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TITLE PAGE

Resistance to apoptosis underpins corticosteroid-insensitivity of group 2 innate lymphoid cells

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Declaration of all sources of funding: NIHR Oxford Biomedical Research Centre Programme (PK, GO, IDP, LX and SG), Wellcome Trust (PK-WT109965MA), MRC (GO).

Conflict of interest: The authors have declared that no conflict of interest exists.

Capsule Summary

Out of all type-2 immune cells, only ILC2s withstand corticosteroid treatment due to a reduced pro-apoptotic machinery. The findings may advance the understanding on the mechanism in steroid-resistant diseases and facilitate the development of improved therapies.

Key Words

Asthma, corticosteroids, ILC2, Th2, Tc2, apoptosis, Bcl-2, mitochondria

47

48 **Abbreviations used**

49 CRTH2: Chemoattractant receptor-homologous molecule expressed on Th2 cells

50 GR: glucocorticoid receptor

51 HSP: heat shock protein

52 ILC2: Group 2 innate lymphoid cells

53 MDR1: multi-drug resistance protein 1

54 MMP: mitochondrial membrane potential

55 MTG: MitoTracker Green

56 MTO: MitoTracker Orange

57 OCS: oral corticosteroids

58 Tc2: type-2 $CD8^+$ cytotoxic T cells

59 Th2: type-2 $CD4^+$ T helpers

To the Editors:

Steroid-insensitivity is one of the major clinical problems in severe asthma, and a better understanding of the mechanisms is important.[1] Given that type-2 cells, including type-2 $CD4^+$ T helpers (Th2), type-2 $CD8^+$ cytotoxic T cells (Tc2) and group 2 innate lymphoid cells (ILC2s) are all contributors to the pathogenesis of asthma,[2,3] it is important to define both their relative resistance to corticosteroids and the underpinning mechanisms.[4] We characterised the effects of corticosteroids on three major types of human type-2 cells ($CD3^+CD4^+CRTH2^+$ Th2, $CD3^+CD8^+CRTH2^+$ Tc2 and $Lin^-CD45^{high}CD127^+CRTH2^+$ ILC2) *in vivo* and *in vitro*. We further defined the potential mechanisms contributing to the insensitivity.

We first compared the blood counts of type-2 cells in patients with severe eosinophilic asthma before and after oral corticosteroid (OCS) treatment with prednisolone (Fig. 1A-D, Table E1). After a period (7-33 days) of OCS, the blood eosinophil counts were markedly reduced (from 477.9 ± 184.2 x10^6 to 66.4 ± 87.9 x10^6 cells/L, $p<0.0003$), while the neutrophil counts increased. The Th2 and Tc2 cell counts were decreased from 37.1 ± 45.5 x10^6 to 12.6 ± 18.6 x10^6 cells/L ($p=0.02$) and from 45.9 ± 79.8 x10^6 to 6.8 ± 6.2 x10^6 cells/L ($p=0.027$), respectively (Fig. 1A,C). In contrast, ILC2s were increased, from 0.4 ± 0.43 x10^6 to 0.57 ± 0.49 x10^6 cells/L ($p=0.019$), representing an increase in the proportion of ILC2s from 1.4% to 8.3% of total blood type-2 cells (Fig. 1D).

To determine if ILC2s' tolerance to corticosteroids is cell-autonomous, we tested the effect of corticosteroids on apoptosis induction in cultured type-2 cells from healthy blood *in vitro*. Incubation with dexamethasone for 16 hours induced apoptosis in all three cell types in a dose-dependent manner but the maximal ILC2 response to dexamethasone was significantly less than Th2/Tc2 cells (Fig. 1E).

To understand whether ILC2s' resistance to apoptosis induction is specific to corticosteroids, cells were exposed to staurosporine (Fig. 1F,G). The ILC2 apoptotic response to incubation with staurosporine for 4 hours was significantly less than Th2/Tc2 cells (Fig. 1F). When cells were treated with staurosporine (10 μM) for longer periods, ILC2s showed a significantly delayed response compared with Th2/Tc2 cells (Fig. 1G). By 9 hours, similar ratios of apoptosis were detected in all three cell types, suggesting that ILC2s are as capable of undergoing apoptosis as the other type-2 cells, but their apoptotic efficiency is reduced.

Corticosteroid-insensitivity of ILC2s appeared to be restricted to apoptotic pathways, as dexmethasone attenuated chemotaxis induced by prostaglandin D_2 (Fig. E1A) or IL-13/5 production induced by prostaglandin D_2 or PMA/Ionomycin (Fig. E1B,C) similarly in all three cell types.

ILC2 resistance to corticosteroid-induced apoptosis was not due to a reduced expression of the glucocorticoid receptor (GR), as no significant difference of GR expression between the cell types was detected at either protein or mRNA (*NR3C1*) levels in blood *ex vivo* or cultured cells (Fig. E2A-C). It has been suggested that heat shock protein (HSP) 90 and 70 molecular chaperones are required for GR function, and HSP70 is also an anti-apoptotic protein.[5] However, no difference in HSP90 expression was detected (Fig. E2A-C), whereas HSP70 expression in ILC2s detected by flow cytometry was significantly lower than in Th2/Tc2 in fresh blood (Fig. E2A right panel); however, HSP70 levels were equivalent in cultured cells (Fig. 2D,E and E2B,C right panels). Thus, it is unlikely that HSP70 contributes to resistance of ILC2s to dexamethasone-induced apoptosis. Reduced expression of multi-drug resistance protein 1 (MDR1) could potentially reduce the efficacy of steroids by transporting drugs out of cells.[6] However, no significant difference in

MDR1 protein expression was detected between the three cell types (Fig. E2D). Importantly, the MDR1 inhibitors, Tariquidar or Zosuquidar, failed to alter the sensitivity of ILC2s to dexamethasone (Fig. E2E).

We next characterised the cell-intrinsic apoptotic pathways in type-2 cells.[7] The level of anti-apoptotic protein Bcl-2, detected by flow cytometry in fresh blood, was slightly higher in ILC2s compared to Th2 and Tc2 cells (Fig. 2A). Anti-apoptotic Bcl-xL expression was not significantly different among the cell types. In contrast, the relative expression of the major pro-apoptotic Bcl-2 proteins BAX and BAK were significantly lower in ILC2s. In cultured cells, mRNA expression of *BCL2* and *BCL2L1* (gene for Bcl-xL) were higher in ILC2s, but no differences were detected in *BAX1* and *BAK1* (Fig. 2B). Comparison of Bcl-2 family expression within each cell type showed that the ratios of pro-apoptotic to anti-apoptotic Bcl-2 mRNAs were significantly lower in ILC2s (Fig. 2C, E3A). These differences were further confirmed by proteome profiler array (Fig. 2D, E3B). In keeping with slightly higher expression of Bcl-2 and less BAD/BAX, the expressions of cleaved (active)-caspase 3, HSP27 and HSP60 were also lower in ILC2s compared to Th2 cells (Fig. 2E). No differences in the expression of inhibitor of apoptotic proteins (IAPs including cIAP-1, cIAP-2 and XIAP) were detected between the cell types. Similar to our qPCR results, the ratios of BAD or BAX to Bcl-2 were significantly lower in ILC2s (Fig. 2F). Dexamethasone (treated for 8 h) did not significantly change the ratios of pro-/anti-Bcl2 proteins in the cells (Fig. E3C-F), indicating that their expression is not directly determined by GR signaling, but is rather an intrinsic property of these cells.

To test whether the altered ratios of pro-/anti-apoptotic Bcl2 proteins play a functional role in apoptosis resistance, we employed Bcl-2 inhibitors.[8] ABT263 or ABT737, at 1 nM, which did not induce apoptosis alone (Fig. E4), was sufficient to

sensitize ILC2s to dexamethasone-induced apoptosis (Fig. 2G) as well as to staurosporine (Fig. 2H), confirming that the low anti-/pro-apoptotic Bcl-2 family protein ratio determines the reduced response of ILC2s to dexamethasone.

BAX and BAK function by permeabilizing the mitochondria to release apoptotic proteins,[7] and mitochondrial content is strongly correlated with apoptosis sensitivity.[9] Interestingly, mitochondrial content measured with MitoTracker Green (MTG, a mitochondrial membrane potential (MMP)-independent dye, Fig. E5A) or an antibody against the outer membrane protein TOMM20 showed lower mitochondrial mass in ILC2s compared with Th2/Tc2 cells in both *ex-vivo* blood and cultured cells (Fig. 2I,J), which was also consistent with the reduction of HSP60, a mitochondrial chaperonin, in ILC2s (Fig. 2D,E). Low MTG staining was not due to loss of MMP, verified using MitoTracker Orange (MTO, a MMP-dependent dye, Fig. E5B). Therefore, fewer mitochondria may act in concert with lower pro-/anti-apoptotic Bcl-2 protein ratios to cause corticosteroid-resistance in ILC2s.

To understand whether corticosteroid-resistance and the reduced propensity to apoptosis are unique features of ILC2s, we also investigated other related cells (Fig. E6). As expected, T lymphocyte counts in blood samples were significantly reduced after OCS (Fig. E6A); however, similar to ILC2s, other ILCs were not (Fig. E6B). The expression profiles of Bcl-2 proteins (Fig. E6C,D) and the mitochondrial content (Fig. E6E,F,G) in CD3/4/8 cells were similar to those of Th2/Tc2 cells. However, CRTH2$^-$ILCs (ILC1/3s) were similar to ILC2s in that pro-apoptotic BAX and BAK to Bcl-2 ratios, and mitochondrial mass were lower compared to T cells.

Collectively, our data show that ILC2 survival is corticosteroid-insensitive both *in vivo* and *in vitro*, which is associated with intrinsically reduced pro-apoptotic machinery including pro-apoptotic Bcl-2 proteins and mitochondria. Our

160 observations provide a better understanding of the molecular mechanisms
161 mediating corticosteroid-resistance in ILC2s. We also revealed for the first time the
162 corticosteroid-tolerance features of other ILCs. These new findings are of potential
163 clinical value and provide further insight into treatment of ILC2 (and ILC)-mediated
164 inflammatory diseases.
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Figure Legends

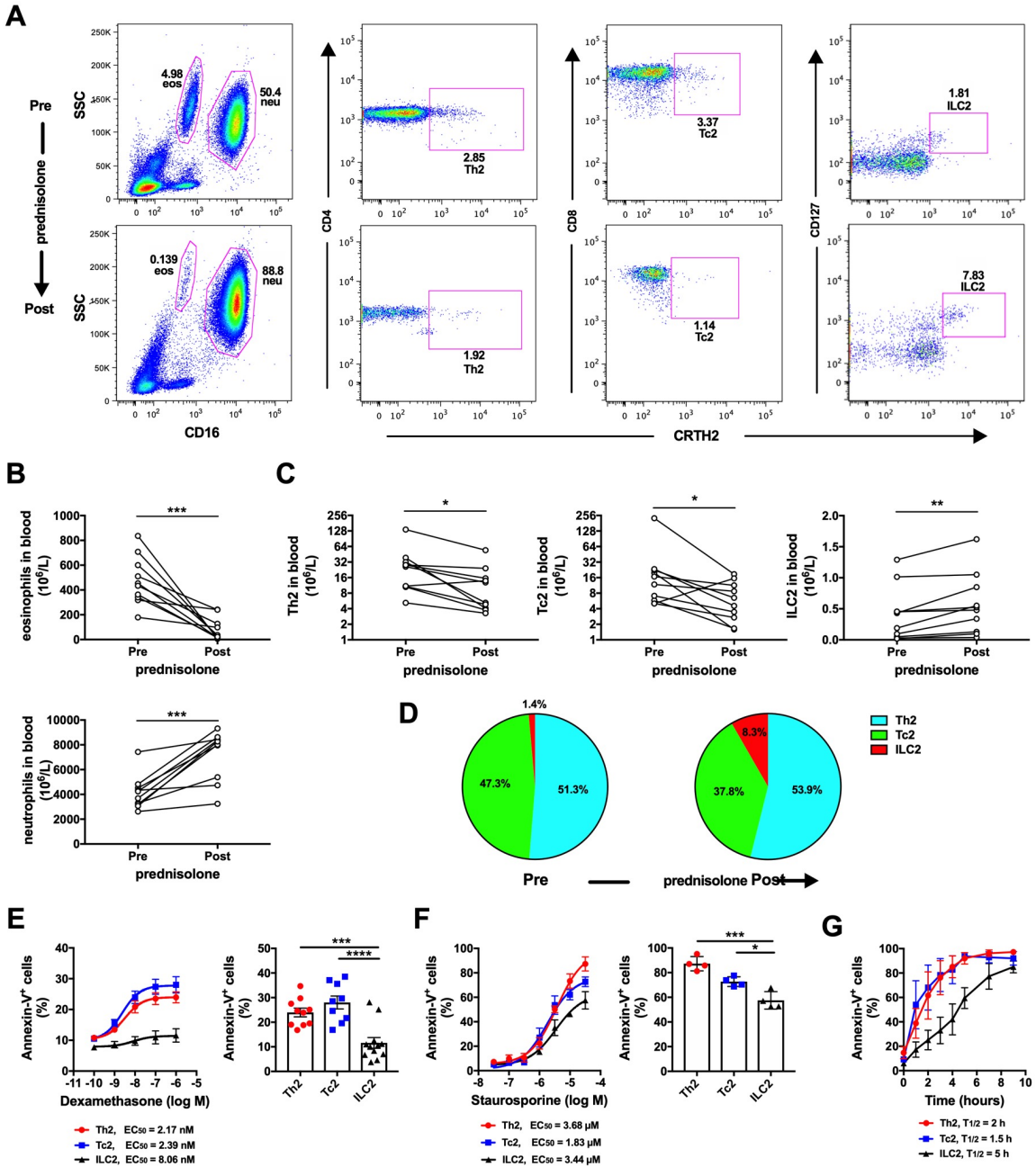


FIG 1. ILC2s were less sensitive than Th2 and Tc2 cells to corticosteroids in clinical treatment and *in vitro* apoptosis assay. (**A**) A representative flow cytometric gating example to compare the frequencies of Th2 ($CD3^+CD4^+CRTH2^+$), Tc2 ($CD3^+CD8^+CRTH2^+$) and ILC2 ($Lin^-CD45^{high}CD127^+CRTH2^+$) cells in the blood from a patient with severe asthma before and after oral prednisolone treatment. Eosinophils (EOS) and Neutrophils (NEU) were used as control. (**B,C**) Comparison

of blood counts of (**B**) eosinophils and neutrophils, (**C**) Th2, Tc2 and ILC2s before and after prednisolone-treatment. (**D**) Average proportions of Th2, Tc2 and ILC2 in total type-2 cells before and after prednisolone-treatment. Paired *t* tests with two tailed. (**E-G**) Annexin-V^+ apoptotic cells in cultured Th2, Tc2 and ILC2 cells in response to (**E**) 16 h-treatment of various concentrations (left panel) or 1 μM (right panel) of dexamethasone, or (**F**) 4 h-treatment of various concentrations (left panel) or 30 μM (right panel) of staurosporine, or (**G**) different length of time incubation with 10 μM staurosporine detected with flow cytometry. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$; n=10-12 for **D** and **E,** n=4 for **F**, n=3 for **G**.

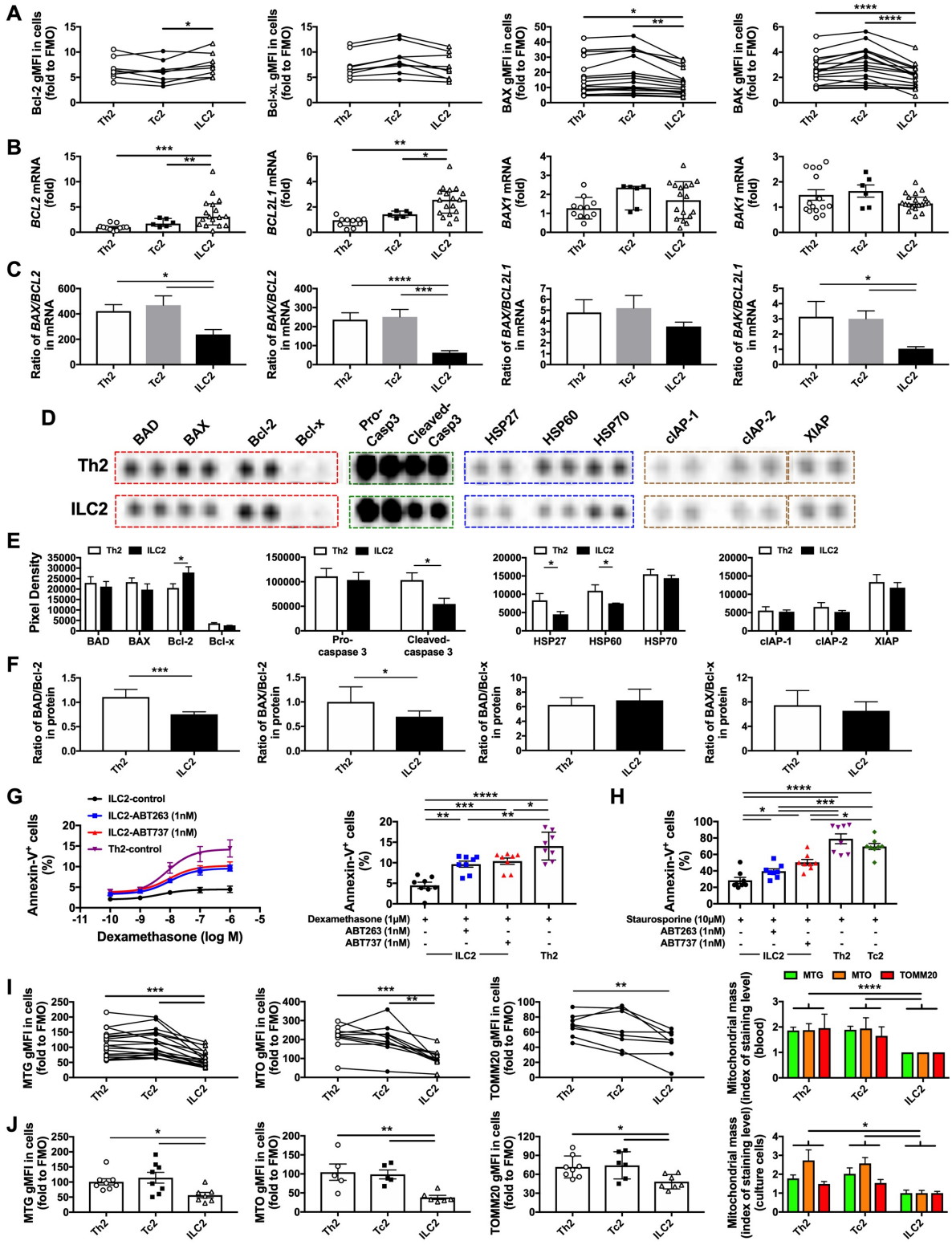


FIG 2. ILC2s had reduced pro-apoptotic machinery including pro-apoptotic Bcl-2 proteins and mitochondria compared with Th2 and Tc2 cells. (**A**) Levels of Bcl-2 family proteins including Bcl-2, Bcl-xL, BAX and BAK in blood type-2 cells compared with flow cytometry. (**B**) mRNA levels of Bcl-2 family members in cultured type-2

cells compared with qPCR. (**C**) Comparison of ratios of pro- to anti-apoptotic Bcl-2 family genes in cultured type-2 cells. (**D**) Expression of apoptosis-related proteins including BAD, BAX, Bcl-2, Bcl-xL, pro-caspase 3 (Casp3), cleaved-Casp3, HSP27/60/70, cIAP-1/-2 and XIAP in cultured Th2 and ILC2s detected with Proteome profiler array. (**E**) Quantitative comparison of these proteins. (F) Comparison of ratios of pro- against anti-apoptotic Bcl-2 proteins in Proteome Profiler Array. (**G,H**) Enhancement of ILC2 apoptosis in response to 16 h-treatment of various concentrations (**G** left panel) or 1 μM (**G** right panel) dexamethasone, or 3 h-treatment of 10 μM staurosporine (**H**) by Bcl-2 inhibitors, ABT263 and ABT737. (**I,J**) MTG, MTO or anti-TOMM20 staining for flow cytometry in blood type-2 cells (**I**) or cultured type-2 cells (**J**). The three types of staining were compared by converting the data to index of the staining levels (**I**,**J** right panels). ****p<0.0001, *** p<0.001, ** p<0.01, * p<0.05; n=6-13 for **C**, n=3 for **D**-**F**, n=7 for **G**, n=5-18 for **I** and **J**.