Online Data supplement

Novel Mechanisms of Action Contributing to Benralizumab's Potent Anti-Eosinophilic Activity

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SUPPLEMENTARY METHODS

Antibodies

Benralizumab (formerly MEDI-563) was produced at AstraZeneca, Gaithersburg, MD, USA, according to good manufacturing practices, via a fucosyltransferase-deficient producer CHO line (5). α-fucosylated R347 IgG1 served as a negative isotype-matched control. Anti–TNF-α (clone mAb11, BioLegend), anti-TNFR (clone mAb625, R&D Systems), and isotype-matched control antibody (MOPC21, BioLegend) were used for blocking experiments.

Human Primary Cell Isolation and Culture

Human whole blood was collected following informed consent for healthy volunteers recruited by the AstraZeneca blood donor program. Healthy volunteers consisted of AstraZeneca employees who were anonymously enrolled in the AstraZeneca Research Specimen Collection Program. Donors with HIV infection, hepatitis B or C virus, human T-lymphotropic virus, or syphilis were excluded. Blood eosinophils ranged 0.5–6%. Eosinophils and NK cells were isolated by immunomagnetic negative selection from whole blood with MACSxpress® Whole Blood Eosinophil Isolation Kit (Miltenyi) and EasySep™ Direct Human NK Cell Isolation Kit (STEMCELL Technologies), respectively, which routinely yielded >97% purity. Peripheral blood mononuclear cells were obtained with Ficoll-Paque density centrifugation for monocyte isolation with EasySep™ Human Monocyte Enrichment Kit without CD16 Depletion (STEMCELL Technologies). Macrophages were differentiated from primary blood monocytes by culturing for 6 days in RPMI-1640 medium supplemented with 10% FBS, 50 U/mL penicillin-streptomycin, 2 mM L-glutamine, and 50 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF; PeproTech).

Antibody-Dependent Cell-Mediated Cytotoxicity

Primary human eosinophils (10^6 cells/mL) were labeled with red lipophilic tracer ($DiIC_{12}$[3]), then incubated (45 minutes, 37°C) with 1 μg/mL of benralizumab or its parent fucosylated anti–IL-5Rα or afucosylated R347 antibodies. Cells were washed and dispatched into 96-well plates (5 x 10^4 cells/well) and coincubated with autologous green-labeled NK cells with SP-$DiOC_{18}$(3) dye, at target:effector ratio of 1:3 for 6 hours at 37°C in serum free medium. Prior to coincubation, NK cells were loaded with green LysoTracker. Eosinophil apoptosis was based on incorporation of PO-PRO-1 dye (1 μM; Thermo Fisher, USA). In real-time imaging of caspase activation, eosinophils were labelled with red dye and NK cells were stained with far-red dye. Caspase activation was monitored with green CellEvent Caspase-3/7 detection reagent (1 μM; Thermo Fisher, USA). For illustration Figures 1d and Video S3, NK cells and caspase activation were depicted green and white colors respectively. Cells were live imaged for 6 hours at 37°C in serum free medium, and apoptotic eosinophils were scored on a Zeiss LSM 880 confocal microscope (Zeiss, Germany). After 6 hours, cells were labeled separately with cell surface markers, then fixed/permeabilized and intracellularly stained for FACS analysis.

Antibody-Dependent Cellular Phagocytosis

Human blood monocytes (100,000 cells/well) were dispensed into eight-chambered borosilicate coverglasses (Thermo Fisher, USA) and differentiated into macrophages in RPMI-1640 medium supplemented with 10% FBS, 50 U/mL penicillin-streptomycin, 2 mM L-glutamine, and 50 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF; PeproTech). After day 6 of differetiation, adherent $CD16^+$ macrophages, counted 10,000 cells, were stained with green

fluorescent, lipophilic SP-DiOC$_{18}$(3) dye (Thermo Fisher, USA). Autologous eosinophils were labeled with far-red lipophilic tracer (DiIC$_{18}$[5]-DS), then incubated with 1 μg/mL of benralizumab, parent fucosylated anti–IL-5Rα, or afucosylated R347 antibodies. Labeled eosinophils were resuspended in RPMI-1640 medium supplemented with 50 ng/mL M-CSF and added (50,000 cells/well) on top of green-labeled macrophages alone or mixed with red-labeled NK cells with DiIC$_{12}$(3) dye (150,000 cells/well). Cells were either fixed after 4 hours and stained for FACS analysis or live imaged for 6 hours at 37°C. Phagocytized cells were then scored on a Zeiss LSM 880 Airyscan confocal microscope (Zeiss, Germany).

Flow Cytometry

For the detection of granzyme B, perforin, TNF-α, and IFN-γ, brefeldin A was included in the assays during the incubation time. ADCC or ADCP assays were stopped after 6 hours by putting plates on ice and replacing the medium with PBS/BSA, to which cell surface antibodies were added for 30 minutes at 4°C in PBS+2% FBS+2mM EDTA. For intracellular staining, cells were fixed in 2% PFA (10 minutes), washed, permeabilized, and stained with the antibodies listed in **Supplementary Table S1**, and then analyzed by flow cytometry (LSRII, BD Bioscience).

Live Cell Imaging

Eosinophils were incubated with 1 μg/mL of benralizumab, parent fucosylated anti–IL-5Rα, or afucosylated R347 antibodies for 45 minutes at 37°C, then labeled with far-red lipophilic tracer. Primary NK cells and macrophages were stained with red lipid membrane and SP-DiOC18, respectively. NK cells (150,000 cells) were mixed with eosinophils (50,000 cells) in 300 μL phenol red-free RPMI medium/PO-PRO-1 and imaged alone for 6 hours or added on top of labeled

macrophages in the eight-well chambered borosilicate coverglasses. For macrophage live imaging, medium was supplemented with 50 ng/mL M-CSF. Imaging was performed on a Zeiss LSM 880 microscope equipped with a 40x water immersion objective and heated stage.

Antibody-Dependent Complement Activation

Eosinophils were plated at 20,000–50,000 cells per well in U-bottom 96-well plates in normal human serum media (NHSM) or heat-inactivated human serum media (HINHSM). Benralizumab, the parent fucosylated anti–IL-5Rα, and negative control antibodies (afucosylated R347 IgG1 or rituximab) were titrated down from 10 μg/mL to 0.03 μg/mL and coincubated with human blood–derived eosinophils under NHSM or HINHSM conditions. CDC activity of rituximab, an anti-CD20 antibody, has been described (1). Daudi cells, expressing CD20, were incubated with decreasing rituximab concentrations (10–0.01 μg/mL) as a positive control for the assay. After 4 hours of incubation at 37°C, cells were washed in PBS and stained with fixable blue live/dead stain for 15 minutes at 4°C. After quenching the dye with PBS+5% BSA (FACS buffer), cells were fixed with 4% paraformaldehyde for 15 minutes at 4°C, washed, and analyzed on LSRII flow cytometer.

Cytokine Quantification by ELISA

ADCC and ADCP assays were stopped after 4 hours by putting plates on ice, and supernatants were collected. Secreted TNF-α was assessed with the human TNF-α quantikine ELISA according to the manufacturer's instructions (R&D Systems).

Statistical Analysis

All data were analyzed via Prism 6 (GraphPad software) and presented as mean ± SEM. *P*-values <0.05 were considered to indicate statistically significant differences. Details of the tests performed to determine statistically significant differences are described in **Supplementary Table S2**.

REFERENCE

1. Rudnicka D, et al. Rituximab causes a polarization of B cells that augments its therapeutic function in NK-cell-mediated antibody-dependent cellular cytotoxicity. *Blood.* 2013;121(23):4694–4702.

SUPPLEMENTARY TABLES

Supplementary Table S1. Antibodies

Antibody	Source	Catalog number	Clone
GranzymeB-FITC	BioLegend	372206	GB11
CD56-BUV395	BD Bioscience	563554	NCAM16
CD94-PE	BioLegend	305506	DX22
CD94-APC F750	BioLegend	305518	DX22
CD94-BUV737	BD Bioscience	748787	3D9
Perforin-Alexa Fluor 700	BioLegend	353324	B-D48
CD107a-BV 711	BioLegend	328638	H4A3
CD107a-BV650	BioLegend	328640	H4A3
CD137-APC Cy7	BioLegend	309834	4-1BB
CD137-BV711	BioLegend	309832	4B4-1
Siglec-8-APC	BioLegend	347105	7C9
Siglec-8-PE Cy7	BioLegend	347112	7C9
TNF-α-BV421	BioLegend	502932	Mab11
TNF-α-BV510	BioLegend	502950	Mab11
TNF-α-APC Cy7	BioLegend	502944	Mab11
CD11b-BV785	BioLegend	301346	ICRF44
CD11b-BV510	BioLegend	562950	M1/70
IFN-γ-BV650	BioLegend	502538	4S.B3
CD66b-Alexa Fluor 700	BioLegend	305114	G10F5
TNFR1-PE	R&D Systems	FAB225P	16803
TNFR2-PE Cy7	Biolegend	358412	3G7A02
Cytochrome C-Alexa Fluor 647	BD Biosciences	558709	6H2.B4
CD163-BV605	BioLegend	333616	GHI/61
SIRPα/β-PerCP/Cy5.5	BioLegend	323812	SE5A5
SIRPα/β-PE Cy7	BioLegend	323808	SE5A5
CD64-PE 594	BioLegend	305032	10.1

Supplementary Table S2. Statistical tests performed to assess significance

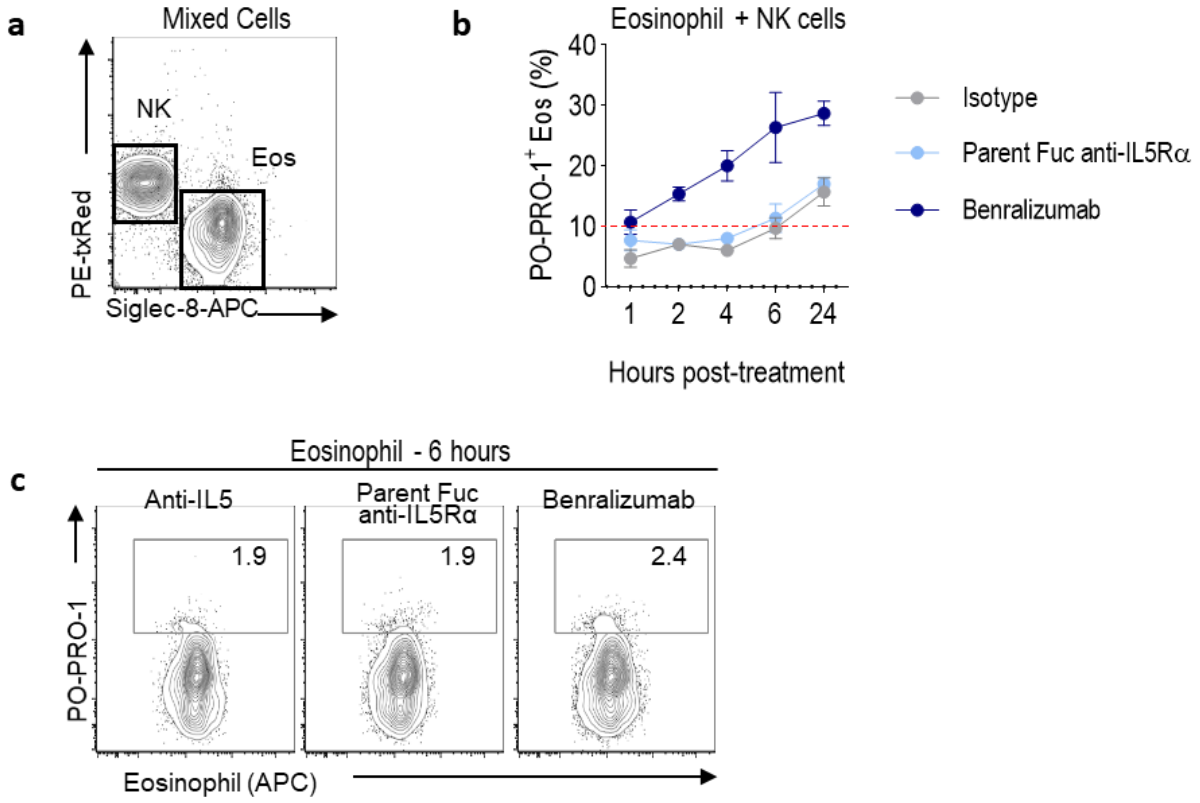
Figure #	Statistical test	Exact value
1a	Two-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ eosinophils; Benralizumab vs. anti–IL-5; 0.0006
1a	Two-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ eosinophils; Benralizumab vs. parent fucosylated anti–IL-5Rα; 0.0049
1b	Two-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ Caspase 3/7+ eosinophils; Benralizumab vs. anti–IL-5; 0.0002
1b	Two-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ Caspase 3/7+ eosinophils; Benralizumab vs. parent fucosylated anti–IL-5Rα; 0.0083
1c	Two-way ANOVA, Tukey's multiple comparisons	Cytochrome C+ eosinophils; Benralizumab vs. anti–IL-5; <0.0001
1c	Two-way ANOVA, Tukey's multiple comparisons	Cytochrome C+ eosinophils; Benralizumab vs. parent fucosylated anti–IL-5Rα; 0.0002
2b	Two-way ANOVA, Tukey's multiple comparisons	CD137+ CD107a+ activated NK cells, benralizumab vs. parent fucosylated anti–IL-5Rα; 0.0034
2b	Two-way ANOVA, Tukey's multiple comparisons	Granzyme B+ Perforin+ activated NK cells, benralizumab vs. parent fucosylated anti–IL-5Rα; 0.0175
3c	One-way ANOVA, Tukey's multiple comparisons	Number of eosinophil engulfed by individual macrophage; benralizumab eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; 0.0065
3d	One-way ANOVA, Tukey's multiple comparisons	Engulfed and PO-PRO-1+ eosinophils; benralizumab eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; 0.0003
3d	One-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ Caspase 3/7+ eosinophils; benralizumab eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; <0.0001
4a	Two-way ANOVA, Sidak's multiple comparisons	Number of eosinophil engulfed by individual macrophage; benralizumab eos+mac vs. benralizumab eos+mac+NK; 0.0001
4a	Two-way ANOVA, Sidak's multiple comparisons	Number of eosinophil engulfed by individual macrophage; benralizumab eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; 0.0448
4a	Two-way ANOVA, Sidak's multiple comparisons	Number of eosinophil engulfed by individual macrophage; benralizumab eos+mac+NK vs. parent fucosylated anti–IL-5Rα eos+mac+NK; 0.0103
4b	Two-way ANOVA, Tukey's multiple comparisons	Engulfed and PO-PRO-1+ eosinophils; benralizumab eos+NK vs. parent fucosylated anti–IL-5Rα eos+NK; <0.0001
4b	Two-way ANOVA, Tukey's multiple comparisons	Engulfed and PO-PRO-1+ eosinophils; benralizumab eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; <0.0001
4b	Two-way ANOVA, Tukey's multiple comparisons	Engulfed and PO-PRO-1+ eosinophils; benralizumab eos+mac vs. benralizumab eos+mac+NK; 0.0190

4c	Two-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ Caspase 3/7+ eosinophils; benralizumab eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; 0.0211
4c	Two-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ Caspase 3/7+ eosinophils; benralizumab eos+mac+NK vs. parent fucosylated anti–IL-5Rα eos+mac+NK; 0.0002
4c	Two-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ Caspase 3/7+ eosinophils; benralizumab eos+mac vs. benralizumab eos+mac+NK; 0.0005
5a	Two-way ANOVA, Tukey's multiple comparisons	CD64+ CD107a+ activated macrophages; benralizumab eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; 0.0003 CD163 SIRPα/β activated macrophages; benralizumab eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; 0.0011
5a	Two-way ANOVA, Tukey's multiple comparisons	CD64+ CD107a+ activated macrophages; benralizumab eos+mac vs. benralizumab eos+mac+NK; 0.0046 CD163 SIRPα/β activated macrophages; benralizumab eos+mac vs. benralizumab eos+mac+NK; 0.0085
5c	Two-way ANOVA, Tukey's multiple comparisons	TNF; benralizumab mac+eos vs. parent fucosylated anti–IL-5Rα mac+eos; 0.05
5c	Two-way ANOVA, Tukey's multiple comparisons	TNF; benralizumab mac+eos+NK vs. benralizumab mac+eos; 0.0478
5d	Two-way ANOVA, Sidak's multiple comparisons	TNF; benralizumab mac+eos+NK vs. parent fucosylated anti–IL-5Rα mac+eos+NK; 0.0052
5d	Two-way ANOVA, Sidak's multiple comparisons	TNF; benralizumab mac+eos+NK vs. benralizumab mac+eos; 0.0020
6a	Two-way ANOVA, Tukey's multiple comparisons	TNFR1; benralizumab mac+eos vs. parent fucosylated anti–IL-5Rα mac+eos; 0.0138
6a	Two-way ANOVA, Tukey's multiple comparisons	TNFR1; benralizumab mac+eos+NK vs. benralizumab mac+eos; <0.0001
6b	Two-way ANOVA, Sidak's multiple comparisons	TNFR2+ Caspase3/7+ eosinophils; benralizumab mac+eos+NK vs. parent fucosylated anti–IL-5Rα mac+eos+NK; 0.0101
6b	Two-way ANOVA, Sidak's multiple comparisons	TNFR1+ Caspase3/7+ eosinophils; benralizumab mac+eos+NK vs. parent fucosylated anti–IL-5Rα mac+eos+NK; <0.0001
6c	Two-way ANOVA, Tukey's multiple comparisons	JC10-Green high PO-PRO-1+ eosinophils: Isotype vs. benralizumab; <0.0001 Isotype vs. benralizumab+anti-TNF+anti-TNFR1; 0.0127 Isotype vs. benralizumab+anti-TNFR2; <0.0001 Parent fuc anti-IL5Ra vs. benralizumab; <0.0001 Parent fuc anti-IL5Ra vs. benralizumab+anti-TNFR2; <0.0001 Benralizumab vs. benralizumab+anti-TNF+anti-TNFR1; <0.0001 Benralizumab vs. benralizumab+anti-TNFR2; <0.0001 Benralizumab+anti-TNF+anti-TNFR vs. benralizumab+anti-TNFR21; <0.0001
6c	Two-way ANOVA, Tukey's multiple comparisons	JC10-Green low PO-PRO-1+ eosinophils: Isotype vs. benralizumab; <0.0001 Isotype vs. benralizumab+anti-TNFR2; <0.0001 Parent fuc anti-IL5Ra vs. benralizumab; <0.0001 Parent fuc anti-IL5Ra vs. benralizumab+anti-TNFR2; <0.0001

		Benralizumab vs. benralizumab+anti-TNF+anti-TNFR1; 0.0230 Benralizumab+anti-TNF+anti-TNFR vs. benralizumab+anti-TNFR21; <0.0001
6c	Two-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ eosinophils: Isotype vs. benralizumab; <0.0001 Isotype vs. benralizumab+anti-TNF+anti-TNFR1; 0.0009 Isotype vs. benralizumab+anti-TNFR2; <0.0001 Parent fuc anti-IL5Ra vs. benralizumab; <0.0001 Parent fuc anti-IL5Ra vs. benralizumab+anti-TNFR2; <0.0001 Benralizumab vs. benralizumab+anti-TNF+anti-TNFR1; <0.0001 Benralizumab vs. benralizumab+anti-TNFR2; <0.0001 Benralizumab+anti-TNF+anti-TNFR vs. benralizumab+anti-TNFR21; <0.0001
6d	One-way ANOVA, Tukey's multiple comparisons	Cytochrome C+ eosinophils; benralizumab+anti-TNFa+anti-TNFR1 vs. benralizumab+ IgG; 0.0031
6d	One-way ANOVA, Tukey's multiple comparisons	PO-PRO-1+ eosinophils; benralizumab+anti–TNFa+anti-TNFR1 vs. benralizumab+IgG; 0.0329
7a	Two-way ANOVA, Tukey's multiple comparisons	IFN-γ; benralizumab NK+eos±mac vs. parent fucosylated anti–IL-5Rα NK+eos±mac; 0.0008
7b	Two-way ANOVA, Tukey's multiple comparisons	TNF; isotype 10 ng/mL IFN-γ+eos+mac vs. isotype eos+mac; 0.0004
7b	Two-way ANOVA, Tukey's multiple comparisons	TNF; parent fucosylated anti–IL-5Rα 10 ng/mL IFN-γ+eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; <0.0001
7b	Two-way ANOVA, Tukey's multiple comparisons	TNF; benralizumab 10 ng/mL IFN-γ+eos+mac vs. benralizumab eos+mac; 0.0001
7c	Two-way ANOVA, Tukey's multiple comparisons	TNFR1; isotype 10 ng/mL IFN-γ+eos+mac vs. isotype eos+mac; <0.0001
7c	Two-way ANOVA, Tukey's multiple comparisons	TNFR1; parent fucosylated anti–IL-5Rα 10 ng/mL IFN-γ+eos+mac vs. parent fucosylated anti–IL-5Rα eos+mac; <0.0001
7c	Two-way ANOVA, Bonferroni multiple comparisons	TNFR1; benralizumab 10 ng/mL IFN-γ+eos+mac vs. benralizumab eos+mac; <0.0001

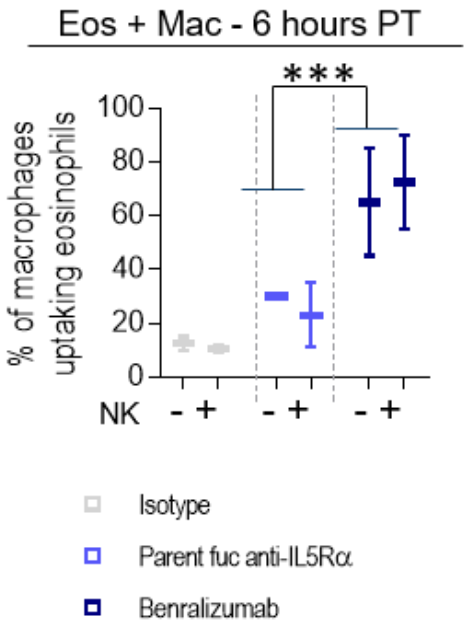
Eos, eosinophil; mac, macrophage; SIRP, signal regulatory protein; TNFR1, TNF receptor 1; TNFR2. TNF receptor 2.

SUPPLEMENTARY FIGURES



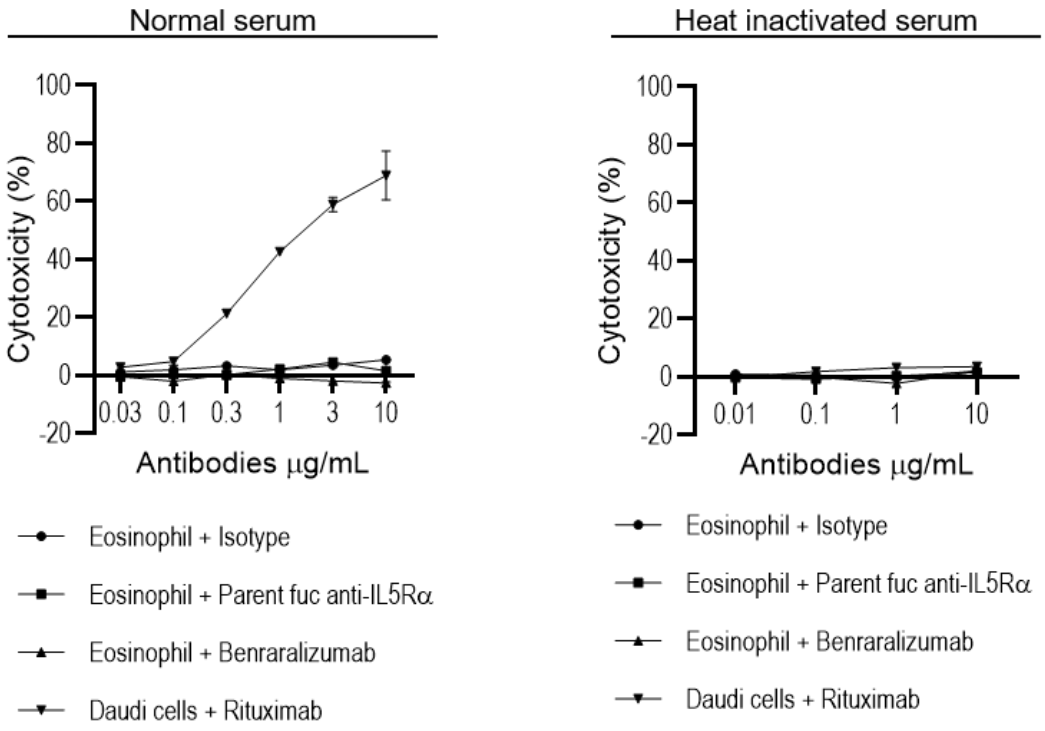
Supplementary Figure S1. Benralizumab treatment in the presence of NK cells significantly increases eosinophil death over time.

(**a**) Gating by FACS on NK cells and eosinophils with lipid membrane labels. (**b–c**) Primary human NK cells and eosinophils isolated from healthy donors were labeled individually. Eosinophils were pretreated with (**b**) 10 nM of indicated antibodies mixed with NK cells or (**c**) antibodies alone for up to 6 hours and analyzed by flow cytometry. Data in B are mean ± SEM.



Supplementary Figure S2. Benralizumab treatment significantly increases the percentage of macrophages uptaking eosinophils.

Quantification of activated macrophages engulfing live or dead eosinophils, presented as the percentage of total macrophages, upon treatment with the indicated antibodies (*n*=4). Data in A are mean ± SEM. Two-way ANOVA test was employed, with Tukey's multiple comparisons. Statistical values are presented in Supplementary Table S2.



Supplementary Figure S3. Benralizumab failed to induce eosinophil depletion through complement activation.

Human eosinophils were isolated from blood and incubated in normal or heat-inactivated sera, with increasing concentrations of indicated antibodies in triplicates for 6 hours (*n*=3). Rituximab-induced $CD20^{+}$ cell cytotoxicity of Daudi B-cells was used as positive control. Data are mean ± SEM. Two-way ANOVA test was employed, with Tukey's multiple comparisons.

SUPPLEMENTARY VIDEO LEGENDS

Videos S1, S2, and S3 are related to Figure 1. Real time video of benralizumab inducing caspase-dependent eosinophil apoptosis through ADCC.

Primary human NK cells and eosinophils were isolated from healthy donors and green-labeled individually. NK cells were loaded with green LysoTracker. To live image benralizumab mediated ADCC, red-labeled-eosinophils were pretreated with 1 μg/mL of parent fucosylated anti–IL-5Rα (**Video S1**) or benralizumab (**Video S2**), then mixed with green-labeled NK cells and PO-PRO-1 for 4 hours. Afterwards, mixed cells were live imaged for 2 hours with confocal microscopy. Images were acquired every 2 minutes. To visualize caspase activation (**Video S3**), CellEvent Caspase-3/7 reagent was added prior to live cell acquisition. Caspase positive cells are depicted in white.

Videos S4 and S5 are related to Figure 2. Real time video of benralizumab mediating eosinophil phagocytosis/efferocytosis by macrophages.

Video S4 highlights the biological check points in assessing NK cell (green) killing of eosinophils (red), starting with the immunological synapse, release of NK-green granules into red-esosinophilic cytosol and ending with eosinophil membrane blebbing and loss of cell integrity, also depicted in **Video S5**. Green-labeled NK lytic granules converged at the immunological synapse and docked at the plasma membrane of red-labeled eosinophils that underwent apoptosis as evidenced by the incorporations of PO-PRO-1 blue nuclear dye.

Videos S6 and S7 are related to Figure 3. Real time video of benralizumab-mediated eosinophil apoptosis via ADCP.

To live image benralizumab-mediated ADCP (**Videos S6** and **S7**), primary monocytes were differentiated into macrophages for 6 days. Then, eosinophils were isolated from the autologous healthy donors and labeled individually. After pretreatment with 1 μg/mL of benralizumab, far-red–labeled eosinophils (white color) were mixed with green-labeled macrophages and blue nuclear dye PO-PRO-1. Afterward, mixed cells were live imaged for 6 hours with confocal microscopy. Images were acquired every 5 minutes.

Videos S8, S9 and S10 are related to Figure 4. Real time movies highlighting NK enhancement of benralizumab-mediated macrophage phagocytosis/efferocytosis, and NK-macrophage immune synapses.

Video S8 illustrates the effect of NK (red) on macrophage (green) phagocytic behavior induced by benralizumab. **Video S9** demonstrates that NK cells were interacting actively with phagocytic macrophages (green) upon benralizumab treatment. Eosinophils are labeled in white and nuclear dye PO-PRO-1 in blue.

Real time **Video S10** demonstrates that white-labeled eosinophil incorporating blue dye, a hallmark of apoposis, was cleared by an active green-labeled macrophage (efferocytosis) that was also able to engulf another red-labeled NK cell.