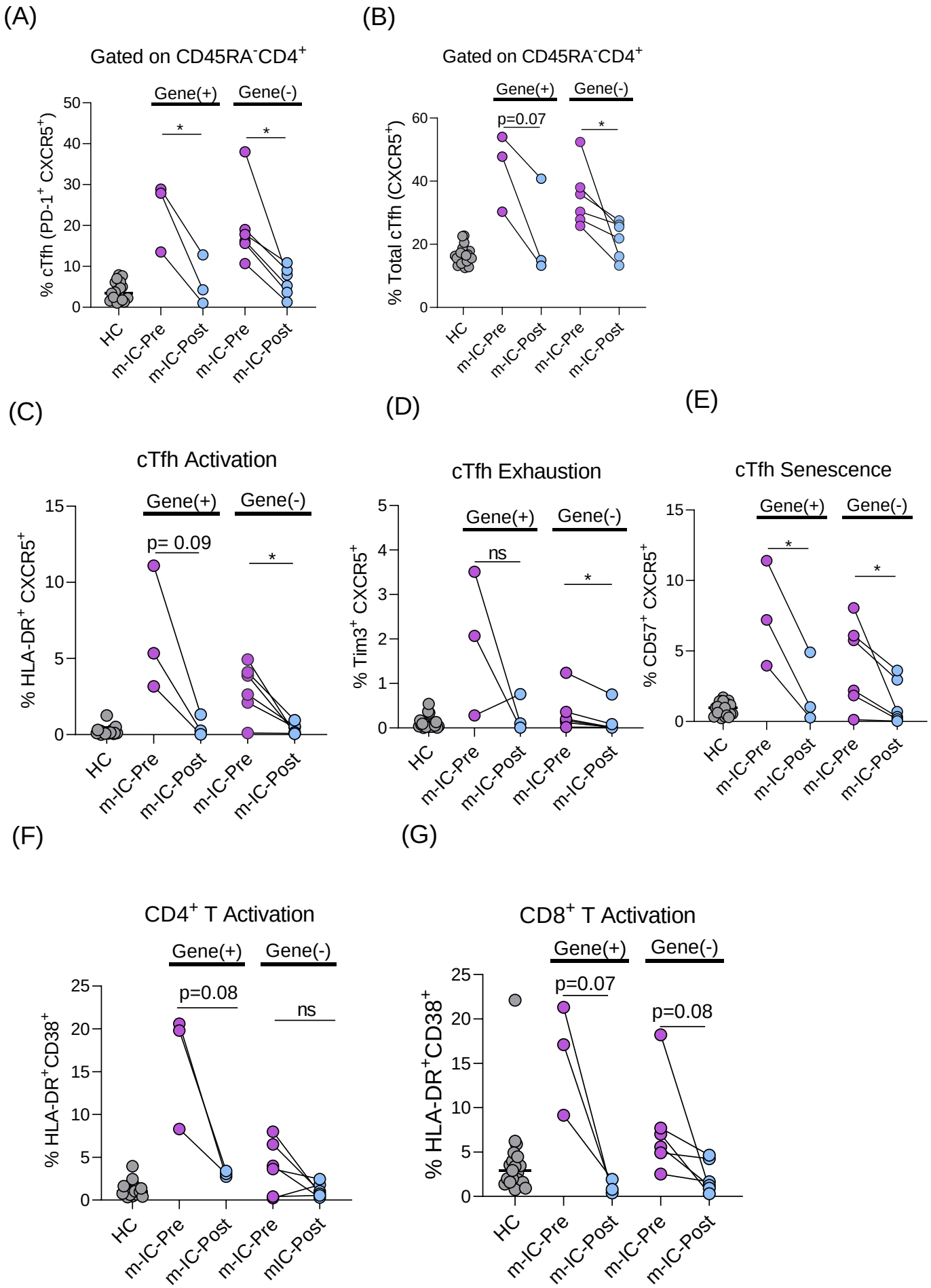
Supplemental Figure 2.



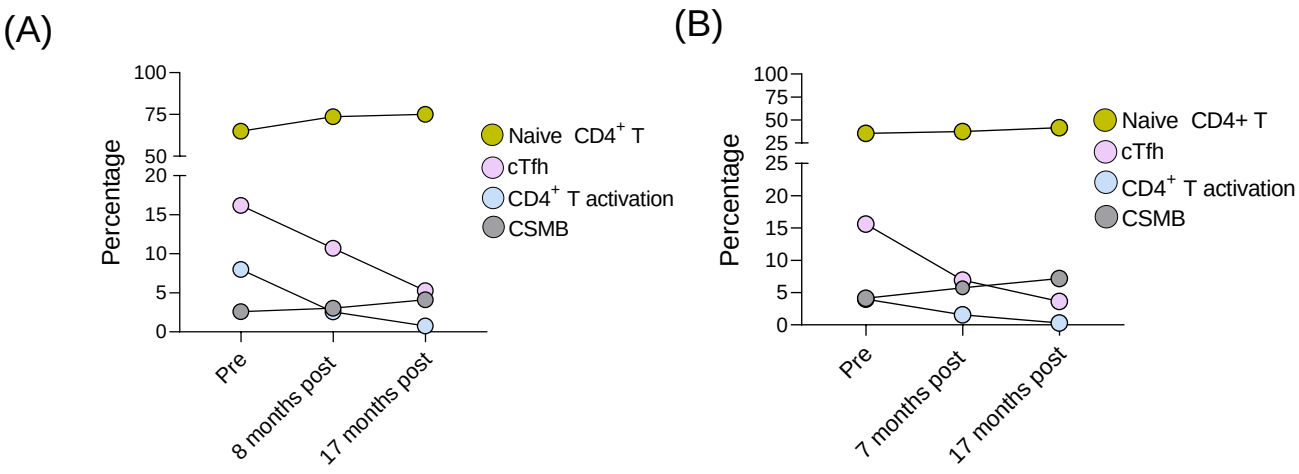
Supplemental Figure 3.



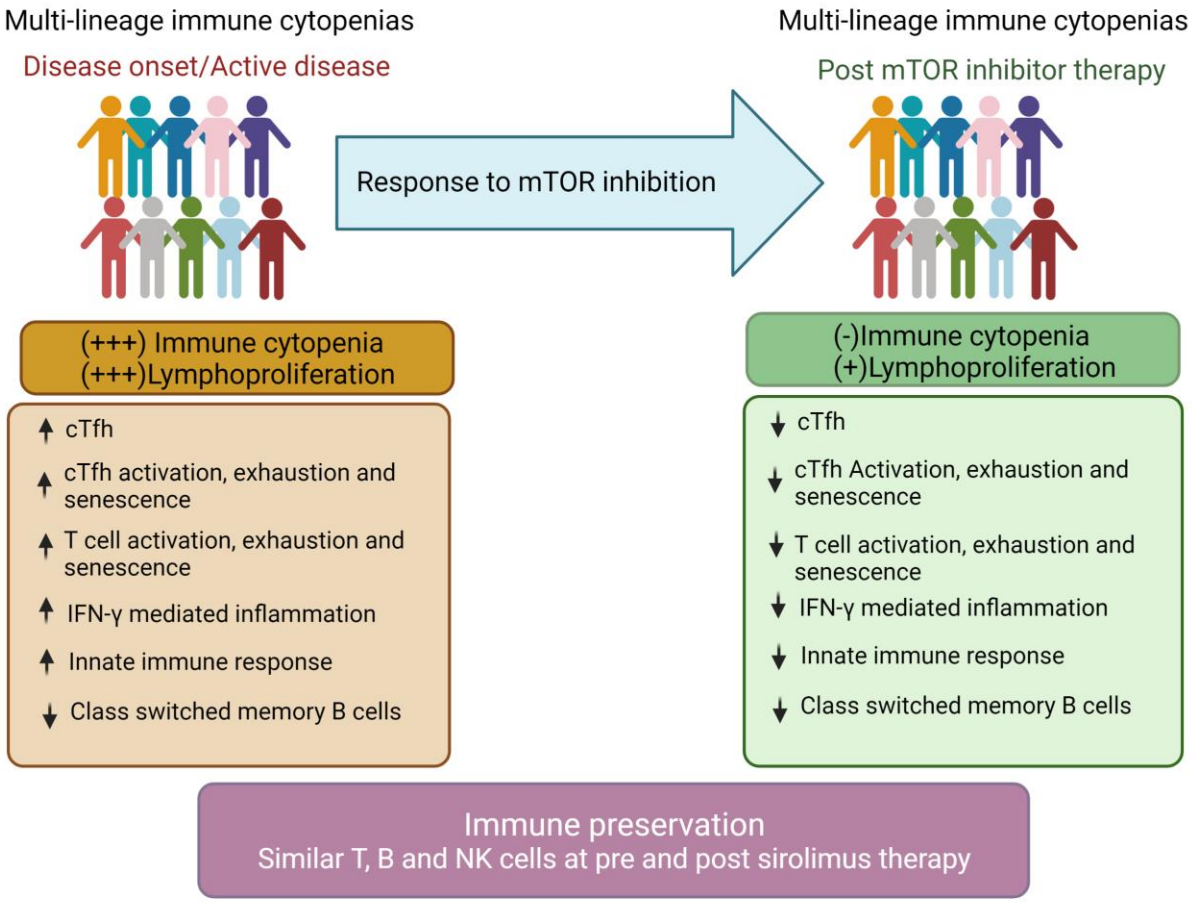
Supplemental Figure 4.



Supplemental Figure 5.



Supplemental Figure 6.



Supplemental Table 1: Clinical characteristics and sirolimus response in patients with m-IC

	Clinical characteristics before sirolimus therapy								Sirolimus response				
ID	**Age(y)/ sex**	**ITP**	**AIHA**	**AIN**	**LAP/Sp**	**ANA (pattern)**	**Vitamin B12 levels (pg/mL), Normal Range (180 - 914 pg/mL)**		**Sirolimus trough (ng/mL) on follow-up**	**Immune cytopenia**	**Lymphoproliferation**	**New autoimmune manifestations**	**Other adverse effects**
1	16/M	+	+	+	+	<1:80	402 (Normal)		7.3	CR	Improved	None	None
2	18/F	+	-	+	+	<1:80	ND		5	CR	Improved	None	None
3	5/F	+	+	+	-	1:80 (speckled)	ND		9.4	CR	N/A	None	Mucositis
4	8/M	+	+	+	-	<1:80	ND		10.1	CR	N/A	None	None
5*	15/M	+	-	+	+	<1:80	453 (Normal)		<2	PR	Persistent	None	None
6	7/F	+	+	-	+	<1:80	924 (High)		11.4	CR	Improved	CNS inflammatory lesion	None
7	9/M	+	+	+	-	1:80 (speckled)	830 (Normal)		5.4	CR	N/A	None	None
8	13/M	+	+	-	+	<1:80	365 (Normal)		9.9	PR	Improved	None	None
9	7/M	+	+	-	+	<1:80	>1500 (High)		3**	CR	Improved	None	Mucositis
10	7/M	+	+	-	+	<1:80	ND		5.3	CR	Improved	None	None
11	5/M	+	+	-	-	<1:80	709 (Normal)		4.9	CR	N/A	None	None
12	15/M	+	+	-	+	<1:80	1388 (High)		4	CR	Improved	None	Mucositis

AIHA-Autoimmune hemolytic anemia; AIN-autoimmune neutropenia; LAP/Sp- Lymphadenopathy or Splenomegaly, ANA -Antinuclear Antibody Test, CR-Complete response, PR-Partial response, N/A- Not applicable, ND- Not done.

* Poor adherence to sirolimus with consistently undetectable levels

** Due to poor tolerance to sirolimus, he was switched to everolimus, the level listed is for everolimus.

Supplemental Table 2: Genetic characteristics of patients with m-IC.

Patient	Variant/s	Protein level mutation	Classification	Zygosity	OMIM
1	*PNP* whole gene dup ……. *PRKCB* c.1763c>A	N/A ……... p. Pro588His	VUS ……... VUS	Homozygous …… Heterozygous	164050 …… 176970
2	*CFHR3* gene deletion	N/A	Risk allele	Homozygous	605336
3	WES negative	N/A	N/A	N/A	N/A
4	*ADA*, Exon 6, c.529G>A	p. Val177Met	VUS	Heterozygous	608958
5	WES negative	N/A	N/A	N/A	N/A
6	*LRBA* c.893del	p. Lys298Serfs*7	Pathogenic	Homozygous	606453
7	WES negative	N/A	N/A	N/A	N/A
8	WES negative	N/A	N/A	N/A	N/A
9	*FAS* c.536T>G	p. Leu179Arg	Likely pathogenic#	Heterozygous	134637
10	*PIK3CD* c.1574A>G	p. Glu525Gly	Likely Pathogenic[1]	Heterozygous	602839
11	WES negative	N/A	N/A	N/A	N/A
12	*FAS* c.386G>A	p. Cys129Tyr	Likely pathogenic	Heterozygous	134637

VUS- Variant of uncertain significance, N/A- Not applicable, OMIM- Online Mendelian Inheritance in Man,

https://www.ncbi.nlm.nih.gov/clinvar/RCV000626571.1/

Reference: -

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Supplemental Table 3. Reagents used for flow cytometry.

Reagent	Cat. No.	Source
Antibodies (Clone)		
PerCP/Cy5.5-anti-CD3 (UCHT1)	300430	Biolegend
PE-Cy7-anti-CD4 (RPA-T4)	560649	BD Biosciences
BUV395-anti-CD8 (RPA-T8)	563795	BD Biosciences
PE-anti-CCR7 (G043H7)	353204	Biolegend
APC-anti-CD45RA (HI100)	304112	Biolegend
BV711-anti-HLA-DR (G46-6)	563696	BD Biosciences
BUV496-anti-CD38 (HIT2)	612947	BD Biosciences
BV605-anti-CXCR5 (J252D4)	356930	Biolegend
BV421-anti-PD-1 (EH12.1)	562516	BD Biosciences
BB515-anti-CD57 (NK-1)	565285	BD Biosciences
APC-Cy7-anti-CXCR3 (G025H7)	353722	Biolegend
BUV737-anti-CCR6 (11A9)	612780	BD Biosciences
BV650-anti-TIM-3 (F38-2E2)	345028	Biolegend
BUV496-anti-CD19 (SJ25C1)	612938	BD Biosciences
APC-Cy7-anti-CD27 (O323)	302816	Biolegend
PE-anti-IgM (MHM-88)	314508	Biolegend
BV421-anti-IgD (IA6-2)	348226	Biolegend
Chemicals		
LIVE/DEAD™ Fixable Aqua Dead Cell Stain	L34966	Thermofisher

Supplemental Table 4. Definitions of T and B cell populations used in this study

Population	Markers	References
T cells		
PD-1$^+$ cTfh/ cTfh	CD4$^+$ CD45RA$^-$ CXCR5$^+$PD-1$^+$	1-4
Total cTfh	CD4$^+$ CD45RA$^-$ CXCR5$^+$	1-4
cTfh1	CD4$^+$ CD45RA$^-$ CXCR5$^+$PD-1$^+$ CXCR3 $^+$ CCR6$^-$	5,6
cTfh2	CD4$^+$ CD45RA$^-$ CXCR5$^+$PD-1$^+$ CXCR3 $^-$ CCR6$^-$	5,6
cTfh17	CD4$^+$ CD45RA$^-$ CXCR5$^+$PD-1$^+$ CXCR3$^-$ CCR6$^+$	5,6
Th1	CD4$^+$ CXCR3 $^+$ CCR6$^-$	7
Th2	CD4$^+$ CXCR3 $^-$ CCR6$^-$	7
Th17	CD4$^+$ CXCR3$^-$ CCR6$^+$	7
Naïve CD4$^+$ T	CD4$^+$ CCR7$^+$ CD45RA$^+$	7
Effector memory (EM) CD4$^+$ T	CD4$^+$ CCR7$^-$ CD45RA$^-$	7
TEMRA CD4$^+$T	CD4$^+$ CCR7$^-$ CD45RA$^+$	7
Effector memory CD8$^+$ T	CD8$^+$ CCR7$^-$ CD45RA$^-$	7
Activated CD4$^+$ T	CD4$^+$CD45RA$^-$CCR7$^-$ HLA-DR$^+$CD38$^+$	8-10
Activated CD8$^+$ T	CD8$^+$CD45RA$^-$CCR7$^-$ HLA-DR$^+$CD38$^+$	8-10
Senescent CD4$^+$ T	CD4$^+$ CD45RA$^-$CCR7$^-$ CD57$^+$	11-14
Senescent CD8$^+$ T	CD8$^+$ CD45RA$^-$CCR7$^-$ CD57$^+$	11-14
Exhausted CD4$^+$	CD4$^+$ CD45RA$^-$CCR7$^-$ PD-1$^+$ Tim3$^+$	15
Exhausted CD8$^+$	CD8$^+$ CD45RA$^-$CCR7$^-$ PD-1$^+$ Tim3$^+$	15
B cells		
Class-switched memory B (CSMB)	CD19$^+$CD27$^+$IgM$^-$IgD$^-$	16

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