The efficacy of combination treatment with elotuzumab and lenalidomide is dependent on crosstalk between natural killer cells, monocytes and myeloma cells

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Supplementary Methods

Flow cytometry based cytotoxicity assay

PBMCs obtained from healthy donors or patients were seeded in a 96-well plate at 100,000 cells per well and co-cultured with 50,000 OPM2 cells pre-labelled with 1:1000 CellTrace™ Violet (Life Technologies). Co-cultures were treated with 10 µg/ml Elo or IgG1 isotype. Len dissolved in DMSO was added at 10 µM and cocultures were incubated for 24 hours at 37^{o}C at 5% CO2. Cells were pelleted at 400 G for 5 minutes and supernatants were transferred to a new 96-well plate and stored at -80^{o}C until analysed by cytokine bead array (CBA). Cells were washed and resuspended in FACS buffer for immunostaining. Counting beads (Quantibrite™ Beads, BD) were added to each well to enumerate remaining target cells for cytotoxicity. Flow cytometry based cytotoxicity assays were acquired on the LSRFortessa X-20 and analysed with FlowJo software.

Chromium release cytotoxicity assay

For labelling target cells with chromium, cells were incubated with 100 µCi Cr51 at 37ÅãC at 5% CO2, in 500 µl of complete RPMI1640 media for 1 hour. Cells were then washed 3x with complete RPMI1640 media. Effector cells were then seeded in a 96-well plate, and serially diluted in complete media to acquire the indicated effector-target ratio. Target cells were added to each well at 10,000 cells/well to reach a 200 µl final volume. Co-cultures were incubated at 37ÅãC for 4 hours in the presence of 10 µg/ml Elo or IgG1 isotype and 10 µM Len or DMSO control. All conditions were set up in triplicate. Cells were then pelleted and 150 ul of supernatants were transferred to microtitre tubes for gamma counting.

Reagents and Antibodies

Elotuzumab and Elo mutant Fc (Elo Fc-neg, CS1.IgG1.1F) were provided by BMS and reconstituted in PBS. This Elo mutant has been described in detail in a prior study by Pazina et al (1). For in vitro experiments, Lenalidomide was reconstituted at 100 µM in DMSO and appropriately diluted in PBS. For in vivo experiments, Lenalidomide was reconstituted in DMSO and diluted with PBS to make a 12.5% DMSO solution at 12.5 mg/ml Lenalidomide. Pomalidomide was similarly reconstituted for in vivo experiments, with a final concentration of 1.25 mg/ml.

Fluorescent-conjugated antibodies used to identify surface molecules for flow cytometry were anti-CD56-PE (5.1H11, Biolegend), anti-CD56-BV395 (NCAM16.2, BD), anti-CD16-BV786 (3G8, Biolegend), anti-CD11a-BV650 (HI111, BD), anti-CD54-ACP (HA58, BioLegend), anti-CD54-BV650 (HA58, BD), anti-CD69-BV510 (FN50, BioLegend), anti-CD107a-AF488 (H4A3,BioLegend), anti-CD25-PE (M-A251, BD), anti-CD3-APC-H7 (SK7, BD), anti-CD14-FITC (M5E2,BD), anti-CD14-APC-Cy7 (MφP9, BD), anti-CD19-ACP-H7 (SJ25C1, BD), anti-NKp30-AF488 (210845, R&D Systems), anti-NKp44-PE (253415, R&D Systems), anti-NKp46-BV605 (9E2,BioLegend), anti-NKG2D-AF700 (149810, R&D Systems), anti-DNAM1-APC (102511, R&D Systems), anti-NKG2A-PE (131221, R&D Systems), anti-TIGIT-APC (MBSA34, eBioscience),anti-KIR2DL1-AF488 (143211, R&D Systems), anti-KIR2DL2/3-PE-Cy7 (DX27, BIoLegend), anti-KIR3DL1-BV421 (DX9, BioLegend), and anti-KIR3DL2-PE (539304, R&D Systems). CellTrace Violet™ (CTV) (Life Technologies) was used to stain target cells at 1:1000. Zombie RedTM fixable viability dye (Biolegend) was used to assess cell viability.

Cytometric bead array (CBA) assays

Cytokine and chemokines were measured in 10 µL of supernatant from human PBMC and OPM-2 co-cultures, incubated at a 2:1 effector:target ratio with Isotype, Elo, Len or combined Elo and Len for 24 hours. Cytokine and chemokine concentration was measured using CBA Flex sets (BD) according to the manufacturer's instructions. CBA Flex sets used are Human IFN-g, TNF, CCL3 (MIP-1a), CCL4 (MIP-1b) and CCL5 (RANTES). Samples were analysed using a FACSVerse cytometer (BD) and FCAP Array software V3 (BD BioSciences).

Immune cell depletion assays

PBMCs were freshly isolated from healthy donors and were labelled with antibodies to CD14 (FITC), CD56 (PE), CD3 (APC-H7), CD19 (ACP-H7) and Zombie Red fixable viability dye. Cells were sorted directly into 90% FCS/10% RPMI1640 media with a FACSAria Fusion 3 (BD), for CD14+ cells, CD14 depleted PBMCs and NK cells. OPM2 cells were labelled with 1:1000 CellTrace Violet™ (Life Technologies) and seeded in a 96-well plate at 5e4 cells per well. 1e5 whole PBMCs, or an equivalent number of CD14 depleted PBMCS, isolated CD14+ cells or NK cells were added to each well. The number of isolated/depleted cells added to each well was equal to the expected number of cells found in whole PBMCs, based on cell percentages in the PBMC phenotype. Cells were co-cultured for 24 hours and were treated with Isotype or 10 µg/ml Elo and 10 µM Len. Flow cytometry was used to analyse killing activity and cell phenotype was analysed on the LSRFortessa™ X-20, BD. 5e3 counting beads (Quantibrite™ BD) were used to enumerate OPM2 cells, and to calculate killing, standardised to spontaneous cell death. For cell phenotypes, cells were stained for CD56 (PE), CD16 (BV786), CD3/19 (APCH7), CD14 (FITC), CD54 (APC), CD11a (BV650) and Zombie Red™ fixable viability dye.

RNA sequencing analysis

3-4e6 PBMCs were cocultured with 1e6 CTV labelled OPM2 cells for 24 hrs in a 24-well plate, and were treated with 10 µg/ml Elo and 10 µM Len. For cell sorting, cells were labelled with antibodies to CD56 (PE), CD16 (BV786), CD3/19 (APCH7), CD14 (APC) and Zombie Red™ viability dye (BioLegend.). Cells were sorted using the ARIA Fusion 3, directly into 90% FCS/10% RPMI1640, pelleted and snap frozen at -80ÅãC. RNA extraction was performed as per manufacturer's instructions (15596026, Invitrogen, USA). cDNA libraries were prepared using either the SMART-seq v4 low input RNA kit for NK cell preparations (Takara Bio USA), or NEBNext Ultra II (New England Biolabs, USA) for monocyte and OPM2 cells preparations. Libraries were sequenced on the NextSeq 550 sequencing system (Illumina, USA) at the Molecular Genomics Facility at the Peter MacCallum Cancer Centre. Reads were aligned using seqliner v0.7.1 to HG19 reference genome and quantified using Htseq v0.6.1 software. Normalisation and differential expression analysis was performed with Limma-Voom in Rv3.3.3.

Statistics and Bioinformatics analyses

All data were analysed using GraphPad Prism software Version 8 using either ANOVA with post-hoc test or 2-tailed Student t-test, and significance levels are denoted in the figure legends. All experimental data are shown as mean (SD) or median (interquartile range) in the text as well as in the figures. RNAseq data visualization, principle component analysis and t-test volcano plots were performed using Perseus computational platform (version 1.5.6) from the Max Planck Institute of Biochemistry (2). T-test analysis was performed using a two-sided t-test with 250 randomizations, a false discovery rate (FDR) of 0.05 and an S0 of 0.1. Candidate

lists were comprised of genes with P-values < 0.05 and/or an FDR < 0.05. We acknowledge our use of the gene set enrichment analysis, GSEA software, and Molecular Signature Database (MSigDB) according to established protocols (3). An FDR of <0.2 was considered significant for GSEA pathway analysis. Ontology analysis of pre-defined gene lists was performed using Enrichr software (23586463, 23586463) according to standard protocols. Visualisations were performed within R using standard ggplot2 package.

References

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Supplementary figures:

Supplementary Figure 1. In vitro expanded NK cells have an activated phenotype.
NK cells were isolated from healthy donor PBMCs and cultured for 21 days with irradiated feeder cells (Jurkat and LCLs) at a ratio of 1:1:1. In (A) day 0 and expanded NK cells were assessed for CD56 and CD16 expression, CD69, and NK activation and inhibitory receptors (B) Representative chromium release assays for NK cells from days 0, 7 and 21 of expansion. NK cells were co-cultured for 4hrs with OPM2 targets in the presence of 10 µg/ml Elotuzumab (Elo) or an isotype control antibody (Iso), where ns = not significant, * p<0.05, **, p<0.01, *** p<0.001, **** p<0.0001, (Student's T-test, n=3).

Supplementary Figure 2. Combination elotuzumab and IMiD treatment significantly controls myeloma tumor growth.
NSG mice were injected s.c. with 2e6 RPMI8226.hSLAMF7 tumour cells in the right flank. 10e6 expanded NK cells were transferred intravenously when tumours reached 10 mm^2 (width x length) on day 0 and day 1. Mice received 5 mg/kg Elo/Iso, and 50 mg/kg Len/Vehicle (12.5% DMSO), 5 mg/kg pom/vehicle (12.5% DMSO), and 5e4 U IL2 and 2.5 µg IL15 via intraperitoneal injection, 3 doses per week for 3 weeks. Shown are tumour growth curves representative of three separate experiments (6 mice/group, N=3, 2-way ANOVA, * p<0.05, *** p<0.001 **** p<0.0001).

Supplementary Figure 3. RRMM patient PBMCS are distinct for efficient myeloma killers.
Data from Figure 2C was reformatted to show (A) statistical differences between RRMM patient PBMCs characterised with efficient killing activity vs patient PBMCs with minimal killing. 2 way ANOVA with Tukey's test, * p<0.05, **, p<0.01, *** p<0.001, **** p<0.0001. (B) NK cells were analysed for treatment induced changes in NK cell CD16 expression levels using HD, NDMM or RRMM PBMCs. RRMM PBMCs nominated as efficient killers are highlighted in red. RM one-way ANOVA, * p<0.05, *** p<0.001, **** p<0.0001. (C) a representative histogram overlay depicting the change in NK cell CD16 expression when treated with either an isotype control or Elo+Len.

Supplementary Figure 4. Elotuzumab and lenalidmide treatment increased RANTES secretion in myeloma co-cultures with RRMM PBMCs.
PBMCs from healthy donors (HD, n=9), patients with newly diagnosed multiple myeloma (NDMM, n=12) or refractory relapsed multiple myeloma (RRMM, n=11) were co-cultured with OPM2 cells at a 2:1 ratio for 24 hours in the presence of 10 µg/ml human IgG1 isotype control (Iso), 10 µM Len, 10 µg/ml Elo or Elo + Len combination. Supernatants from triplicate wells of PBMC and OPM2 co-cultures were taken at 24-hours for cytokine bead array analysis. Shown are comparative changes between patient cohorts (HD, NDMM and RRMM patients) for (A) MIP-1a (B) MIP-1b (C) RANTES secretion. 2-way ANOVA, * p<0.05.

Supplementary Figure 5: Transcriptomic analysis of natural killer cells from myeloma patients.
(A) Venn diagram of overlap in significantly upregulated genes (p-value < 0.05, log2 fold-change>2) in two sample types (healthy donor and patient) sorted by three treatments groups (Elo, Len, Elo and Len) compared to isotype control. Boxes indicate gene sharing between relevant groups. (B) Principle component analysis of RNA expression in all NK cell

samples following treatment. (C) Gene set enrichment analysis of highly enriched NK cell-related canonical pathways using triplicate data. Only significant enrichments are shown (FDR<0.2). Normalised z-score heatmaps of GO:0032814:Regulation of NK cell activation pathway genes according to treatment for (D) healthy donor and (E) RRMM patient samples.

Supplementary Figure 6. Elo or Elo plus Len treatment upregulates CD11a on OPM2 and NK cells.

OPM2 cells were co-cultured 1:2 in the presence or absence of PBMCs from healthy donors and RRMM patients (efficient killers red triangles in Fig 2B). Co-cultures were treated for 24 hours with 10 μg/ml human IgG1 isotype control (Iso) or 10 μg/ml Elo and 10 μM Len and analysed via flow cytometry for change in CD11a expression following drug treatment on (A) OPM2 cells and (B) NK cells. (2-way ANOVA, ns = not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, n=3).

Supplementary Figure 7. Elo and Len treatment upregulated CD54 on OPM2 cells was not dependent on T cells.

PBMC (or $CD4^+$ or $CD8^+$ T cell depleted PBMCs) and OPM2 cell co-cultures (2:1) were treated for 24 hours with 10 μg/ml human IgG1 isotype control (Iso) or 10 μg/ml Elo and 10 μM Len and analysed via flow cytometry for (A) the % killing of OPM2 cells, and (B) change in OPM2 myeloma cell CD54, (C) CD11a expression level following drug treatment. (2 way ANOVA, * $p<0.05$, **, $p<0.01$, *** $p<0.001$, **** $p<0.0001$, n=3). Representative histogram overlays depicting Elo+Len-induced changes in OPM2 CD54 (D) and CD11a (E).

Supplementary Figure 8. Elo and Len treatment upregulated CD11a on OPM2 cells was dependent on the presence of monocytes and NK cells.

PBMC (or $CD4^+$ or $CD8^+$ T cell depleted PBMCs) and OPM2 cell co-cultures (2:1) were treated for 24 hours with 10 μg/ml human IgG1 isotype control (Iso) or 10 μg/ml Elo and 10 μM Len. OPM2 cell CD11a expression level was analysed via flow cytometry in (A) following depletion of monocytes of NK cells (B) following the addition of purified NK cells, monocytes or combination NK cells + monocytes. In (C) OPM2 CD54 expression after treatment for 24 hours with conditioned media harvested from Elo+Len treated PBMC:OPM2 co-cultures. (2 way ANOVA, * $p<0.05$, **, $p<0.01$, *** $p<0.001$, **** $p<0.0001$, n=3). Representative histogram overlays depicting Elo+Len-induced changes in OPM2 CD54 (D) and CD11a (E).

Supplementary Figure 9: Drug treatment changes in myeloma, NK cells and monocyte transcript levels and gene ontology analysis.

Related to Figure 6D and 6E. Ontological enrichment analysis using GO: Molecular Functions database of overexpressed genesets for either (A) monocytes, or (B) OPM2 cells. Only significantly enriched ontologies are shown (adjusted p-value < 0.05). Genes contributing to each ontology are shown. No enrichments for lenalidomide treatment were identified. Genes contributing to each group are shown in boxed. Heatmaps of absolute and relative expression differences between four treatment groups in triplicate samples for (C) NK cells, (D) monocytes and (E) OPM2 cells

Supplementary Figure 10. Cross talk between NK cells, monocytes and myeloma target cells is required for optimal anti-tumour activity in response to Elo and Len.
A schematic illustrating the mechanisms for cross talk between NK cells, monocytes and myeloma cells. This conceptual figure is divided into panels depicting molecular interactions between (A) NK and myeloma cells treated with Len (B) NK cells, monocytes and myeloma cells (C) NK cells and myeloma cells treated with Elo or Elo and Len (D) NK cells and myeloma cells treated with Elo in the presence of monocytes (E) NK cells and myeloma cells treated with Elo and Len in the presence of monocytes. Changes in NK cell CD11a/CD54 and CD69 levels were observed when Elo was present (C), whereas increased myeloma cell CD11a/CD54, NK activating ligands (CD155 and MIC-A), or chemokines and receptors (CCR7, CXCR3, CCL5, CXCL10,CXCL11) were only observed in condition (E).

Supplementary Figure 11. Graphical abstract.
Co-culture of myeloma patient PBMCs and myeloma cells with Elo+Len treatment increased myeloma cell MHC-II, CD11a/CD54 and *CXCL10-11, CCL5* expression. This change in myeloma cell profile was associated with myeloma cell death. In this context, myeloma cell death required monocytes and NK cells which collectively secreted increased IFN-γ, TNF and CCL5. In contrast, if no change in myeloma cell profile occurred, the myeloma cells were resistant to NK and monocyte effector mechanisms in the presence of Elo+Len treatment.

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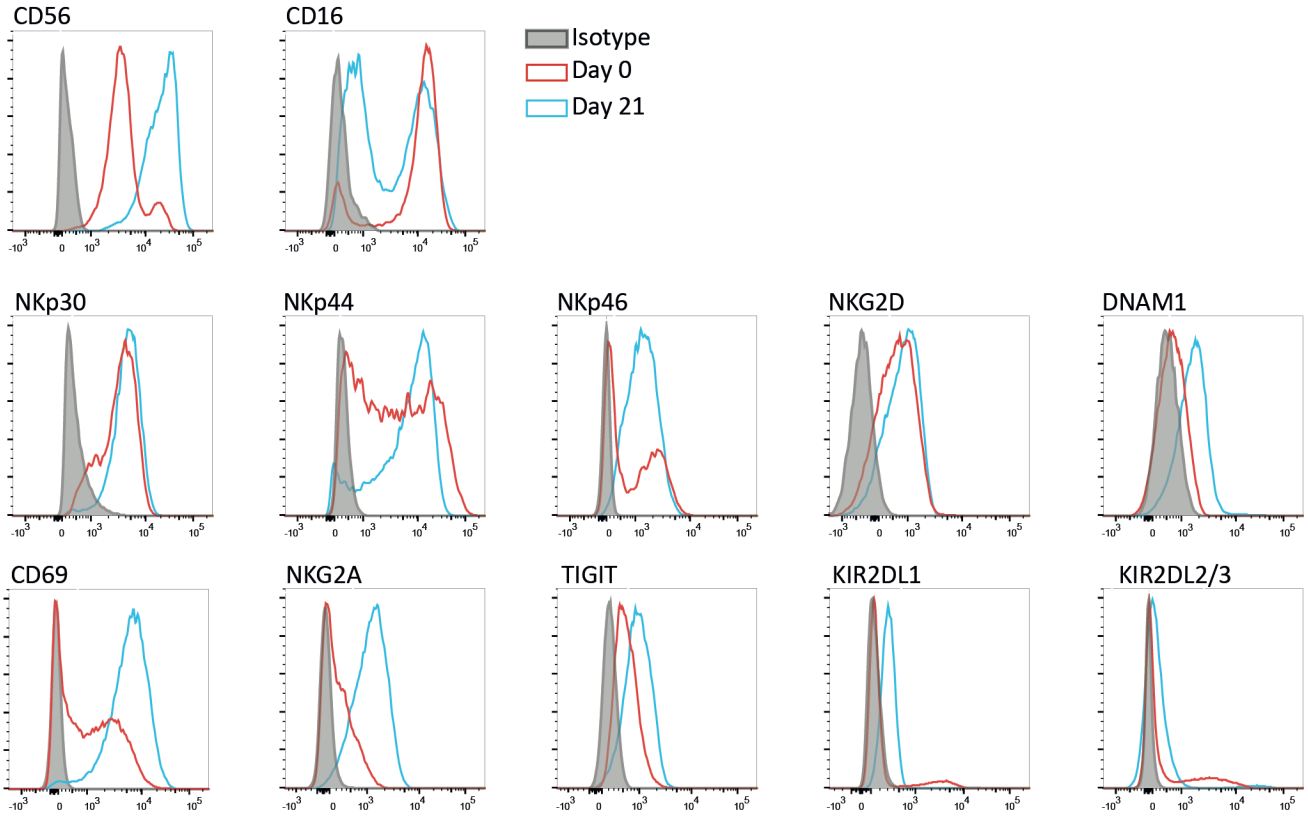
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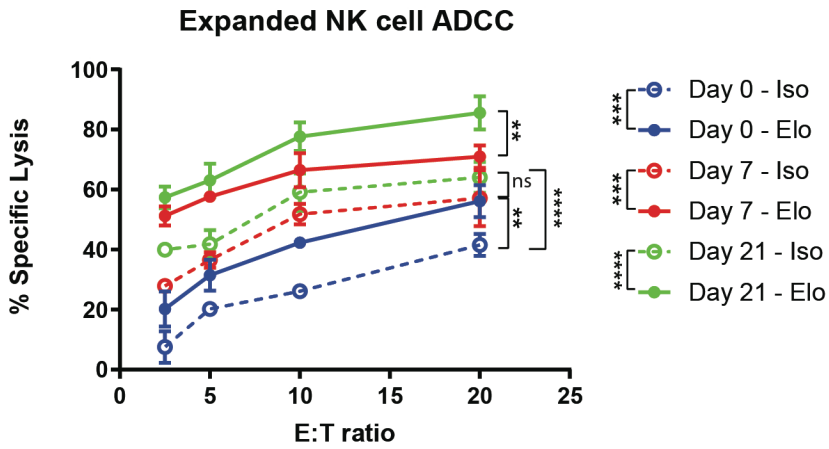
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Day 0 - Elo
Day 7 - Iso
Day 7 - Elo
Day 21 - Iso
Day 21 - Elo
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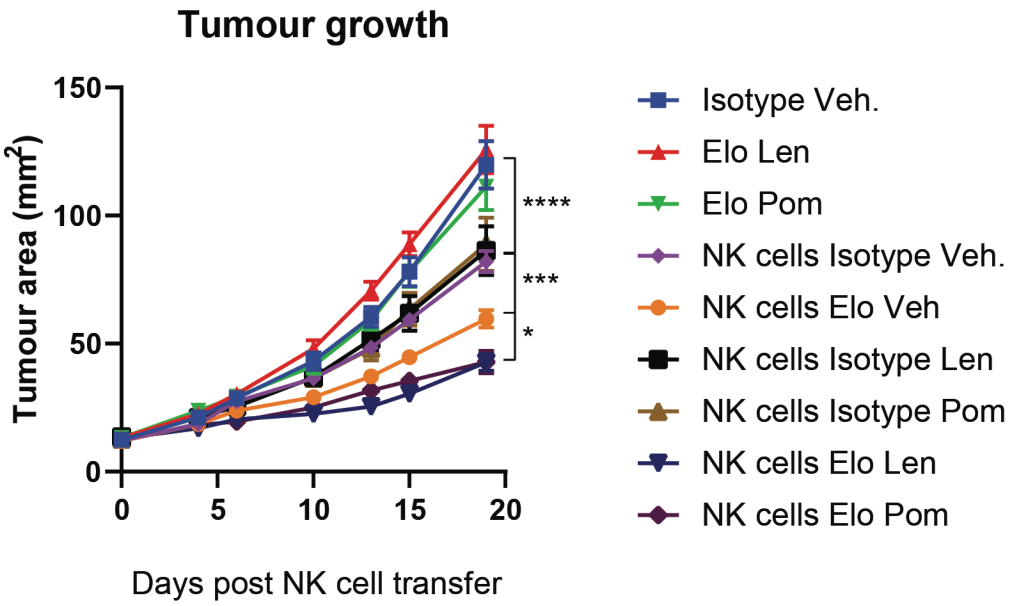
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Tumour area (mm2)
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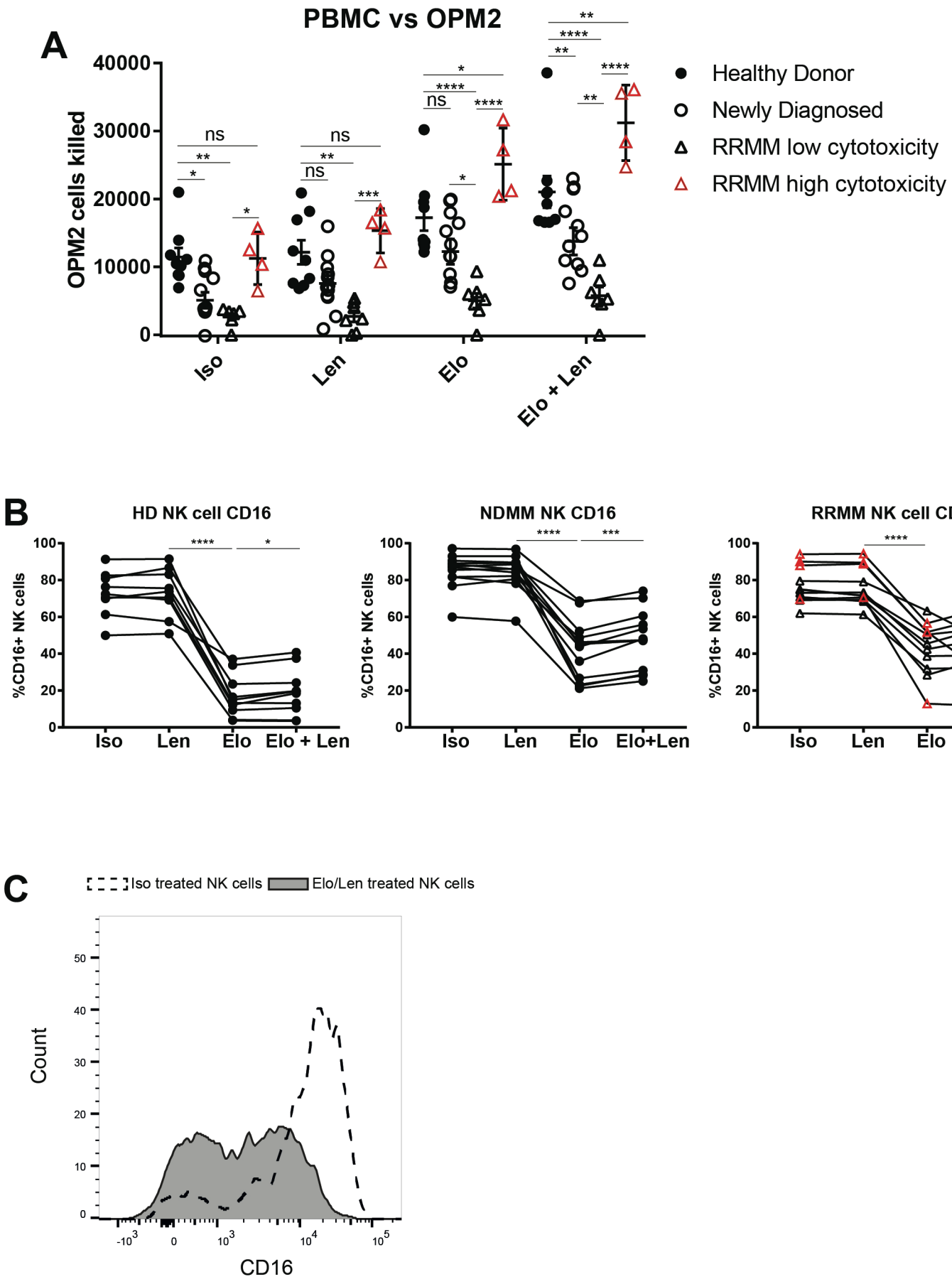
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RRMM low cytotoxicity
RRMM high cytotoxicity
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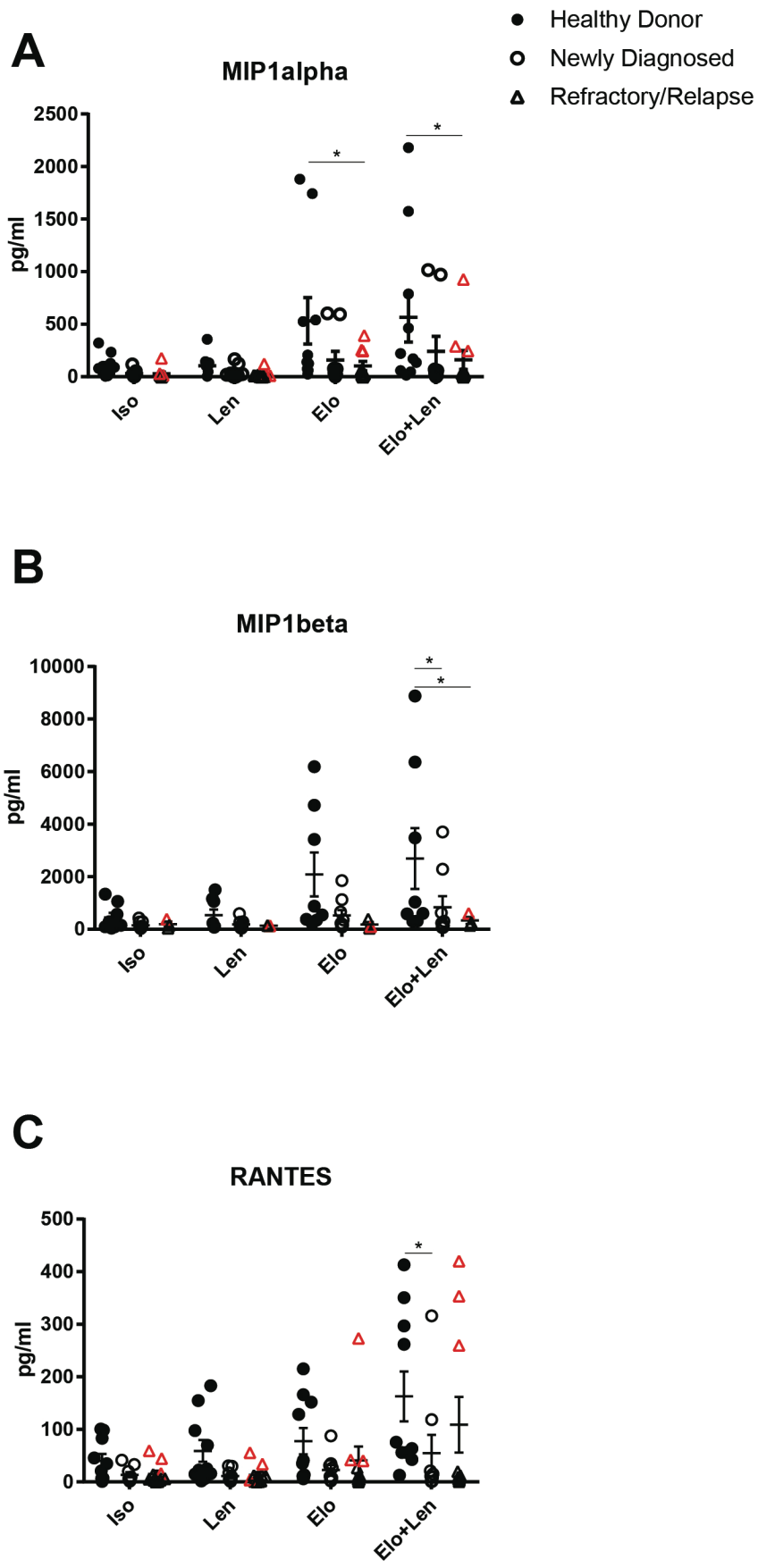
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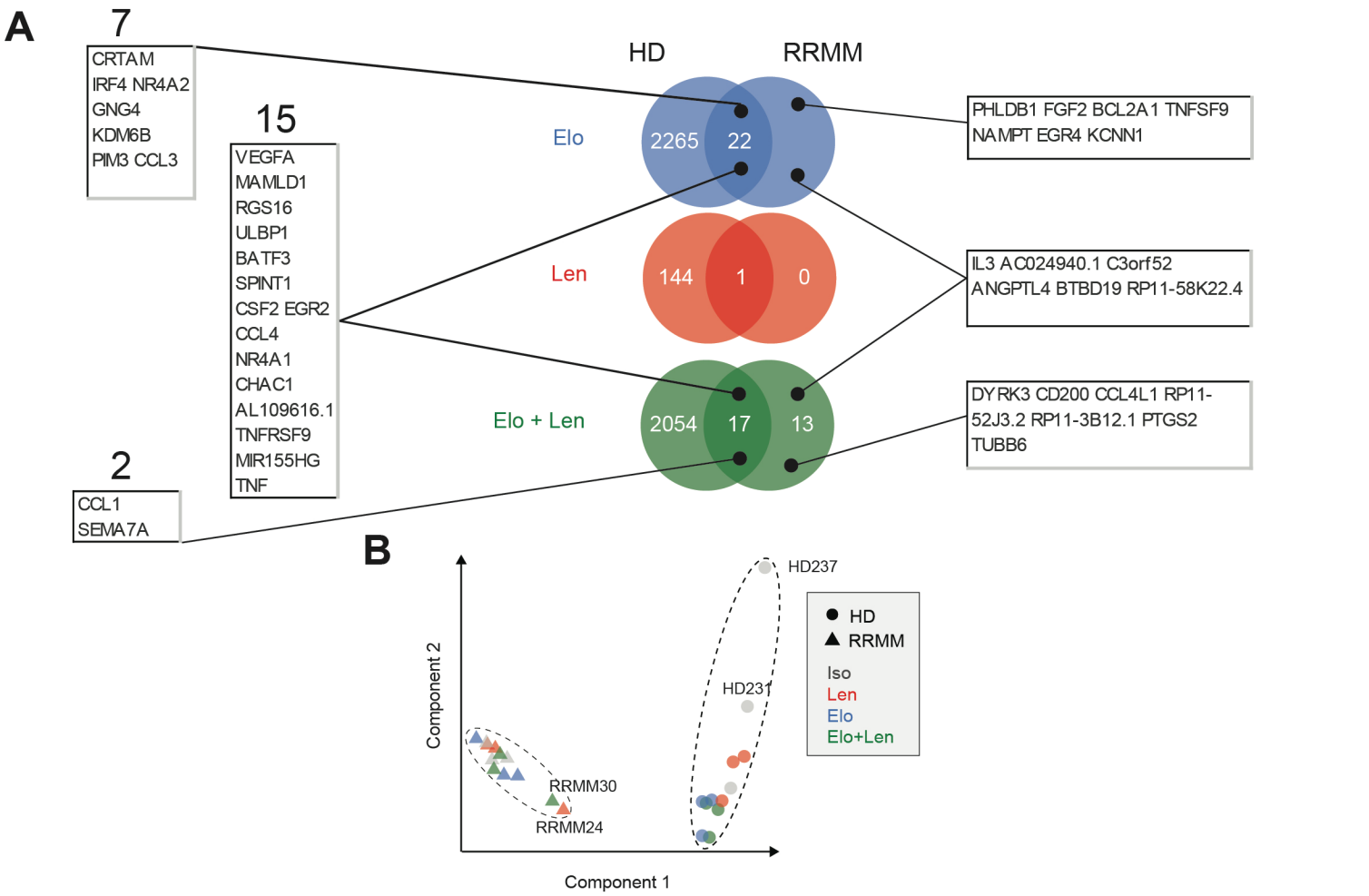
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TNFRSF9
MIR155HG
TNF
2
CCL1
SEMA7A
HD
RRMM
Elo
2265
22
Len
144
1
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Elo + Len
2054
17
13
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IL3 AC024940.1 C3orf52
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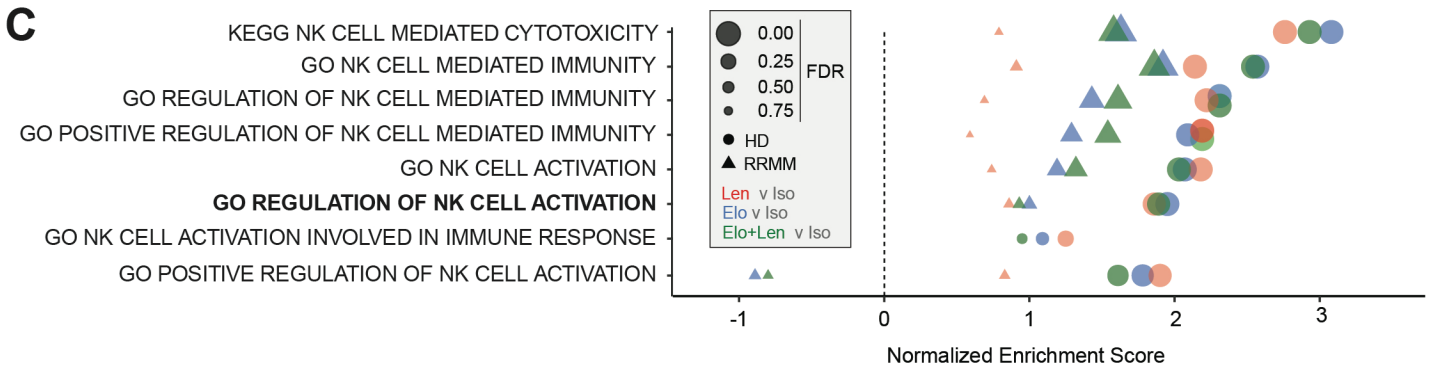
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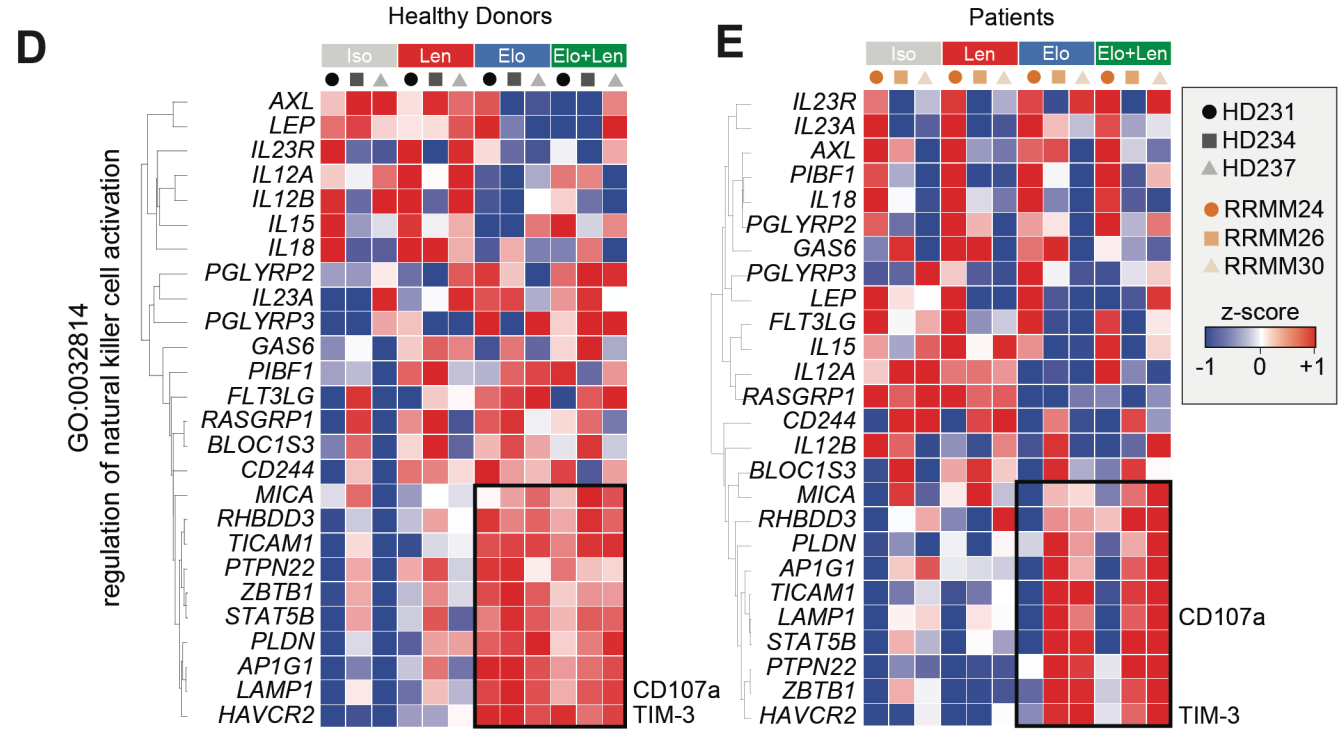
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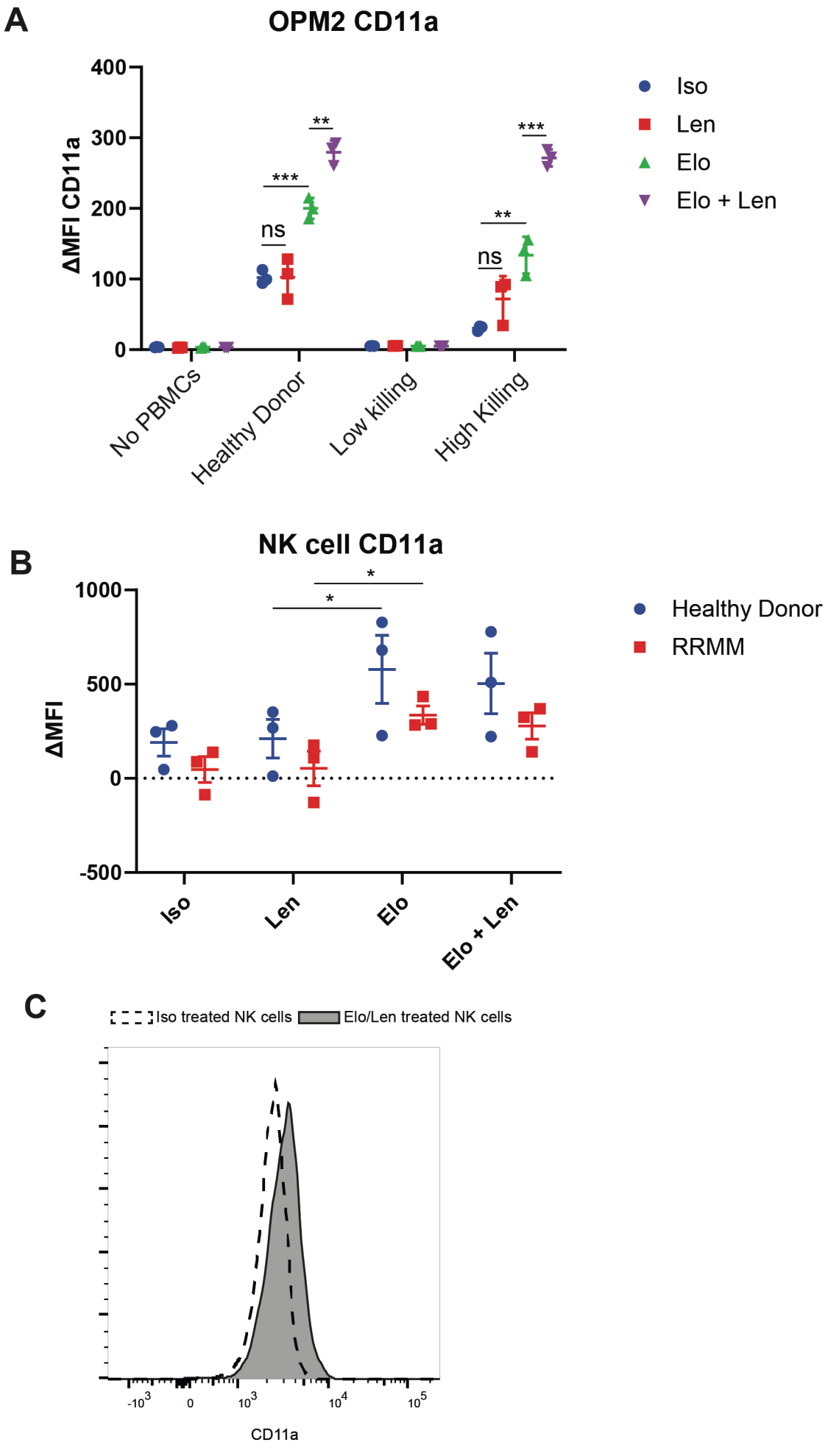
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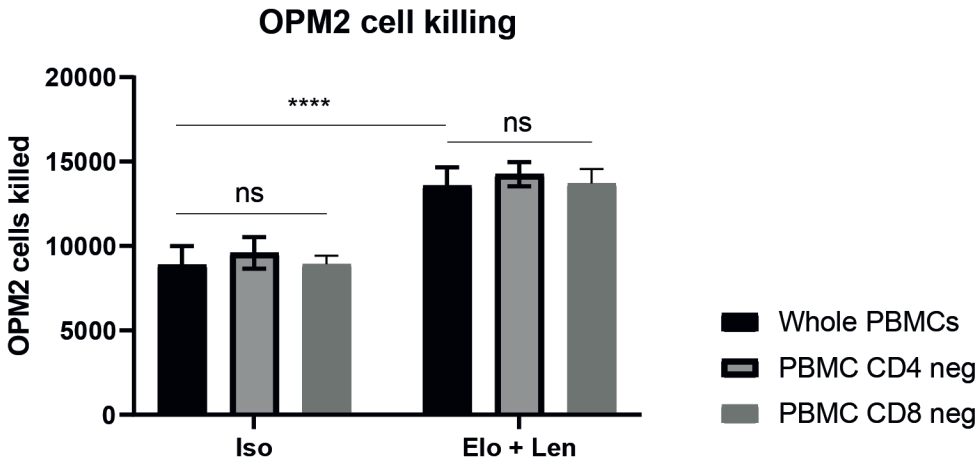
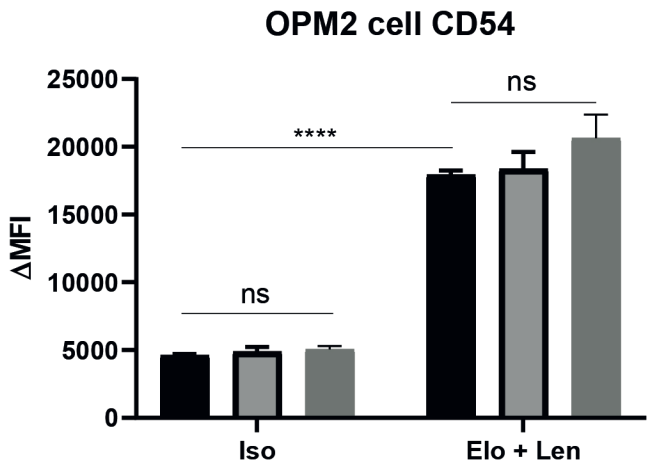
CD11a

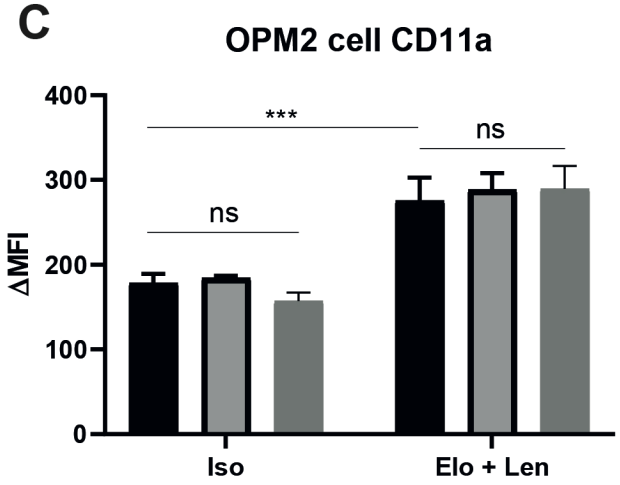
A


OPM2 cell killing
OPM2 cells killed
20000
15000
10000
5000
0
****
ns
ns
Iso
Elo + Len
Whole PBMCs
PBMC CD4 neg
PBMC CD8 neg

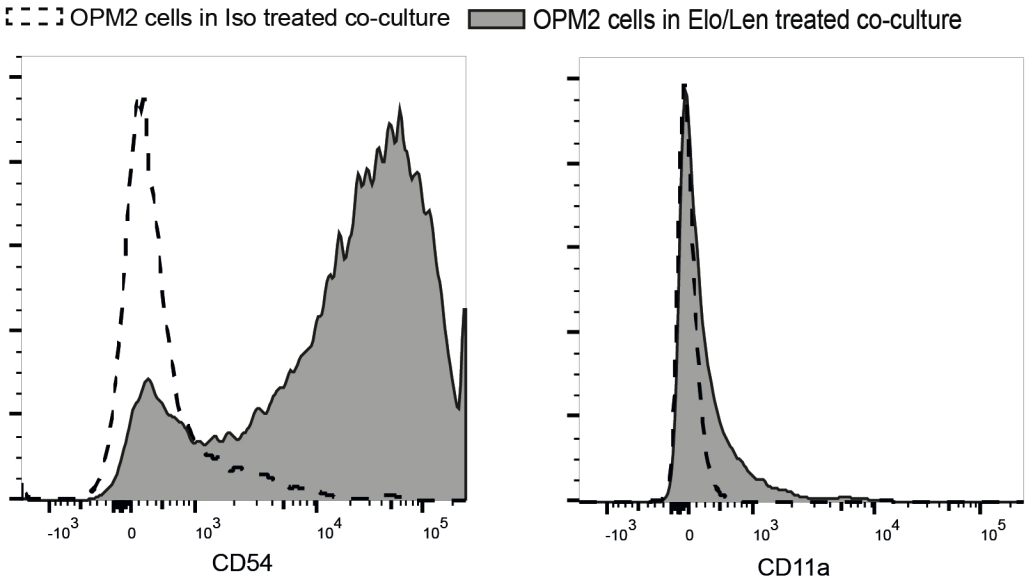
B

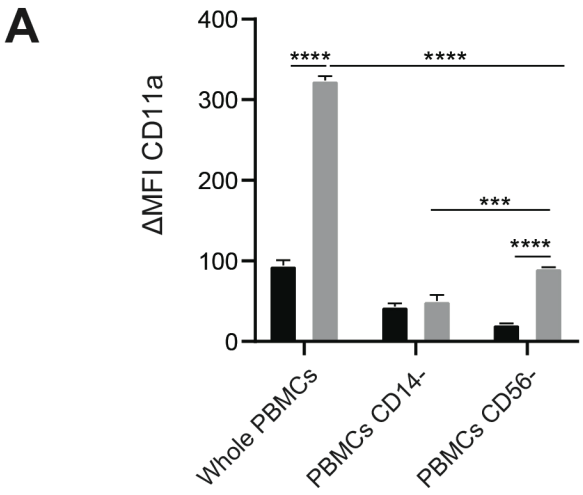

OPM2 cell CD54
ΔMFI
25000
20000
15000
10000
5000
0
****
ns
ns
Iso
Elo + Len

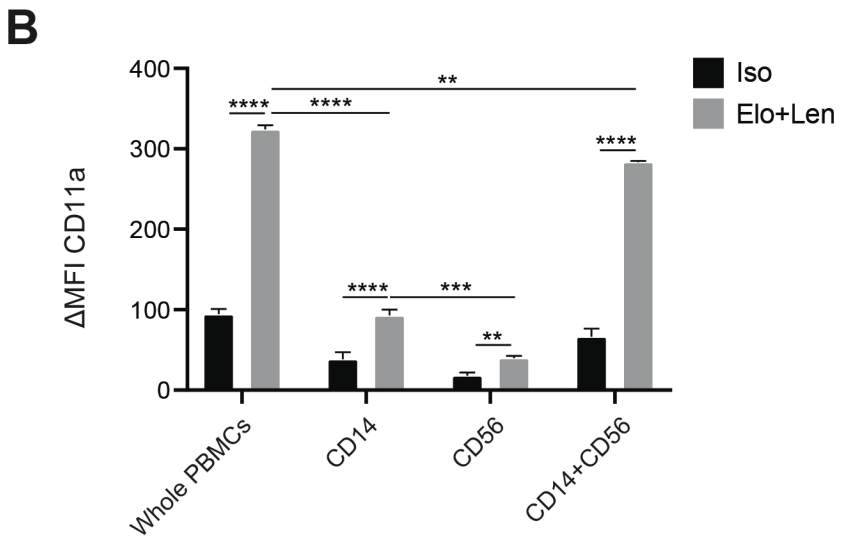
C

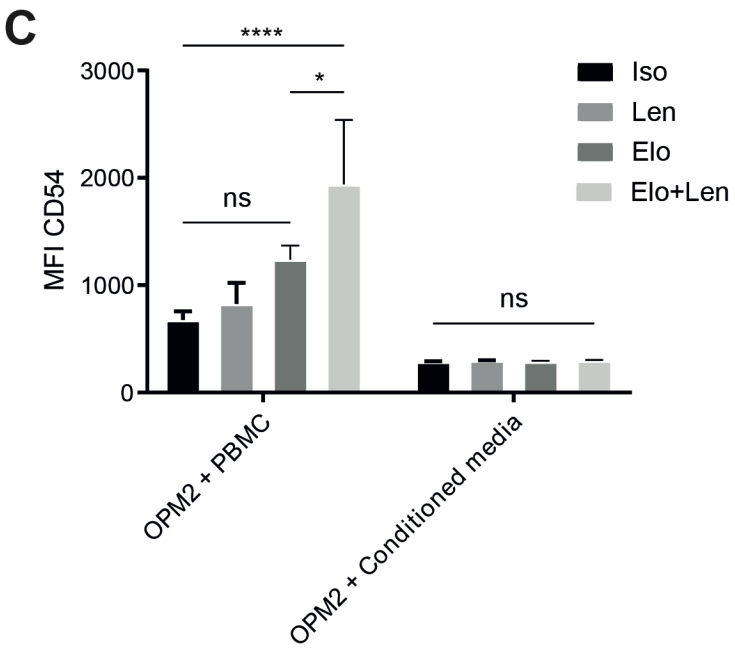

OPM2 cell CD11a
ΔMFI
400
300
200
100
0
***
ns
ns
Iso
Elo + Len

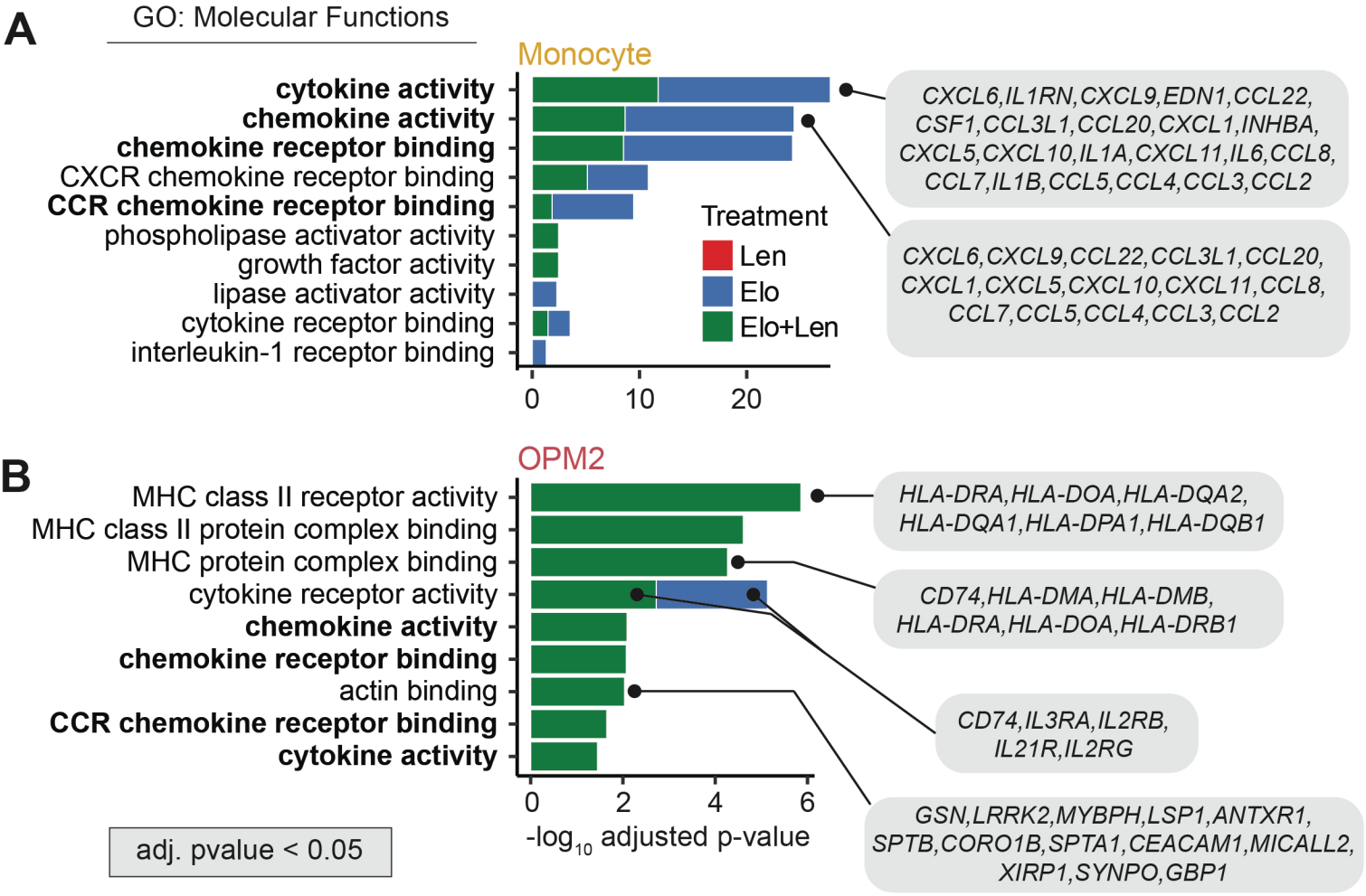
D

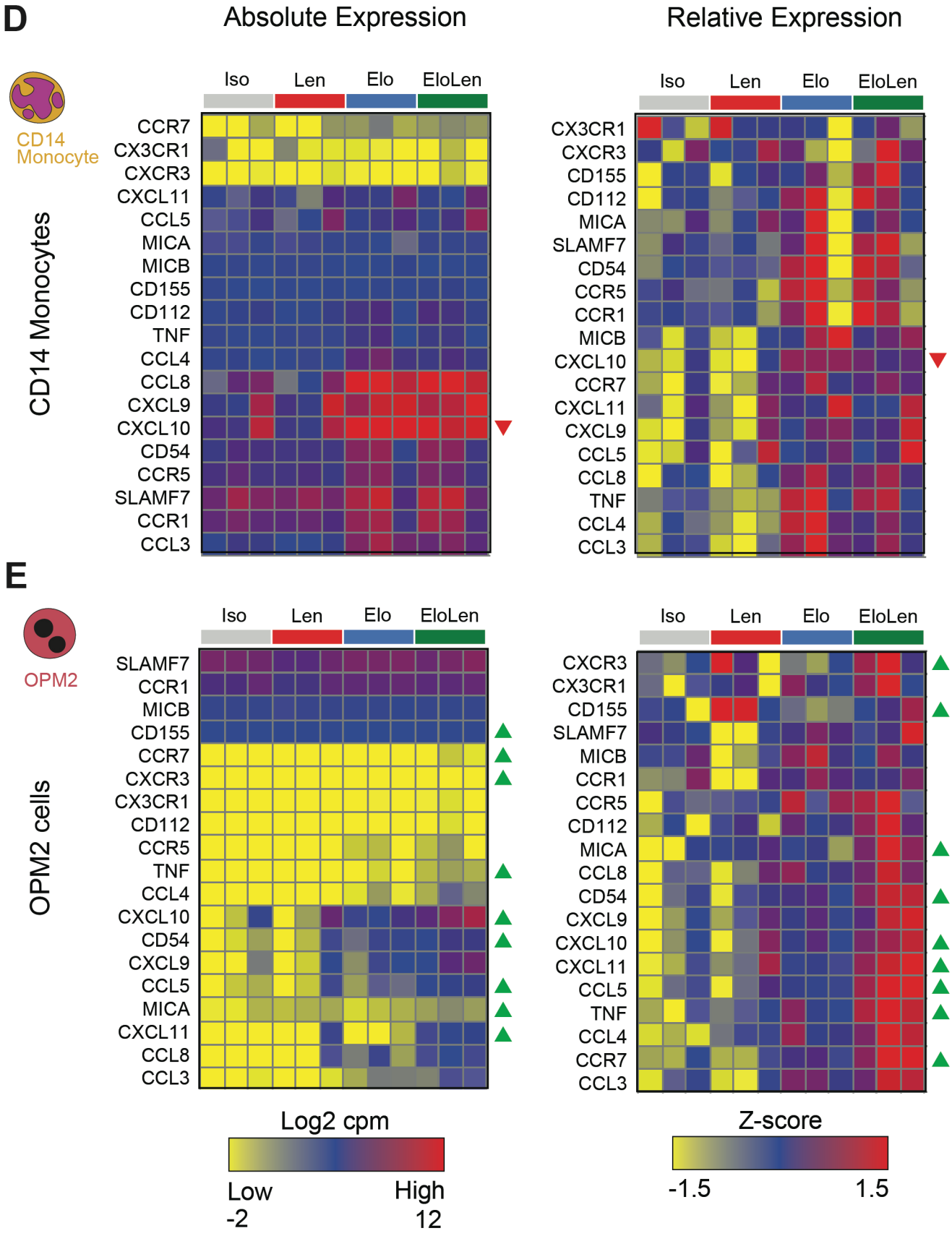

OPM2 cells in Iso treated co-culture
OPM2 cells in Elo/Len treated co-culture
-10^3
0
10^3
10^4
10^5
CD54
CD11a

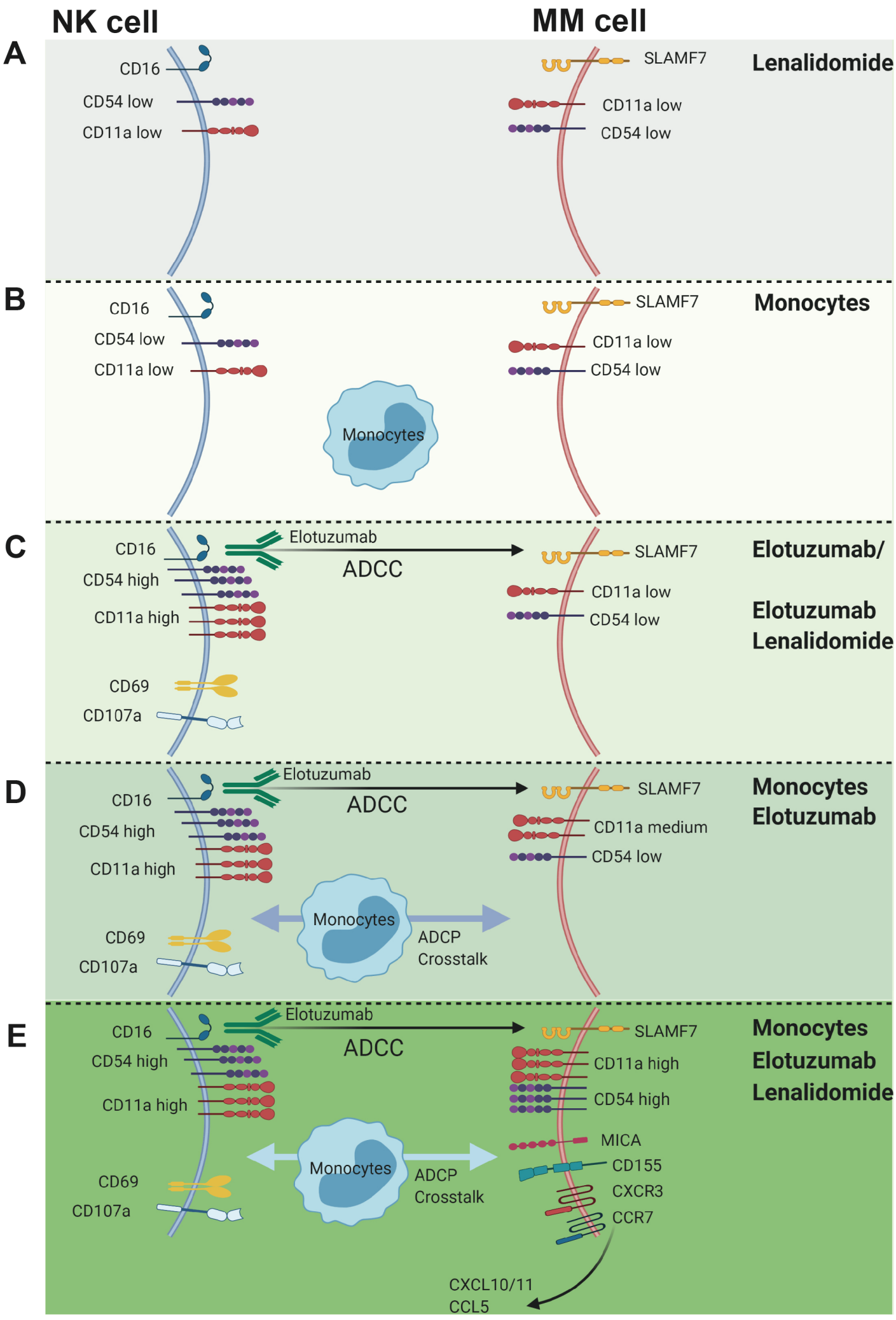

A
400
300
200
100
0
ΔMFI CD11a
****
****
***
****
Whole PBMCs
PBMCs CD14-
PBMCs CD56-

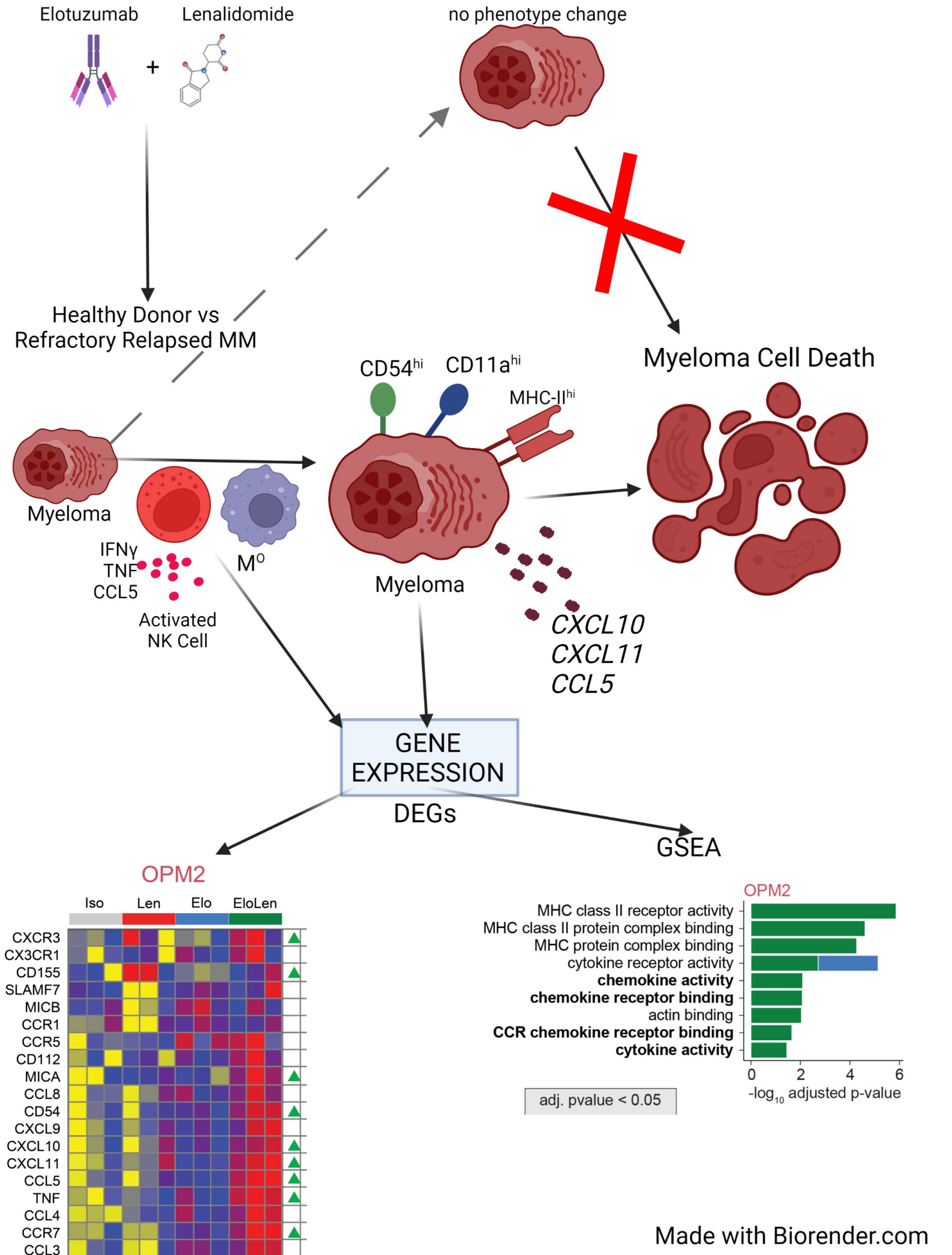

B
400
300
200
100
0
ΔMFI CD11a
**
****
****
****
****
***
**
Iso
Elo+Len
Whole PBMCs
CD14
CD56
CD14+CD56


C
3000
2000
1000
0
MFI CD54
****
*
ns
ns
Iso
Len
Elo
Elo+Len
OPM2 + PBMC
OPM2 + Conditioned media


A
GO: Molecular Functions
Monocyte
cytokine activity
chemokine activity
chemokine receptor binding
CXCR chemokine receptor binding
CCR chemokine receptor binding
phospholipase activator activity
growth factor activity
lipase activator activity
cytokine receptor binding
interleukin-1 receptor binding
Treatment
Len
Elo
Elo+Len
0
10
20
CXCL6,IL1RN,CXCL9,EDN1,CCL22,
CSF1,CCL3L1,CCL20,CXCL1,INHBA,
CXCL5,CXCL10,IL1A,CXCL11,IL6,CCL8,
CCL7,IL1B,CCL5,CCL4,CCL3,CCL2
CXCL6,CXCL9,CCL22,CCL3L1,CCL20,
CXCL1,CXCL5,CXCL10,CXCL11,CCL8,
CCL7,CCL5,CCL4,CCL3,CCL2
B
OPM2
MHC class II receptor activity
MHC class II protein complex binding
MHC protein complex binding
cytokine receptor activity
chemokine activity
chemokine receptor binding
actin binding
CCR chemokine receptor binding
cytokine activity
0
2
4
6
-log10 adjusted p-value
adj. pvalue < 0.05
HLA-DRA,HLA-DOA,HLA-DQA2,
HLA-DQA1,HLA-DPA1,HLA-DQB1
CD74,HLA-DMA,HLA-DMB,
HLA-DRA,HLA-DOA,HLA-DRB1
CD74,IL3RA,IL2RB,
IL21R,IL2RG
GSN,LRRK2,MYBPH,LSP1,ANTXR1,
SPTB,CORO1B,SPTA1,CEACAM1,MICALL2,
XIRP1,SYNPO,GBP1
C
Absolute Expression
Relative Expression
Iso
Len
Elo
EloLen
NK
NK cells
CD112
CXCL10
MICB
CD155
CXCL11
CXCL9
CCR1
CCR7
MICA
CCR5
CCL8
CD54
CXCR3
TNF
SLAMF7
CCL5
CX3CR1
CCL4
CCL3
CXCL11
CXCL9
CD155
CXCL10
CD112
CCR5
CCL8
CXCR3
CX3CR1
CCL5
CCR7
CCR1
MICB
CD54
SLAMF7
MICA
TNF
CCL4
CCL3


D
Absolute Expression
Relative Expression
CD14 Monocyte
CD14 Monocytes
Iso
Len
Elo
EloLen
CCR7
CX3CR1
CXCR3
CXCL11
CCL5
MICA
MICB
CD155
CD112
TNF
CCL4
CCL8
CXCL9
CXCL10
CD54
CCR5
SLAMF7
CCR1
CCL3
CX3CR1
CXCR3
CD155
CD112
MICA
SLAMF7
CD54
CCR5
CCR1
MICB
CXCL10
CCR7
CXCL11
CXCL9
CCL5
CCL8
TNF
CCL4
CCL3
E
OPM2
OPM2 cells
SLAMF7
CCR1
MICB
CD155
CCR7
CXCR3
CX3CR1
CD112
CCR5
TNF
CCL4
CXCL10
CD54
CXCL9
CCL5
MICA
CXCL11
CCL8
CCL3
CXCR3
CX3CR1
CD155
SLAMF7
MICB
CCR1
CCR5
CD112
MICA
CCL8
CD54
CXCL9
CXCL10
CXCL11
CCL5
TNF
CCL4
CCR7
CCL3
Log2 cpm
Low
-2
High
12
Z-score
-1.5
1.5


NK cell
MM cell
A
CD16
CD54 low
CD11a low
SLAMF7
CD11a low
CD54 low
Lenalidomide
B
CD16
CD54 low
CD11a low
Monocytes
SLAMF7
CD11a low
CD54 low
Monocytes
C
CD16
CD54 high
CD11a high
CD69
CD107a
Elotuzumab
ADCC
SLAMF7
CD11a low
CD54 low
Elotuzumab/
Elotuzumab
Lenalidomide
D
CD16
CD54 high
CD11a high
CD69
CD107a
Elotuzumab
ADCC
SLAMF7
CD11a medium
CD54 low
Monocytes
ADCP
Crosstalk
Monocytes
Elotuzumab
E
CD16
CD54 high
CD11a high
CD69
CD107a
Elotuzumab
ADCC
SLAMF7
CD11a high
CD54 high
MICA
CD155
CXCR3
CCR7
Monocytes
ADCP
Crosstalk
CXCL10/11
CCL5
Monocytes
Elotuzumab
Lenalidomide


Elotuzumab
Lenalidomide
+
no phenotype change
Healthy Donor vs
Refractory Relapsed MM
Myeloma Cell Death
CD54hi
CD11ahi
MHC-IIhi
Myeloma
IFNγ
TNF
CCL5
Activated
NK Cell
M0
Myeloma
CXCL10
CXCL11
CCL5
GENE
EXPRESSION
DEGs
GSEA
OPM2
Iso
Len
Elo
EloLen
CXCR3
CX3CR1
CD155
SLAMF7
MICB
CCR1
CCR5
CD112
MICA
CCL8
CD54
CXCL9
CXCL10
CXCL11
CCL5
TNF
CCL4
CCR7
CCL3
OPM2
MHC class II receptor activity
MHC class II protein complex binding
MHC protein complex binding
cytokine receptor activity
chemokine activity
chemokine receptor binding
actin binding
CCR chemokine receptor binding
cytokine activity
0
2
4
6
-log10 adjusted p-value
adj. pvalue < 0.05
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