

our results, and further proteomics and functional studies are needed to elucidate the role these genes play in relation to changes in *Staphylococcus* species and skin barrier dysfunction in general.

As a general conclusion, we postulate that the explanation for AD flare development and exacerbation is a complex interaction between the skin microbiota, barrier, and immune system. Interaction points between different components of human skin might be the key to development of therapeutic approaches to allergic skin diseases.

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FcεRI cross-linking and IL-3 protect human basophils from intrinsic apoptotic stress



To the Editor:

Basophils are armed with proinflammatory mediators that are detrimental during IgE-mediated allergic reactions. Stimuli present in an allergic inflammatory milieu, such as IL-3, are known for extending the lifespan of basophils and eosinophils.¹ Several studies have suggested that induction of apoptosis, which is critical for the resolution of inflammation, is poorly managed in allergic airway inflammation, such as in the case of eosinophils in patients with acute asthma.²

In this study we aimed to investigate the underlying mechanism regulating survival in human basophils under noninflammatory and allergic conditions, respectively. We focused on the study of antiapoptotic B-cell lymphoma 2 (Bcl-2) family members, which control the intrinsic pathway of apoptosis. The relevance of key antiapoptotic Bcl-2 family members in primary human basophils was evaluated by using small-molecule inhibitors termed Bcl-2 homology domain 3 (BH3) mimetics, which specifically inhibit Bcl-2 (ABT-199/venetoclax [Vencloxtax]), myeloid cell leukemia 1 (Mcl-1; A-1210477), or Bcl-2 and B-cell lymphoma-extra large (Bcl-xL; ABT-263/navitoclax).^{3,4} Because all 3 types of granulocytes are developmentally related but differ in their expression profiles of Bcl-2 family members (see Fig E1, A, and the Methods section in this article's Online Repository at www.jacionline.org), eosinophils and neutrophils were used as comparisons.

Our data showed that unlike eosinophils and neutrophils, basophils are highly susceptible to the inhibition of Bcl-2 (Fig 1, A). This confirmed our previously published study in which we demonstrated that survival of mouse basophils was also highly dependent on Bcl-2.⁵ Simultaneous inhibition of Bcl-2 and Bcl-xL further increased the cell death rate in basophils but also efficiently induced apoptosis in eosinophils (Fig 1, A). In basophils titration experiments demonstrated that ABT-199 was more potent in inducing cell death than ABT-263, with the half-maximal effective concentration being 9 and 35 nmol/L, respectively (see Fig E2, A, in this article's Online Repository at www.jacionline.org and the Methods section in this article's Online Repository). Accordingly, eosinophil survival was reduced by 50% in response to 28 nmol/L ABT-263 (see Fig E2, A).

Short-term evaluation of BH3 mimetics on basophils revealed that, although less potent, ABT-263 triggered cell death more rapidly than ABT-199, as evidenced by a significant induction of cell death after only 2 hours (see Fig E2, B). This was coherent with reduced protein levels of X-linked inhibitor of apoptosis (XIAP), an endogenous inhibitor of initiator and effector caspases,⁶ in ABT-263-treated basophils compared with cells exposed to ABT-199 (Fig 1, C). Compared with basophils, ABT-263 induced cell death in eosinophils with a certain delay, which was unexpected because eosinophils are known to have a shorter spontaneous lifespan than basophils (see Fig E2, B). None of the tested inhibitors significantly accelerated cell death of neutrophils (Fig 1, A, and see Fig E2, C), and hence the variable endogenous expression of antiapoptotic Bcl-2 family members among granulocytes reflects their susceptibility to these BH3 mimetics.

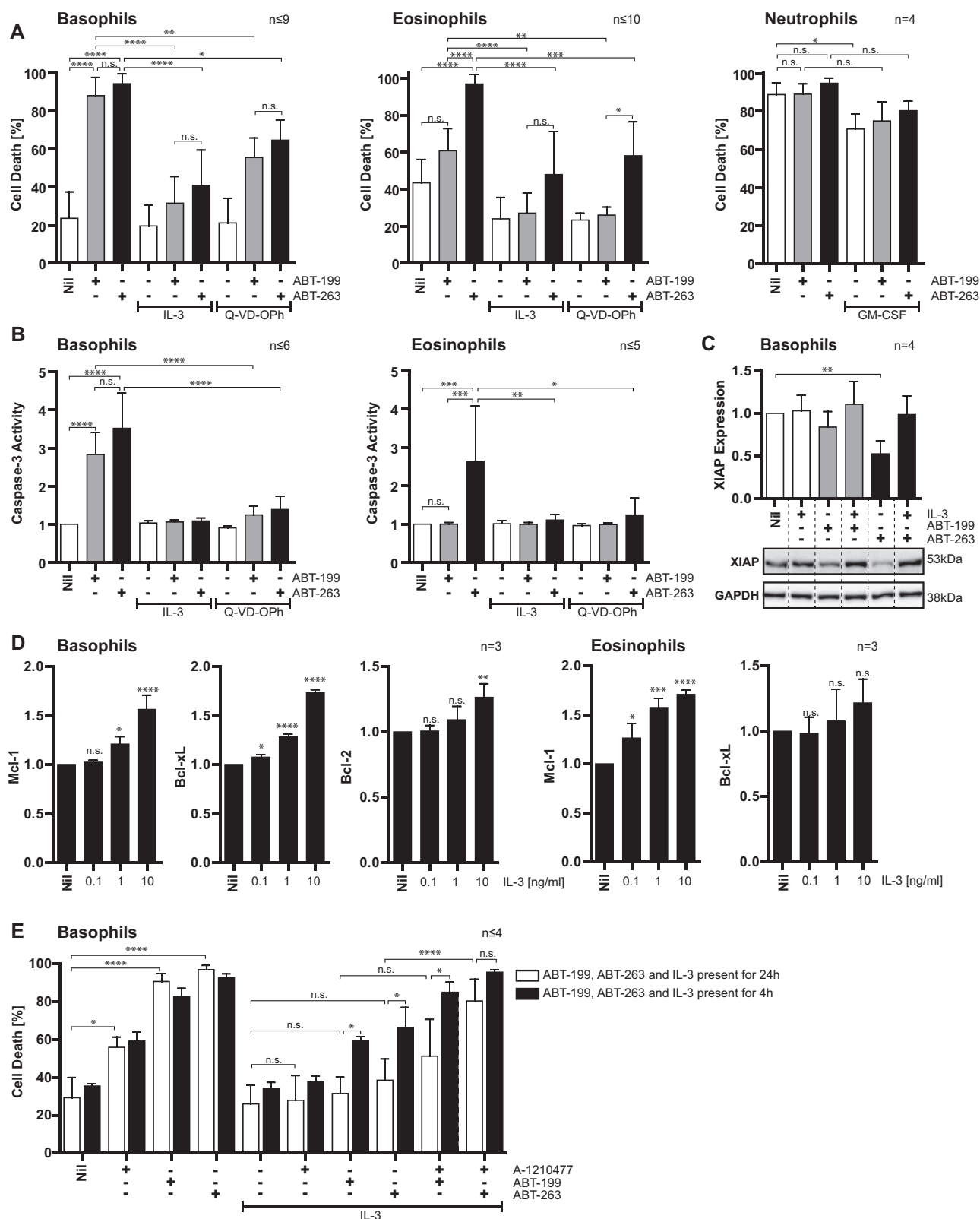


FIG 1. Survival of resting basophils depends greatly on endogenous Bcl-2, whereas IL-3-activated cells rely on Mcl-1 and Bcl-xL. For technical details, see the [Methods](#) section in this article's Online Repository. **A** and **E**, Percentages of cells positive for Annexin V, PI, or both are shown. **B** and **D**, Fold change of median fluorescence intensity (MFI; Fig 1, *B*) and Δ MFI (stained minus unstained cells; Fig 1, *D*) normalized to medium alone (*Nii*) is shown. **C**, Representative Western blotting and quantified X-linked inhibitor of apoptosis (*XIAP*) bands using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for normalization. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$. n.s., Not significant.

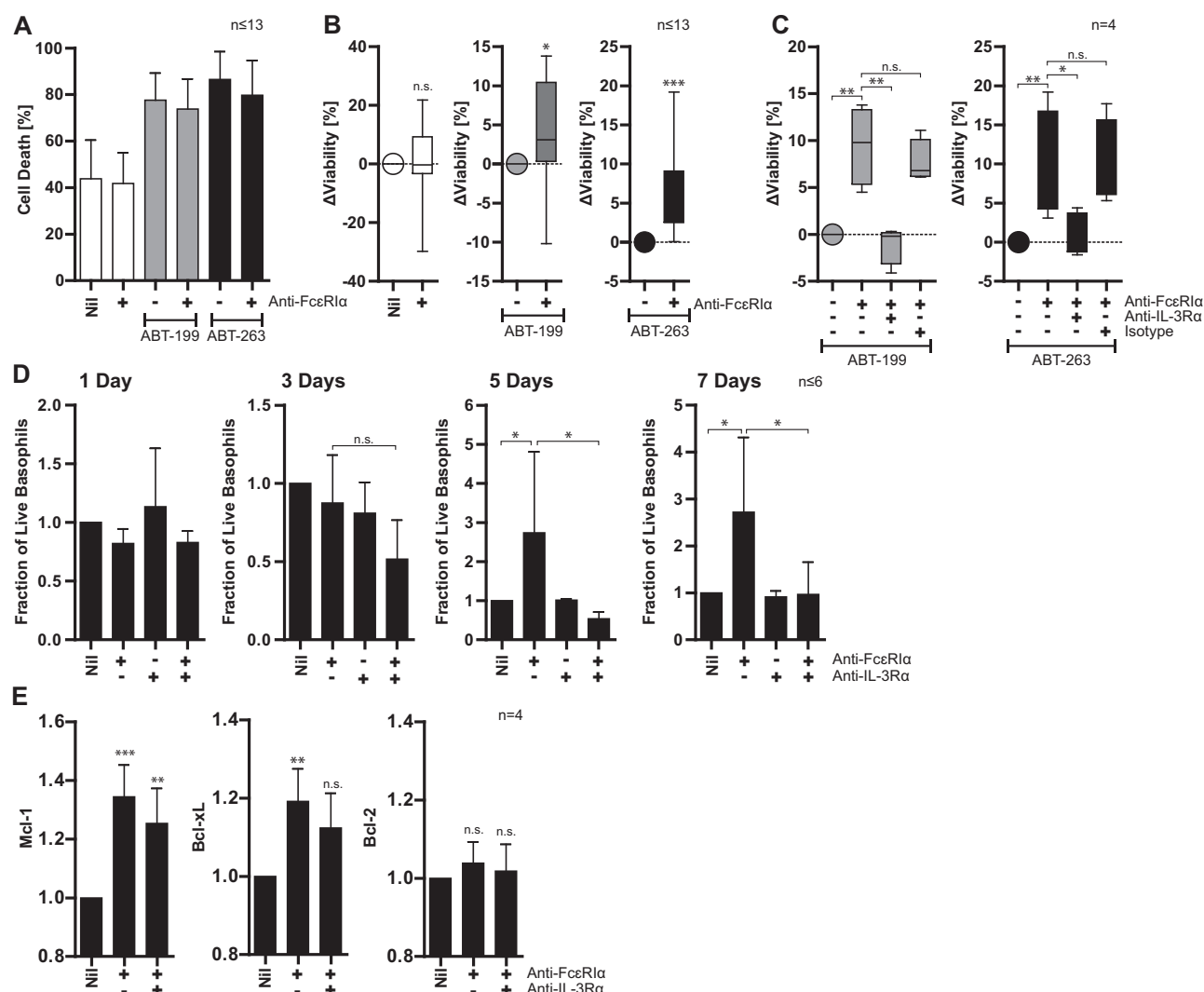


FIG 2. FcεRI receptor cross-linking renders human basophils less susceptible to intrinsic apoptosis through IL-3-dependent and independent signaling. For technical details, see the Methods section in this article's Online Repository. **A–C**, Percentage of cell death (Fig 2, A) and difference in cell viability of basophils treated with or without ABT compounds compared with their respective FcεRI cross-linking treatment groups (Fig 2, B and C) or the fraction of basophils negative for both cell death markers (absolute cell number multiplied by survival in percentage). Basophil viability was assessed by using Annexin V/PI (Fig 2, A–C) or Hoechst 33342/PI staining (D). **E**, Shown is the Δ median fluorescence intensity (stained minus unstained cells) of Bcl-2, Bcl-xL, and Mcl-1 normalized against medium alone (Nil). **P* < .05, ***P* < .01, and ****P* < .001. n.s., Not significant.

To confirm apoptosis as an underlying mechanism, we analyzed proteolytic activation of caspase-3 using Western blotting (early time points; see Fig E1) and flow cytometry (late time points; Fig 1, B). In basophils both ABT-199 and ABT-263 triggered increased levels of activated caspase-3 (Fig 1, B, and see Fig E1, A and B), whereby ABT-263 was more efficient than ABT-199.

Consistent with the survival data, ABT-263 caused cleavage of caspase-3 in eosinophils but not in neutrophils (Fig 1, B, and see Fig E1, A and B). Members of the GM-CSF family are well known for their capacity to prolong survival of human granulocytes but also play a crucial role in granulocyte activation. IL-3 significantly and sustainably protected basophils and eosinophils from ABT-mediated apoptosis, as evidenced by the inhibition of cell death and caspase-3 activity,

respectively (Fig 1, A and B, and see Fig E2, D). Like IL-3, the pancaspase inhibitor Q-VD-OPh efficiently suppressed caspase-3 activity (Fig 1, B) but reduced cell death only partially (Fig 1, A). An explanation for this discrepancy could be that although IL-3 prevents caspase activation indirectly and upstream of mitochondrial depolarization, Q-VD-OPh blocks caspase activity directly but does not prevent leakage of apoptogenic and cytotoxic proteins from mitochondria into the cytoplasm.⁷ Indeed, although Q-VD-OPh-treated granulocytes exhibited reduced phosphatidyl serine externalization, propidium iodide (PI) inclusion, which is not exclusive for apoptosis, was still increased (data not shown). In IL-3-treated basophils and eosinophils, Mcl-1 was rapidly upregulated (see Fig E1, C) in a dose-dependent manner (Fig 1, D).

Expression of Bcl-xL and, to a much lesser degree, expression of Bcl-2 were increased in basophils but not in eosinophils (Fig 1, D), thus confirming and extending data published by Didichenko et al.¹ Because coinhibition of Bcl-2 and Bcl-xL did not overcome IL-3-mediated survival, we tested the selective Mcl-1 inhibitor A-1210477 in the presence or absence of ABT-199, ABT-263, IL-3, and combinations thereof, as indicated (Fig 1, E). A-1210477 increased spontaneous cell death up to 60% and was thus less efficient than the ABT compounds. Coinhibition of Mcl-1/Bcl-2/Bcl-xL was able to overcome the protective nature of IL-3, which was not the case when Bcl-2 together with Mcl-1 or individual antiapoptotic proteins were targeted (Fig 1, E, white columns). Previously, we reported that sustained functional and phenotypic changes in human basophils depend on continuous IL-3 signaling.⁸ The prosurvival effect was attenuated in all tested conditions on IL-3 removal (Fig 1, E, black columns), indicating that continuous IL-3 signaling is required to provide sufficient replenishment of antiapoptotic proteins.

In reference to our data demonstrating that IL-3 efficiently protects against BH3 mimetic-induced apoptosis and to the finding that basophils produce IL-3 in response to FcεRI cross-linking,⁹ we investigated the effect of FcεRI cross-linking and autocrine IL-3 signaling. In the absence of proapoptotic stimuli, FcεRI cross-linking had an ambiguous effect on cell viability (Fig 2, A and B). In the presence of ABT compounds, FcεRI cross-linking reduced cell death, which became obvious when calculating the difference in cell viability of basophils treated with ABT compounds compared with their respective FcεRI cross-linking treatment group (Fig 2, B). Importantly, this effect was abrogated by addition of an antagonistic anti-IL-3 receptor α (IL-3Rα) mAb (Fig 2, C).

In a further step, we mimicked apoptotic stress by culturing basophils over a time period of 7 days. Our data revealed that FcεRI-activated basophils had a survival advantage compared with nonactivated cells after 5 days and that this effect was fully dependent on IL-3 (Fig 2, D). Thus, we assumed that in the absence of proapoptotic stimuli, the effect of autocrine IL-3 signaling on cell viability is marginal because of the high constitutive expression of Bcl-2 and its consequential low rate of spontaneous cell death within this timeframe. In contrast to high concentration of recombinant IL-3 (10 ng/mL), which resulted in a pronounced increase in expression of both Mcl-1 and particularly Bcl-xL, the addition of the cross-linking antibody resulted in a substantial increase in Mcl-1 expression but a comparably modest induction of Bcl-xL expression, whereas Bcl-2 expression was not affected (Fig 2, E). Surprisingly, induction of Mcl-1 and Bcl-xL was not completely abolished by pretreatment with IL-3 receptor antagonist, suggesting that an IL-3-independent pathway might be involved as well. Taken together, this study delivers insight into the mechanisms underlying survival at the level of the Bcl-2 proteins of primary human basophils and eosinophils in different activation states.

We show that FcεRI-activated basophils have a survival advantage compared with resting basophils, which manifests itself through modulation of the Bcl-xL and Mcl-1 expression, enabling human basophils to outlast degranulation, to resist proapoptotic stimuli, and thus to maintain allergic inflammations.

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Primary immunodeficiency disorder caused by phosphoinositide 3-kinase δ deficiency



To the Editor:

Phosphoinositide 3-kinase δ (PI3Kδ) participates in signaling transduction downstream of diverse receptors of the immune system, including the T-cell receptor, the B-cell receptor, and costimulatory receptors.¹ It is a heterodimeric protein composed of a catalytic subunit (p110δ) and a regulatory subunit (p85). The consequences of PI3Kδ deficiency have been extensively characterized in mice, providing much of our knowledge on the biological role of PI3Kδ, such as its involvement in early development of B cells and regulation of adaptive immune responses.² Heterozygous gain-of-function mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit δ gene (*PIK3CD*; the gene encoding p110δ) cause a primary immunodeficiency disorder characterized by both immunodeficiency and immune dysregulation (activated PI3Kδ syndrome). Evidence on the consequences of PI3Kδ deficiency in human subjects remains scarce. Here we present an autosomal recessive primary immunodeficiency disorder caused by PI3Kδ deficiency.

METHODS

Isolation and culture of primary human granulocytes

This study was approved by the ethics committee of the state of Bern (no. 108/14) and conducted according to the Declaration of Helsinki. Blood samples ($n = 66$) were obtained from voluntary adult donors after receiving informed consent. Isolation of primary human granulocytes and assessment of absolute cell numbers and purity were performed, as described previously.^{E1} Purity of isolated basophils, eosinophils, and neutrophils was 95.7% (SD, 4.1%), 97.6% (SD, 1.2%), 95% (SD, 2.2%), respectively. Freshly isolated granulocytes were cultured at 37°C in a humidified atmosphere with 5% CO₂ for indicated time periods at a density of 1×10^6 cells/mL, as described elsewhere.^{E1}

Statistical analysis

The α significance level was set to .05 for all experiments. Group comparisons were performed by using the 2-tailed unpaired *t* test (Fig 2, B) or ordinary 1-way ANOVA followed by Bonferroni *post hoc* test (Fig 1, A, B, and E, and Fig 2, D) or Dunnett *post hoc* test (Fig 1, C and D, and Fig 2, C and E). Statistical analysis was performed with GraphPad Prism 7.00 software (GraphPad Software, San Diego, Calif). All values are presented as means $\pm$ SDs.

Inhibitors, cytokines, and functional antibodies

The following inhibitors were used: the selective Bcl-2 inhibitor ABT-199 (Venclaxta), the Bcl-2 and Bcl-xL inhibitor ABT-263 (navitoclax), the selective Mcl-1 inhibitor A-1210477 (all from Selleck Chemicals, Houston, Tex), and the pancaspase inhibitor Q-VD-OPH (SM Biochemicals, Anaheim, Calif). Recombinant human IL-3 (Novartis, Basel, Switzerland) and GM-CSF (PeproTech, Rocky Hill, NJ) were used. Cross-linking of the high-affinity IgE receptor was performed with the anti-Fc ϵ R1 α mAb 29C6 (Hoffmann-La Roche, Basel, Switzerland). Blocking of the IL-3R α chain was done with the anti-IL-3R α mAb CSL360 (CSL Behring, King of Prussia, Pa), a humanized IgG₁ mAb derived from clone 7G3.^{E2} hBM4, which was produced under the same conditions as CSL360, was used as a matched isotype control and kindly provided by CSL Behring. ABT-199, ABT-263, A-1210477, and Q-VD-OPH were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St Louis, Mo), whereas IL-3, GM-CSF, 29C6, CSL360, and hBM4 were reconstituted in $1 \times$ PBS supplemented with 0.1% BSA. Dimethyl sulfoxide vehicle controls were used at corresponding concentrations to exclude off-target effects of the solvent during BH3 mimetic treatment (compare Fig E1). The order of addition of inhibitors was as follows.

For Fig 1, A to D, granulocytes were cultured for 24 hours (Fig 1, A, B, and D) or 4 hours (Fig 1, C) in medium alone or in the presence of ABT-199 or ABT-263 (300 nmol/L each) without or with 0.1, 1 (Fig 1, D), and 10 ng/mL (Fig 1, A-C) IL-3, 10 ng/mL GM-CSF (Fig 1, A), or Q-VD-OPH (50 μ mol/L; Fig 1, A and B), as indicated. Q-VD-OPH was added 2 hours before and cytokines were added 30 minutes after addition of BH3 mimetics.

For Fig 1, E, human basophils were cultured with or without ABT-199 or ABT-263 (300 nmol/L each) for the first 30 minutes and subsequently exposed to medium only or 10 ng/mL IL-3 for another 4 hours. After 4 hours, culture medium was replaced with plain medium (black columns) or left untouched (white columns), whereupon the selective Mcl-1 inhibitor A-1210477 (3 μ mol/L) was added. Cells were cultured for 24 hours in total.

For Fig 2, basophils were cultured for 24 hours with or without the Fc ϵ R1 α cross-linking mAb 29C6 (100 ng/mL) in the presence or absence of ABT-199 and ABT-263 (10 and 30 nmol/L, respectively, added 30 minutes after 29C6; Fig 2, A-C) and with the anti-IL-3R α mAb CSL360 or its corresponding isotype control hBM4 (10 μ g/mL each; Fig 1, C-E).

Flow cytometry

Cell death was assessed in 1×10^5 cells per condition by means of dual staining with PI (Sigma-Aldrich) and Annexin-V (BD Biosciences, Allschwil, Switzerland) or Hoechst 33342 (Thermo Fischer Scientific AG, Reinach, Switzerland). Annexin/PI staining was performed according to the manufacturer's instructions. Since eosinophils exhibit strong auto-fluorescence in the fluorescein isothiocyanate (FITC)-channel, Annexin-V allophycocyanin (APC) was used for this particular cell type, whereas basophils and neutrophils were stained with Annexin-V FITC. Cell death is shown as the percentage of cells positive for either Annexin V or PI or both. Fraction of live basophils is the product of the number of total gated cells in the sample and the percentage of basophils that are negative for Hoechst 33342 and PI. Total number of cells within flow cytometry samples was calculated using CountBright Absolute Counting Beads (ThermoFischer Scientific AG). Caspase-3 activity was assessed by using the CaspGLOW Red Active Caspase-3 staining kit (Bio-Vision, Milpitas, Calif), which consists of a caspase-3 inhibitor conjugated to sulforhodamine (Red-DEVD-FMK). To detect intracellular antiapoptotic Bcl-2 family members, freshly isolated and cultured cells were permeabilized by using BD FACS Permeabilizing Solution 2 (BD Biosciences) and stained with anti-Bcl-2-FITC (Clone 100; BD Bioscience), Bcl-xL-phycoerythrin (PE) (clone 54H6), and Mcl-1-PE (clone D2W9E; both from Cell Signaling Technology, Danvers, Mass) for 30 minutes, as recommended by the manufacturer.

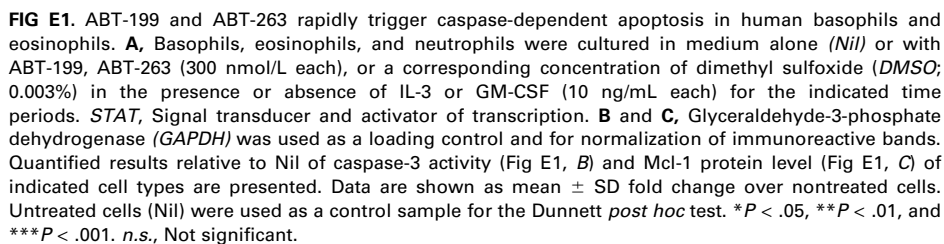
Data were acquired with the FACSCalibur or FACSCanto II (both from Becton Dickinson, Heidelberg, Germany). Data analysis was performed with FlowJo software 10.1r5 (TreeStar, Ashland, Ore).

Western blot analysis

Cell lysates of 0.4×10^6 human granulocytes per condition were prepared for Western blotting, as previously described.^{E3} The following primary antibodies with indicated dilutions were used: anti-Bcl-2 (clone 124; 1:100; DakoCytomation, Baar, Switzerland), anti-Bcl-xL (clone H-5; 1:100; Santa Cruz Biotechnology, Santa Cruz, Calif), anti-Mcl-1 (pAb; 1:200; Santa Cruz Biotechnology), anti-XIAP (clone 3B6; 1:1000) anti-caspase-3 (clone 8G10; 1:1000), anti-phospho-STAT3 (Tyr705; clone D3A7; 1:1000), and anti-phospho-STAT5 (Tyr694; pAb; 1:1000; all from Cell Signaling Technology). Anti-GAPDH (clone 6C5; 1:500; Millipore, Zug, Switzerland) served as a loading control. Goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated IgG antibodies (Bio-Rad, Reinach, Switzerland) were used as secondary antibodies. Blots were developed by using Super Signal West Pico Chemiluminescent Substrate or Super Signal West Femto Maximum Sensitivity Substrate (both from Thermo Fischer Scientific AG, Reinach, Switzerland), and immunoreactive bands were visualized with the Luminescent Image Analyzer LAS-3000 (Fujifilm, Tokyo, Japan). For quantification, immunoreactive bands were normalized against GAPDH and quantified with the AIDA Image Analyzer V3.52 (Fujifilm, Tokyo, Japan).

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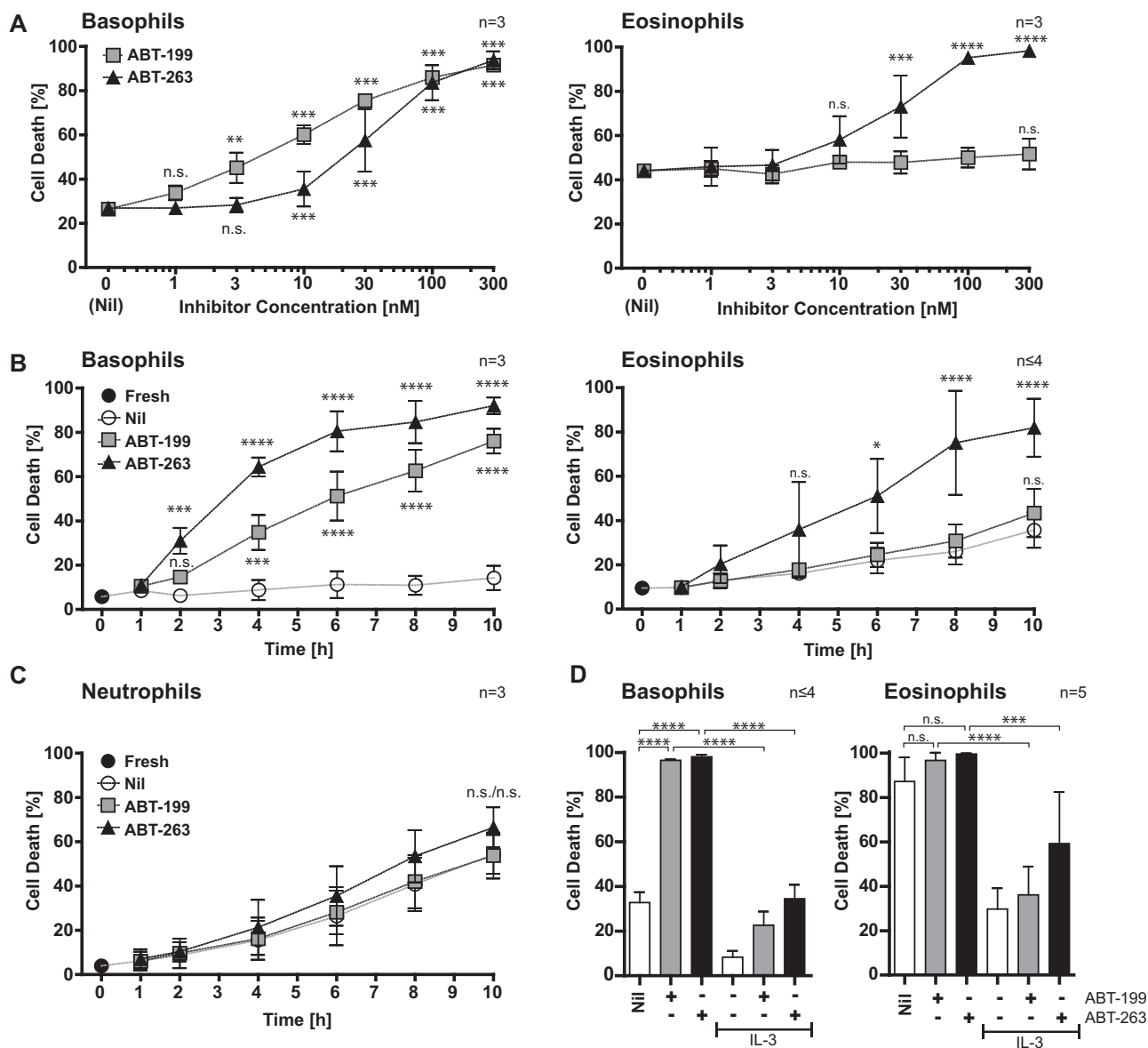


FIG E2. The Bcl-2 inhibitor ABT-199 and the Bcl-2 and Bcl-xL inhibitor ABT-263 mediate potent and rapid induction of cell death in human basophils and eosinophils. Basophils and eosinophils (**A**, **B**, and **D**) and neutrophils (**C**) were cultured for 1 to 10 hours (Fig E2, **B** and **C**), 24 hours (Fig E2, **A**), or 72 hours (Fig E2, **D**) in medium alone (*Nil*) or in the presence of ABT-199 or ABT-263 used at indicated concentrations (Fig E2, **A**) or at 300 nmol/L each (Fig E2, **B-D**). IL-3 was used at 10 ng/mL (Fig E2, **D**). Percentages of cells that were single and/or double positive for Annexin V and PI are depicted. Untreated cells after 24 hours (*Nil*; Fig E2, **A**) or untreated cells after corresponding time points (*Nil*; Fig E2, **B** and **C**) served as a control sample for the Bonferroni multiple comparison posttest for ordinary 1-way ANOVA. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$. *n.s.*, Not significant.