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Increased proportion of CD16+ NK cells in the colonic lamina propria of IBD patients but not after Azathioprine treatment.

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4 Increased proportion of CD16⁺ NK cells in the colonic lamina
5 propria of IBD patients but not after Azathioprine treatment.
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45 **Short running head**

46 CD16⁺ Natural Killer cells in IBD
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52 **Long running head**

53 Azathioprine depletes CD16⁺ NK cells in IBD
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Abstract

Background: Distinct functional subsets of natural killer(NK) cells potentially contribute to the pathology of inflammatory bowel diseases(IBD).

Aim: To report the phenotypic and functional characteristics of NK cells in blood and lamina propria(LP) of IBD patients, and the effect of azathioprine.

Methods: NK cells from blood and LP of healthy controls(HC) or patients with Crohn's disease(CD), or ulcerative colitis(UC) were studied by flow cytometry. Activation, cytokine production, proliferation and apoptosis of NK cell subsets were studied *in-vitro*.

Results: CD16⁺NK cells are increased in frequency in the LP comparing CD or UC with HC. Azathioprine therapy was associated with a reduction of total NK cells in blood and LP, preferentially of the CD16⁺ subset. **Azathioprine therapy did not impair NK degranulation, but reduced natural and cytokine activated cytotoxicity and interferon-gamma(IFN- γ) production.** Culture of resting PBMC with azathioprine resulted in loss of NK cells and inhibition of activation and IFN- γ production. Azathioprine preferentially inhibited proliferation of CD16⁺ NK cells and induced apoptosis in resting but not in pre-activated NK cells.

Conclusions: NK cells with cytolytic potential are enriched in the colonic LP of individuals with IBD. Azathioprine is associated with a reduction in these cells and a normalisation of NK cell populations.

INTRODUCTION

Peripheral blood human natural killer (NK) cells have been classically defined into two major functional subpopulations (1). CD56⁺CD16⁺ NK cells constitute the most abundant blood NK cell population, expressing high levels of intracellular perforin, being enriched for cells expressing Killer Cell Immunoglobulin-like receptors (KIR) and having high levels of cytotoxic activity against target cell lines. CD56⁺CD16⁻ NK cells represent a minor population of NK cells in the blood, have little or no expression of KIR and perforin and exhibit poor natural cytotoxicity whilst being effective in the production of cytokines, including IFN- γ , TNF- α , IL-10 and IL-13 (1). Previous studies from our group and others have, in contrast, demonstrated that tissue NK cells, including those detected in the human gastrointestinal tract, are predominantly of the latter CD56⁺CD16⁻ phenotype, and have helper rather than cytotoxic function (2-5).

Our previous studies demonstrated that chronic infection with HIV-1 resulted in an increase in the representation of CD56⁺CD16⁺ NK cells in colonic lamina propria, which are virtually absent in HIV-1 negative healthy control individuals, possibly reflecting increased NK cell differentiation under conditions of chronic infection (4).

Natural Killer cells have been implicated both in the pathogenesis of and recovery from inflammatory bowel diseases. Early studies indicate that only weak natural cytotoxicity can be detected lamina propria in patients with either Ulcerative Colitis (UC) or Crohn's disease (CD), implying a lack of conventional cytotoxic NK cell involvement (6, 7). Furthermore, in the

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4 peripheral circulation there is impaired NK cytotoxicity in CD, but no change in
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6 total numbers of NK cells found (8, 9). An increased frequency of colonic
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8 dendritic cells producing IL-6 and IL-12, has been observed in human Crohn's
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10 disease, suggesting an increased potential for dendritic cell (DC) mediated
11
12 NK cell stimulation (10).

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15 An increase in NK cell markers has been demonstrated in the colonic mucosa
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17 of patients with active UC (11). However, TCR- $\alpha\beta^+$ and TCR- $\gamma\delta^+$ subsets also
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19 express NK cell markers in the colonic mucosa, and have subsequently been
20
21 demonstrated to play an important role in driving the Th-2, IL-13 dominated
22
23 pattern of cytokine production in UC (12). Conversely, a murine model of
24
25 colitis, in which IL-10 $^{-/-}$ mice are susceptible to a Th-1 mediated pathology,
26
27 demonstrates a role for NK cells in controlling disease (13).

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29
30 There is some indication that IBD therapies can impact on NK cell function.
31
32 Corticosteroids have clearly been demonstrated to impede NK cytotoxicity (14,
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34 15). Azathioprine (and its orally active metabolite mercaptopurine) are thought
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36 to impair the cytotoxicity of peripheral NK cells in treated CD and rheumatoid
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38 arthritis patients (16-18). The other main classes of drugs used to treat IBD
39
40 are aminosalicylates that have not been shown to affect NK cell numbers or
41
42 function, and the recently adopted monoclonal anti TNF biologic therapies
43
44 which have yet to be specifically linked to changes in NK cell frequency or
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46 function.

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49 We therefore investigated whether chronic inflammatory conditions of the
50
51 gastrointestinal tract (UC and CD) would, like chronic infectious inflammation
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53 influence the composition of functional NK cell subsets in the blood and
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colonic lamina propria and whether treatment of these diseases with immunomodulatory therapy (azathioprine) affects these cell populations.

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MATERIALS AND METHODS

This is a cross sectional *ex-vivo* and *in-vitro* study of blood and intestinal mucosa in UC and CD to determine NK subset phenotype and function and the effect of azathioprine therapy. The study was approved by the relevant Research Ethics Committee with written informed consent obtained from all subjects.

Study subjects.

For *ex vivo* studies 28 healthy control patients were selected from patients attending for endoscopic examination because of: personal history of colonic polyps, familial history of polyposis or for investigation of rectal bleeding where endoscopic examination was unremarkable. All healthy controls had no overt pathology on endoscopic examination. Studies were performed on colonic biopsy and blood samples from a total of 17 Individuals CD and 30 patients with UC with disease previously defined as extending at least to the splenic flexure. Patients on current or recent (four weeks) corticosteroid therapy and those ever treated with biologic therapies were excluded. All subjects receiving azathioprine therapy had done so at a stable dose for more than six months. Levels of thiopurine methyltransferase (TPMT) prior to commencement of azathioprine therapy were not routinely recorded at the time of this study.

To determine the effect of disease activity UC patients were scored using the Ulcerative Colitis Severity Score (UCSS), with a range 0-12 and a score of ≤ 6 corresponding to inactive/mildly active disease (19). For CD patients the

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3 Harvey Bradshaw Index (HBI) was used with a score of ≤ 6 corresponding to
4
5 inactive/mildly active disease (20). Since both of these measures include
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7 components that are subjective we also independently evaluated C reactive
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9 protein levels (CRP). Further details of patient characteristics are shown in
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11 table 1.
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17 **Biopsy sampling and processing**

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19 Biopsies for evaluation of LP NK populations were taken from the sigmoid
20
21 colon (x6) circumferentially at 30cm from the anus, avoiding areas of
22
23 ulceration where necrotic cells and fibroblastic proliferation predominate.
24
25 Biopsy forceps used were Olympus FB 242 radial jaw with external diameter
26
27 of 3.7mm (Keymed, Southend-on-Sea, UK). Parallel blood samples were
28
29 taken into Lithium Heparin Vacutainers (Becton Dickinson, Oxford, UK) and
30
31 peripheral blood mononuclear cells were separated by density gradient
32
33 centrifugation on Histopaque-1077 (Sigma, Poole, UK).
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41 Biopsies obtained during colonoscopy were collected in ice-cold Dutch
42
43 modification of RPMI 1640 supplemented with antibiotics. Epithelium and
44
45 associated lymphocytes were removed by repeated treatment with EDTA and
46
47 washing and lamina propria lymphocytes (LPL) were prepared either by
48
49 enzymatic digestion or by 'walk out' methods as previously described (10).
50
51 Briefly, for phenotypic analysis, epithelium and intra epithelial lymphocyte
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53 (IEL)-free tissue was digested with 1 mg/ml collagenase D (Roche Molecular
54
55 Products, Basel, Switzerland) in Dutch modification of RPMI 1640 containing
56
57 20 mg/ml DNase I (Roche Molecular Products) and 2% FCS to release lamina
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3 propria lymphocytes. For functional assays, epithelium and IEL free tissue
4
5 explants were left overnight in complete medium to permit emigration of LPL.
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10 **Flow cytometric analysis of NK cell subsets.**

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12 Total NK cells were identified within PBMC or LPL as CD3⁻CD56⁺ cells and T
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14 cells as CD3⁺ cells. CD16⁺CD56⁺ and CD16⁺CD56⁻ NK cell subsets were
15
16 identified within PBMC or LPMC by flow cytometry after labelling with
17
18 Fluorescein (FITC) conjugated anti-CD45 (Sigma, Poole, UK), phycoerythrin
19
20 (PE) conjugated anti-CD16, allophycocyanin (APC) conjugated anti-CD56
21
22 (both from Beckman Coulter, Marseille, France) and PerCP conjugated anti-
23
24 CD3 (Becton-Dickinson, UK). For analysis, lymphocytes were identified as
25
26 CD45⁺ side scatter^{lo} cells and NK cells as CD3⁻CD56⁺CD16⁺ or CD3⁻
27
28 CD56⁺CD16⁻ cells. Further flow cytometric analysis of activating and inhibitory
29
30 NK cell receptor expression was performed using the following phycoerythrin
31
32 conjugated antibodies: anti-CD161, anti-KIR3DL-1, anti-NKG2D (Becton
33
34 Dickinson); anti-KIR2DL-1/2 (AbD Serotec, Oxford, UK) anti-NKG2C (R&D
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36 systems, Abingdon, UK); anti-NKp30, anti-NKp44 and anti-NKp46 (Beckman
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38 Coulter).
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50 **Degranulation assay.**

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52 NK cell specific degranulation was measured using CD107a as previously
53
54 described (21). Biopsy material was cultured after removal of epithelial cells in
55
56 complete medium with 20ng/ml IL-15 (R&D systems). After 18 hours cells
57
58 were removed into polypropylene cell culture tubes (BD Falcon, Oxford, UK)
59
60 and cultured with anti-CD107a antibody and with K562 cells at a ratio of 2

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2
3 lamina propria mononuclear cells (LPMC) or peripheral blood mononuclear
4 cells (PBMC) per target cell for one hour. Brefeldin A and monensin were
5
6 added and the samples incubated for a further 4 hours. Samples were then
7
8 washed once in complete medium and stained for flow cytometric analysis.
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12 13 14 15 **Cytotoxicity assay**

16
17 For natural cytotoxicity, PBMC were tested immediately after isolation. For
18 cytokine activated cytotoxicity cells were cultured overnight in IL-2 (100U/ml)
19 and IL-15 (20ng/ml) before testing. Natural and cytokine activated cytotoxicity
20 were measured using a non-radioactive cytotoxicity assay using PBMC
21 effectors and K562 target cells at ratios titrated from 50:1 to 5:1 (Biorad,
22 Southampton, UK). Specific lysis of target cells was calculated using the
23 following formula:
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$$33 \text{ \% Cytotoxicity} = \frac{\text{Experimental} - \text{Effector spontaneous} - \text{Target spontaneous}}{\text{Target maximum} - \text{Target spontaneous}} \times 100$$

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42 **Studies on the effect of Azathioprine *in-vitro***

43 Resting PBMC from healthy control individuals were incubated with
44 azathioprine at a concentration range between 1 and 50 μ M and the absolute
45 number and proportion of NK cell and T cell subsets remaining after 6 days *in-*
46 *vitro* were assessed by viable cell counting and flow cytometry. Previous
47 studies have defined azathioprine concentrations of 5 μ M to be
48 pharmacologically relevant (22, 23).
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58 To test the impact of azathioprine on NK cell activation and expansion PBMC
59 were stimulated with 100U/ml recombinant human IL-2 (R&D systems,
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3 Abingdon, UK) in combination with 25ng/ml recombinant human IL-15
4
5 (Peptotec, London, UK) in the presence of varying concentration of
6
7 azathioprine (Imuran, GlaxoSmithKline, UK). The impact of azathioprine on
8
9 earlier cellular activation was measured by the expression of CD69 (anti-
10
11 CD69 PE, Becton Dickinson) on NK cells after overnight incubation. The
12
13 distribution of NK cell subsets was measured after 6 days of incubation.
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17 Interferon gamma production in gated CD56⁺CD16⁻ and CD56⁺CD16⁺ NK cell
18
19 subsets was measured by intracellular staining and flow cytometry using a
20
21 FITC conjugated anti-human IFN- γ monoclonal antibody (BD Biosciences).
22
23

24
25 Apoptosis was measured in azathioprine treated unstimulated or cytokine
26
27 activated NK cell subsets using FITC conjugated Annexin V (Invitrogen,
28
29 Molecular Probes, Paisley, UK) after gating out dead cells with a fixable live-
30
31 dead cell discriminatory dye (RVID, Invitrogen). Apoptotic cells were defined as
32
33 Annexin V⁺/RVID negative.
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37 Cell proliferation was measured after labelling cells with carboxyfluorescein
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39 diacetate succinimidyl ester (CFSE, Invitrogen) (24) and stimulating with
40
41 cytokines in the presence or absence of Azathioprine as above. The
42
43 proportion of proliferating cells was calculated by assessing the proportion of
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45 CFSE negative/low cells within gated NK cell and T cell subsets.
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50 51 **Statistical analysis.**

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53 Statistical analyses were performed after consultation with an on-site
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55 statistician. Comparison of continuous variables was performed using non-
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57 parametric tests. Mann–Whitney U test was used for comparison between
58
59 groups and Wilcoxon signed-rank test was used for intra-group comparisons.
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3 Comparison of discrete variables was performed using a Chi-squared test.
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5 StatView 5.01 software was used (Abacus, Berkley, California, USA) and p
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8 values<0.05 were considered as significant.
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RESULTS

Increased proportion of CD16⁺ NK cells in the colonic lamina propria of CD patients and reversal by azathioprine.

The proportions of total CD3⁺CD56⁺ NK cells within gated CD45⁺ lymphocytes were first compared within blood and colonic biopsies from healthy control individuals and CD and UC patients (Figure 1a, b). No significant differences were observed in the proportion of total CD3⁺CD56⁺ NK cells between HC (blood: median 7.5%, range 2.2-17.0%; colon: median 4.8%, range 1.7-17.4%) and IBD patients with either CD (blood: median, 4.4%, range 1.3-10.9%; colon: median 4.2%, range 2.3-8.8%) or UC (blood: median 9.4%, range 1.3-23.2%; colon: median 3.6%, 1.4-13.6%) (Figure1b).

Furthermore, no significant difference was observed in the subset distribution of NK cells, as determined by the proportion of CD16⁺ NK cells, within total blood NK cells from untreated IBD patients compared to healthy control individuals (HC, median 91.1%, range 81.3-96.9%, CD, median 86.9%, range 64.3-97.7%, UC median, 89.7% range 70.6-98.2%) (Figure 1c). A significant increase was, however, observed in the representation of CD56⁺CD16⁺ NK cells within colonic LPNK cells in CD (median, 14.7%, range 3.6-48.9%, p=0.04) and UC patients (median 18.3%, range 0.1-49.5%, p=0.037) compared to control individuals (median 5.7%, range 0.1-30.2%) (Figure 1c).

A reduction in the overall proportion of blood NK cells was observed in IBD patients receiving azathioprine (blood: CD median 1.7%, range 0.3-6.2%; UC median 1.4%, range 0.5-10.9%), compared to healthy control individuals (CD, p=0.001; UC, p=0.001) and to untreated individuals (CD, p=0.013; UC, p=

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3 0.004). A similar tendency towards a reduction in total NK cells was observed
4 within colonic lamina propria NK cell populations (colon: CD median 3.2%,
5 range 0.6-4.6%, $p=0.04$ vs untreated; UC median 3.5%, range 0.9-9.4%,
6 $p=0.123$ vs untreated) (Figure 1b). Analysis of NK cell subsets revealed that
7 azathioprine treatment resulted in preferential reduction in the proportion of
8 CD56⁺CD16⁺ NK cells in the blood of CD (blood: median 63.4%, range 25-
9 84%, $p=0.025$ vs untreated;) and UC patients (blood median 81.1%, range
10 25.5-95.2% $p=0.033$ vs untreated;) (Figure 1c). A similar trend towards a
11 reduction in the proportion of CD56⁺CD16⁺ NK cells in colonic lamina propria
12 comparing patients with or without azathioprine treatment (CD patients
13 median 1.6%, range 0.8-21.9%, $p=0.044$ vs untreated; UC patients colon:
14 median, 10.1%, range 0.1-21.7%, $p=0.089$ vs untreated, Figure 1c). No
15 significant differences were observed comparing confounding variables,
16 including sex, age, time since diagnosis, disease activity (HBI or UCSS), C-
17 reactive protein levels and 5-aminosalicate therapy between azathioprine
18 treated or untreated groups of patients indicating that these variables were
19 unlikely to contribute to differences in NK cell populations observed between
20 Azathioprine treated or untreated patient groups (Table S1). Together these
21 data demonstrate that inflammatory bowel diseases result in a redistribution of
22 NK cell subsets in the colonic lamina propria towards a CD16⁺ phenotype.
23 Azathioprine therapy results in an overall depletion of NK cells with a
24 preferential impact on the CD56⁺CD16⁺ NK cell subset both in the blood and
25 colonic lamina propria.
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27 The expression of activating and inhibitory NK cell receptor families were also
28 examined within gated NK cell populations from blood and colon (Table S2).
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3 The distribution of NK cell receptors on colonic lamina NK cells was
4 significantly different compared to peripheral blood for control and for IBD
5 patients. In addition to a reduced proportion of LPNK cells expressing CD16
6 compared to PBNK ($p < 0.0001$ for HC, CD and UC), significantly fewer LPNK
7 expressed KIR compared to PBNK (HC, $p = 0.040$, CD, $p = 0.043$, UC, $p = 0.4$).
8 Furthermore, the expression of NCR NKp44 was elevated in LPNK compared
9 to PBNK (HC, $p = 0.028$, CD, $p = 0.028$, UC, $p = 0.031$) (Table S2). However,
10 other than the expression of CD16, no significant differences were observed
11 in the expression of these receptors comparing blood or colonic lamina
12 propria between controls and IBD patients or comparing groups of IBD
13 patients untreated or treated with azathioprine (Table S2).
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32 **IL-15 activated NK cells from HC and IBD patients degranulate in** 33 **response to target cells.** 34

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36 The functional capacity NK cells from untreated UC patients and UC patients
37 receiving azathioprine was then tested by measuring their ability to
38 degranulate in response to target cells. Degranulation, as measured by
39 CD107a expression was observed both in peripheral blood and lamina propria
40 NK cells from HC individuals and from UC patients in response to K562 target
41 cells (Figure 2a, 2b). Interestingly, the remaining PBNK and LPNK cells from
42 UC treated individuals receiving azathioprine were also capable of
43 degranulation, indicating that these cells remained functional (Figure 2a, 2b).
44 These data indicate that CD16⁻ NK cells remaining after azathioprine therapy
45 maintain in-vitro responsiveness to target cell lines. Although NK cells from
46 azathioprine treated individuals were capable of degranulating in response to
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3 target cell lines, blood NK cells from Azathioprine treated individuals were less
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5 effective in both natural and cytokine activated cytotoxicity (Figure 2c). Poor
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7 cytotoxicity is consistent with a reduced proportion of perforin expressing
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9 CD16⁺ 'cytotoxic' NK cells in Azathioprine treated individuals compared to
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11 healthy control or untreated individuals (Figure S1)
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17 **Resting and activated NK cells are sensitive to azathioprine in-vitro**

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19 Azathioprine and its metabolites 6-mercaptopurine and 6-thioguanine
20
21 triphosphate have been shown in previous studies to inhibit T cell proliferation
22
23 in-vitro at pharmacologically relevant concentrations (22, 23). In view of the
24
25 selective depletion of CD56⁺CD16⁺ NK cells measured ex-vivo in PBMC or
26
27 LPMC from azathioprine treated CD or UC patients, we tested whether
28
29 similar pharmacologically relevant effects could be observed within resting or
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31 PBNK cells in-vitro activated NK cells in the presence or absence of
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33 azathioprine .
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39 Surprisingly, incubation of resting PBMC with azathioprine for 6 days in
40
41 culture resulted in a significant preferential loss in both the absolute number
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43 and proportion of CD3⁻CD56⁺ NK cells in all donors tested at a
44
45 pharmacologically relevant concentration of azathioprine concentration of 5µM
46
47 whereas no reduction was observed in the proportion of resting CD3⁺ T-cells
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49 even at suprapharmacological concentrations of up to 50µM (Figure 3a). In
50
51 contrast to ex-vivo observations on NK cells from treated patients, in-vitro
52
53 treatment of resting NK cells with azathioprine had no impact on the relative
54
55 proportions of CD56⁺CD16⁻, CD56⁺CD16⁺ or CD56⁺KIR⁺ NK cell subsets
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60 (data not shown). We therefore tested the impact of azathioprine on cytokine

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3 activated NK cells within PBMC. A reduction in the absolute number and
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5 proportion of CD3-CD56+ NK cells was observed only at
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activated NK cells within PBMC. A reduction in the absolute number and proportion of CD3-CD56+ NK cells was observed only at suprapharmacological concentrations of azathioprine (20 μ M or greater), indicating that cytokine activated NK cells were less sensitive to azathioprine than resting NK cells (figure 3b). No difference was observed in the overall proportions of CD56⁺CD16⁻ and CD56⁺CD16⁺ NK cells subsets within the remaining NK cells. However, in contrast to resting T-cells, the proportions of cytokine activated T cells were also reduced in the presence of high concentrations of azathioprine (figure 3b).

Azathioprine preferentially inhibits early activation, IFN- γ production and proliferation of CD16+ NK cells.

To test whether azathioprine impacted on cytokine driven early NK cell activation and proliferation we studied CD69 expression and the distribution of CFSE staining in CD56⁺CD16⁻ and CD56⁺CD16⁺ NK cell subsets. Only a small fraction of NK cells within freshly isolated PBMC expressed CD69 prior to stimulation with cytokines (median 4.9%, range 1.9-11.2%). High levels of CD69 were induced after 16 hours of cytokine stimulation and azathioprine reduced the proportion of NK cells expressing CD69 within both NK cell subsets (Figure 4a,b). The impact of azathioprine was greater on the activation of CD56⁺CD16⁺ NK cells (inhibition at concentrations \geq 5 μ M) compared to CD56⁺CD16⁻ NK cells (inhibition at concentrations \geq 20 μ M)(Figure 4d). The impact on the level of CD69 expression (mean fluorescence activity) gave similar results (data not shown). Azathioprine

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3 significantly inhibited cytokine driven T cell activation only at concentrations of
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6 10 μ M or greater (Figure 4d).

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9 The impact of azathioprine on IFN- γ production by resting and activated NK
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11 cells was also assessed using intracellular cytokine staining. Similarly to the
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13 effects on CD69 expression, azathioprine inhibited IFN- γ production by NK
14
15 cell subsets in a concentration dependent manner, when added
16
17 simultaneously with IL-12 and IL-15 (Figure 4e) whereas no effect was
18
19 observed on IFN- γ production by NK cells which had been previously
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21 activated and then restimulated with IL-12+ IL-15 (Figure 4f).

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25 The proliferative response of NK cells to cytokines was also dramatically
26
27 reduced by azathioprine (Figure 5). CD56⁺CD16⁺ NK cells were particularly
28
29 sensitive to inhibition by azathioprine with a significant reduction in proliferating
30
31 (CFSE low/negative) cells being observed at concentrations of 5 μ M or greater
32
33 (Figure 5b). Significant Inhibition of cytokine driven CD56⁺CD16⁻ NK cell
34
35 proliferation and T cell proliferation was observed only at higher
36
37 concentrations of azathioprine (\geq 10 μ M) (Figure 5b). Taken together these
38
39 data indicate that whilst resting NK cell subsets are equally sensitive to
40
41 azathioprine, activation and proliferation of CD56⁺CD16⁺ NK cells is
42
43 preferentially inhibited, providing a potential mechanism for our *ex-vivo*
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45 observations in azathioprine treated subjects.
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51 52 53 **Azathioprine induces apoptosis in resting NK cells.**

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56 In order to assess whether the impact of azathioprine on resting NK cells was
57
58 associated with the induction of apoptosis, as shown previously by an
59
60 increase in the proportion of apoptotic activated T-cells (22), we measured

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3 apoptosis in unstimulated or cytokine activated NK cell subsets after 5 days of
4 culture (Figure 6). Resting NK cells from both CD56⁺CD16⁻ and CD56⁺CD16⁺
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8 subsets were susceptible to apoptosis in the presence of azathioprine, with a
9
10 progressive increase in Annexin V⁺,RVID⁻ cells being observed at
11
12 concentration of 5mM or greater. However, a higher overall proportion of the
13
14 resting CD56⁺CD16⁺ subset was undergoing apoptosis after 5 days of culture
15
16 compared to CD56⁺CD16⁻ cells. In contrast to resting NK cells, cytokine
17
18 activated NK cells were more resistant to apoptosis, in keeping with a
19
20 maintenance in culture at lower concentrations of azathioprine (5mM or less).
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CONCLUSIONS

Tissue NK cells are universally of a dominant CD56⁺CD16⁻ phenotype as observed in human tonsils, secondary lymphoid tissue, uterine decidua and in the GI tract (2, 4, 25-27). Recent studies have demonstrated a predominant role for mucosal CD56⁺CD16⁻NKp44⁺ NK cells from human tonsils in promoting repair of epithelial tissue via production of growth and anti-apoptotic factors (28). Indeed, in animal models such cells play a role in protection from inflammatory bowel disease (13).

This study demonstrates no significant difference in the overall proportion of NK cells present in the peripheral blood or colonic lamina propria or individuals with either CD or UC compared to unaffected healthy control individuals however, significant enrichment of LPNK cells for the cytotoxic CD16⁺CD56⁺ NK cell subset is observed in IBD patients not on immunomodulatory therapy. The biological impact of this shift in NK cell phenotype remains to be demonstrated although the expression of perforin and enhanced cytotoxic capacity in the CD56⁺CD16⁺ NK cell subset may potentially contribute to tissue damage and inflammatory processes in the GI tract. A relative reduction of CD56⁺CD16⁻ helper NK cells may conversely impact on repair processes in the gastrointestinal tract. **In this respect we observe no direct correlation between disease severity and the proportion of CD16⁺CD56⁺ NK cells present in colonic lamina propria from both CD and UC patients. However, UC patients with active disease have a significantly higher proportion of lamina propria CD16⁺ NK cells compared to those with inactive disease (p=0.035), suggesting that there may be some causal relationship.**

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3 Several reports have established blood CD56⁻CD16⁺ NK cells differentiate
4 into CD56⁺CD16⁺ cells on activation with various stimuli (25, 26, 29, 30). IL-2
5 and IL-15 promotes the differentiation of blood CD56⁺CD16⁻ NK cells into
6 CD56⁺CD16⁺ NK cells that acquire the expression of cytolytic machinery
7 including perforin and granzymes (26, 30, 31). Increased telomere length in
8 CD56⁻CD16⁺ cells compared to CD56⁺CD16⁺ NK cells indicates that these
9 cells are less mature, having undergone fewer cell divisions (30). A fraction of
10 human lamina propria CD56⁺CD16⁻ NK cells express c-kit, a marker of
11 immature progenitor cells and a receptor for stem cell factor (SCF-1) and can
12 be derived from lineage marker negative, c-kit⁺ precursors. (2). IL-15, which is
13 elevated in expression in IBD, could potentially act to promote the
14 differentiation of CD56⁺CD16⁻ into CD56⁺CD16⁺ lamina propria NK cells,
15 although the relationship between these subsets in the lamina propria
16 CD56⁺CD16⁻ and CD56⁺CD16⁺ NK cells. Consistent with such a cytokine
17 induced differentiation of NK cells in the gastrointestinal tract, IL-21, which is
18 also elevated in expression in IBD, was recently reported to induce cytotoxic
19 capacity in colonic LPNK cells and to promote the differentiation of regulatory
20 T cell subsets (32). An increased proportion of cytotoxic type CD56⁺CD16⁺
21 NK cells in the lamina propria during IBD could also, however, result from
22 emigration of peripheral blood NK cells in response to inflammatory signals.
23
24 Here we report a differential effect of azathioprine on NK cell subsets.
25 Although azathioprine resulted in overall reduction in the proportion of NK
26 cells in the blood and GI tract of treated individuals, our *ex-vivo* studies
27 demonstrate a preferential depletion of CD56⁺CD16⁺ NK cells. Azathioprine
28 inhibits human T-cell proliferation *in-vitro* and ablates antigen-specific memory
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3 T-cell responses on extended administration in an *in-vivo* murine model (22).
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5 It is therefore likely that azathioprine acts preferentially on mature
6 lymphocytes while permitting the generation or persistence of immature cell
7 populations. Such an effect is consistent with a precursor role for CD56⁻CD16⁻
8 cells in the development of mature cytotoxic CD56⁺CD16⁺ NK cells.
9
10 Interestingly, we observed that IL-15 stimulated CD56⁺CD16⁻ NK cells from
11 the blood and colonic lamina propria of azathioprine treated UC patients were
12 capable of degranulating in the presence of MHC class I negative target cells,
13 indicating that these cells retained the potential to mature and potentially
14 acquire cytotoxic capability. However, few perforin expressing NK cells are
15 detected within PBMC from azathioprine treated individuals, in contrast to
16 healthy control individuals and UC patients who are not receiving azathioprine,
17 consistent with a preferential depletion CD16⁺ cytotoxic NK cells (figure 3). IL-
18 15 treated NK Cells from azathioprine treated patients also responded
19 similarly to the enterocyte lines HT29 and Caco2, indicating a potential for
20 these cells to interact with intestinal epithelium (data not shown).
21
22 The Azathioprine metabolite 6-thioguanine triphosphate (6-thio-GTP)
23 mediates its proapoptotic effects on CD28 costimulated CD4⁺ T cells via
24 interaction with the small rho dependent GTPase Rac1 (23). Rac 1 plays an
25 important role in NK cell integrin signalling leading to P38 mitogen activated
26 kinase induced chemokine secretion and in PI3-kinase and CD16 dependent
27 NK cell cytotoxicity (33-35). Binding of azathioprine-derived 6-Thio-GTP to
28 Rac 1 could therefore also impact on NK cell function and survival.
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30 Consistent with the depletion of the entire NK cell population by azathioprine
31 in treated patients, we observed that total CD3⁻CD56⁺ NK cells are
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3 progressively lost on culture with increasing concentration of azathioprine *in-*
4 *vitro*. However, no preferential depletion of CD16⁺ NK cells was observed *in-*
5 *vitro*. Global NK cell depletion in azathioprine treated individuals may
6 therefore be compensated by the generation of CD56⁺CD16⁻ cells from bone
7 marrow-derived NK cell precursors and from resident precursors in the gut (2).
8 We observed no preferential impact of azathioprine on overall proportions of
9 IL-2 + IL-15 stimulated CD56⁺CD16⁺ and CD56⁺CD16⁻ NK cell subsets,
10 however, activation and proliferation in CD56⁺CD16⁺ NK cells were more
11 sensitive to azathioprine, again consistent with a preferential effect on mature
12 NK cell subsets. Azathioprine could therefore potentially promote the
13 maintenance of CD56⁺CD16⁻ cells *in-vivo* by preventing the cytokine driven
14 differentiation of CD56⁺CD16⁻ into CD56⁺CD16⁺ NK cells or by inhibiting the
15 proliferation and expansion of CD56⁺CD16⁺ NK cells.

16
17 Further studies on differentiation and function of NK cells within the
18 gastrointestinal tract will clarify the role of these cells in the
19 immunopathogenesis of inflammatory bowel diseases and the potential of
20 therapeutic interventions in preventing aberrant innate immune responses.

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 **Statement of Interests**

47
48 This work was funded by grants from the St Stephen's AIDS Trust, the
49 Campbell Foundation and the trustees of Chelsea and Westminster Hospital,
50 London (to MRG).
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Figure Legends

Figure 1. Increased proportion of CD16⁺ NK cells in the colonic lamina propria of individuals with CD and UC and NK cell depletion in azathioprine treated individuals. (A) Gating strategy for analysis on NK cell populations showing representative plots from patients without and with azathioprine treatment. PBMC (Upper 2 panels) or LPL (lower 2 panels) were gated initially as CD45⁺, side scatter low populations (Left hand panels, R1) and NK cells were subsequently identified within this gate as CD3⁻CD56⁺ cells (Middle panels, R2). The proportions of CD56⁺CD16⁺ cells were then analysed within gated total NK cells (Right hand panels). (B) The proportions of CD3⁻CD56⁺ NK cells within small CD45⁺ lymphocytes from peripheral blood and colonic lamina propria from healthy control individuals (n=28), patients with CD with (n=11) or without (n=6) azathioprine therapy, and in UC patients with (n=20) with or without (n=10) azathioprine therapy. (C) The proportions of total NK cells expressing CD16. Boxplots show medians and interquartile range with 10th and 90th percentiles. * p≤0.05, **p≤0.01, comparing groups using Mann-Whitney U test.

Figure 2. Functional activity of NK cells. The ability of PBNK and LPNK cells from healthy control individuals and UC patients with or without azathioprine therapy to degranulate was assessed by flow cytometric analysis of the expression of CD107a in response to MHC class I negative cell line K562. (A) Representative plots from a healthy control individual and UC patients with or without Azathioprine therapy. (B) Data for 8 healthy control individuals, 3

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3 untreated UC patients and 2 treated UC patients are shown). (C) Natural and
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5 cytokine activated cytotoxicity against K562 target cells were compared in a
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7 further 3 untreated patients and 4 patients receiving azathioprine.
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12 **Figure 3.** In vitro impact of Azathioprine. (A) resting (n=16) or (B) cytokine (IL-
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14 2+IL-15) activated PBMC from healthy control individuals (n=16) were
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16 incubated with Azathioprine for 6 days in vitro and the absolute number and
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18 proportions of CD3⁻CD56⁺ NK cells and CD3⁺ T cells (B) Boxplots show
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20 medians and interquartile range with 10th and 90th percentiles and ranges.
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24 * p≤0.05, **p≤0.01.*** p≤0.001, comparing untreated to azathioprine treated
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26 cultures using Wilcoxon signed-rank test.
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31 **Figure 4.** Azathioprine inhibits early NK cell activation and IFN-γ production.
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33 The impact of azathioprine on IL-2 + IL-15 driven activation of NK cells was
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35 monitored by measuring CD69 expression after 16 hours of culture. Gating
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37 strategy for CD56⁺CD16⁻NK cells(R3), CD56⁺CD16⁺ NK cells(R4) and CD3⁺T
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39 cells(R5) is shown (A). Sample plots are shown for azathioprine inhibition of
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41 CD69 expression within CD56⁺CD16⁻ (B)and CD56⁺CD16⁺ NK cells(C) are
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43 shown. Data are shown for inhibition of CD69 expression in CD56+CD16-NK
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45 cells, CD56⁺CD16⁺ NK cells and CD3⁺T cells in 6 individuals (D). Inhibition of
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47 IL12+IL-15 induced IFN-γ production in freshly stimulated CD56⁺CD16⁻ (open
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49 boxes) and CD56⁺CD16⁺ NK cells (shaded boxes) (E) but not in pre-activated
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51 NK cell subsets (F). Boxplots show medians and interquartile range with 10th
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53 and 90th percentiles and ranges. * p≤0.05, **p≤0.01, comparing untreated to
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55 azathioprine treated cultures using Wilcoxon signed-rank test.
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6 **Figure 5.** Azathioprine inhibits NK cell proliferation. The impact of
7 azathioprine on IL-2 + IL-15 driven proliferation of NK cells was monitored by
8 measuring loss of CFSE after 6 days of culture. Histograms show
9 representative data for CFSE staining from a single individual after gating on
10 total CD3⁻CD56⁺ NK cells, CD56⁺CD16⁻ NK cells, CD56⁺CD16⁺ NK cells and
11 CD3⁺ T cells (A). Data are shown for 6 individuals (B). Boxplots show
12 medians and interquartile range with 10th and 90th percentiles and ranges.
13 * p≤0.05, **p≤0.01, comparing untreated to azathioprine treated cultures using
14 Wilcoxon signed-rank test.
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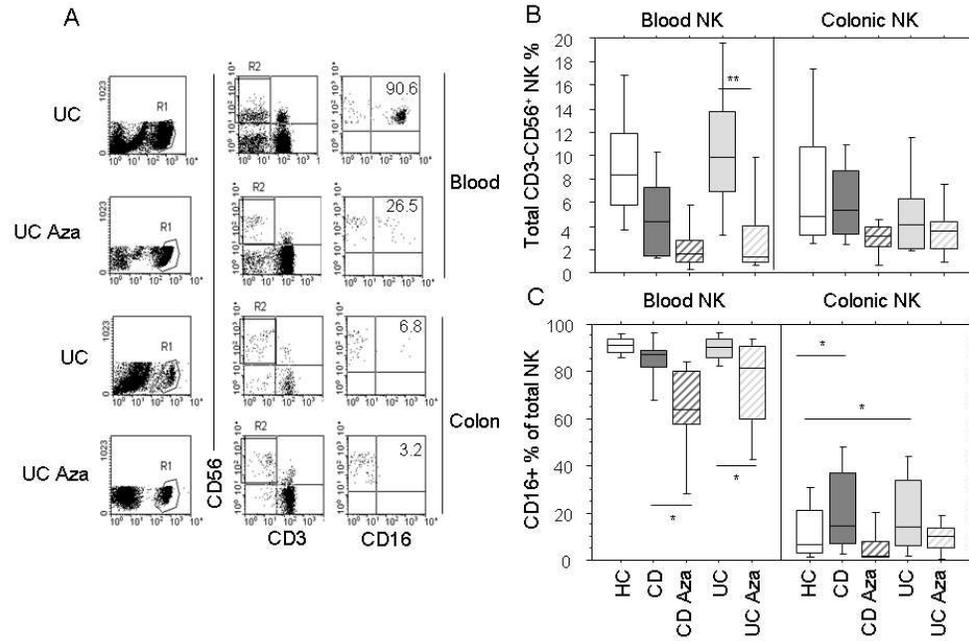
29 **Figure 6.** Induction of apoptosis in CD56⁺CD16⁻ and CD56⁺CD16⁺ NK cell
30 subsets. (A) Resting or (B) IL-2 + IL-15 activated PBMC were incubated with
31 varying concentration of azathioprine for 5 days. Cells were gated on CD3⁻
32 CD56⁺ NK cells for analysis and apoptotic cells defined as R⁺Vid⁻Annexin V⁺
33 for CD56⁺CD16⁻ cells (open boxes) and CD56⁺CD16⁺ cells (shaded boxes).
34 Data are shown from 6 individuals. Boxplots show medians and interquartile
35 range with 10th and 90th percentiles and ranges. * p≤0.05, comparing
36 untreated to azathioprine treated cultures using Wilcoxon signed-rank test.
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Table 1. Patient characteristics for the study.

	Healthy Control (n=28)	Crohn's Disease (n= 17)	Ulcerative Colitis (n=30)
Age,years median (Range)	45 (27-70)	42 (23-74)	42.5 (16-78)
Time since diagnosis,years median (range)	n.a.	12 (0.5-26)	8.5 (0.5-38)
Inactive/mildly active disease n(%)	n.a.	11 (65)	19 (65)
CRP, mg/l median (range)	n.a.	4 (2-37)	4 (2-112)
Azathioprine (% , n)	n.a	35.3 (6)	33.3 (10)

Disease stage for Crohn's disease was assessed using the Harvey Bradshaw Index (HBI) and for Ulcerative Colitis using the Ulcerative Colitis Severity Score. Inactive Crohn's disease was defined as HBI \leq 6, whilst inactive/mildly active UC was defined as UCSS \leq 6

Figure 1.



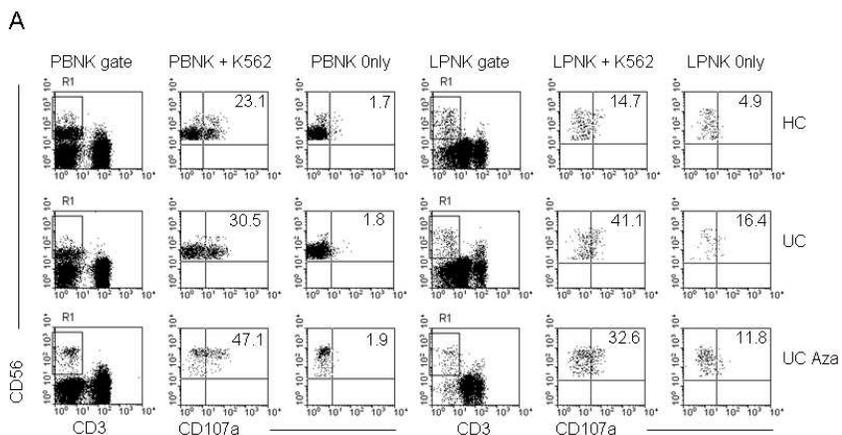
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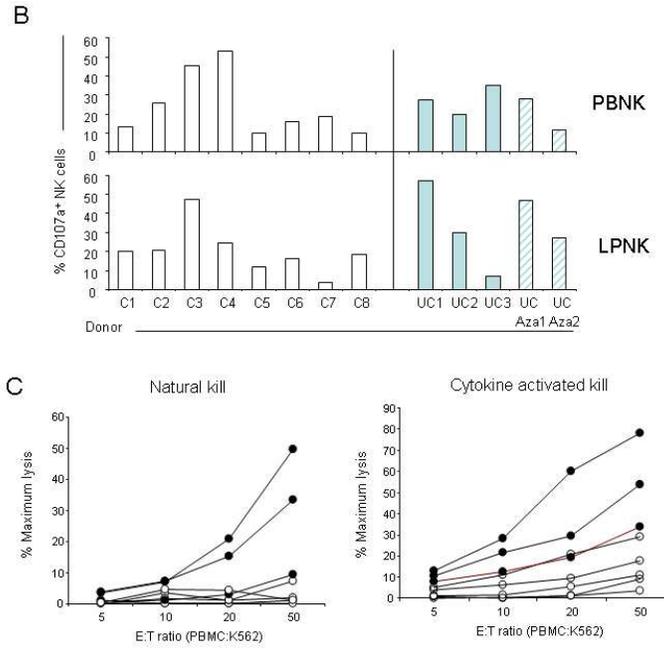
Figure 2



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Review

Figure 2.



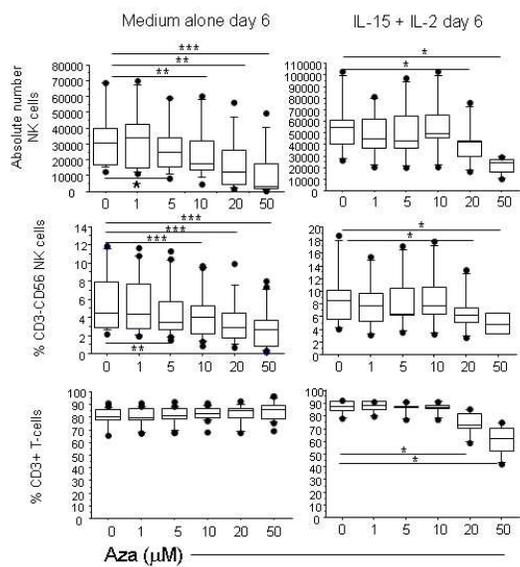
254x190mm (96 x 96 DPI)

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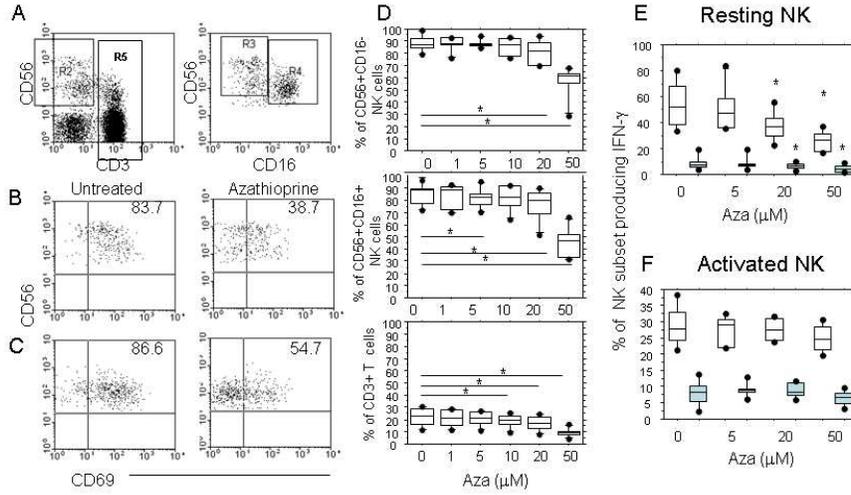
Figure 3.



254x190mm (96 x 96 DPI)

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Figure 4.



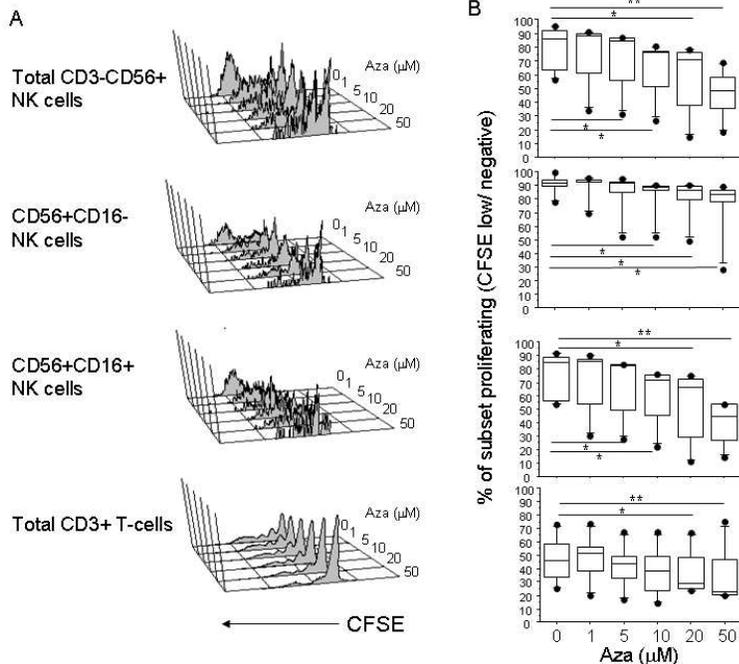
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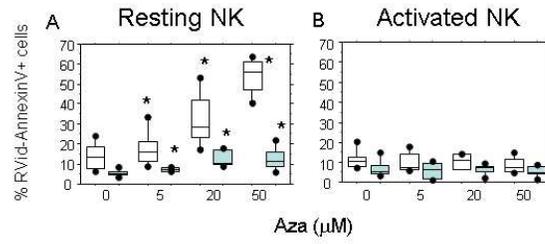
Figure 5.



254x190mm (96 x 96 DPI)

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Figure 6.

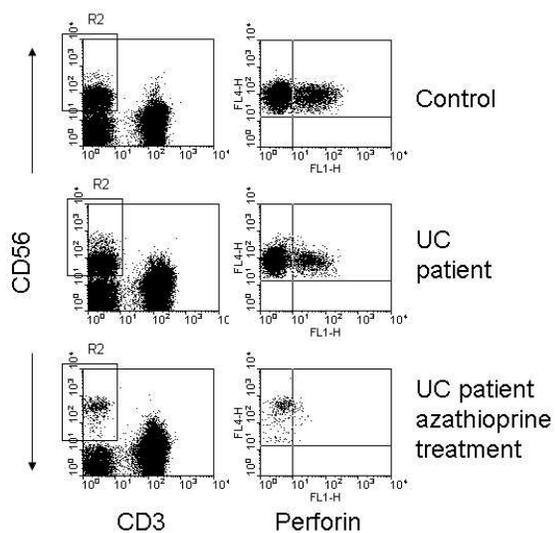


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Figure S1



Supplementary figure 1
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Review

Supplementary Table 1. Comparison of characteristics between Azathioprine untreated and treated individuals.

	CD N=11	CD Azathioprine N=6
Age, median (range)	40 (23-74)	40 (23-58)
Sex (m:f)	5:6	5:1
Time since Diagnosis, Years, median (range)	12 (4-26)	8 (2-18)
CRP, mg/l Median (range)	4 (2-18)	3 (2-37)
Harvey Bradshaw Index. Median (range)	4 (0-8)	4 (0-7)
5-Asa therapy, n(%)	6 (55)	2 (33)
	UC N=20	UC Azathioprine N=10
Age, median (range)	43 (16-75)	46 (21-78)
Sex (m:f)	7:13	5:5
Time since Diagnosis, Years, median (range)	12 (0.5-23)	9 (1-38)
CRP mg/l Median (range)	4 (2-112)	3 (2-77)
Ulcerative Colitis Severity Score, median (range)	3 (2-7)	3 (1-9)
5-Asa therapy n (%)	15 (75)	7 (70)

Table S2: Assessment of NK cell receptor expression in the blood and colon

% of subset, median(range)	Healthy Control		Crohn's Disease		Crohn's disease Azathioprine		Ulcerative Colitis		Ulcerative Colitis Azathioprine		Kruskal Wallis p value	
	PBMC	LP	PBMC	LP	PBMC	LP	PBMC	LP	PBMC	LP	PBMC	LP
Total CD3- CD56+ NK cells	7.5 (2.2-17.0)	4.8 (1.7-17.4)	4.4 (1.3-10.9)	4.2 (2.3-8.8)	1.7 (0.3-6.2)	3.2 (0.6-4.6)	9.4 (1.3-22.0)	3.6 (1.4-13.6)	1.4 (0.5-10.9)	3.5 (0.9-9.4)	0.12	0.51
CD16	91.1 (81.3-96.9)	5.7 (0.1-30.2)	86.9 (64.3-97.7)	14.7 (3.6-48.9)	63.4 (25-84.0)	1.6 (0.8-21.9)	89.7 (70.6-98.2)	18.3 (0.1-49.5)	81.1 (25.5-95.2)	10.1 (0.1-21.7)	0.80	0.01
CD161	11.9 (2.7-80.6)	28 (0.1-81.5)	28.0 (5.2-73.0)	27.7 (15.9-72.4)	nd	nd	6.7 (0.1-34.2)	8.3 (0.7-40.4)	4.3 (2.4-6.2)	nd	0.31	0.70
KIR2DL-1/2	25.6 (16.1-61.3)	4.0 (0.6-16.0)	29.4 (14.6-55.1)	3.6 (1.2-11.1)	nd	nd	43.3 (11.0-50.4)	5.4 (2.4-18.3)	21.7 (18.0-31.1)	8.6 (5.6-11.5)	0.36	0.43
KIR3DL-1	19.2 (0.06-41.6)	1.0 (0.1-5.5)	18.2 (5.0-31.2)	0.8 (0.1-33.3)	nd	nd	17.3 (13.3-22.0)	3.2 (0.9-5.1)	nd	nd	0.81	0.74
NKG2A	44.8 (22.8-84.9)	nd	45.5 (25.9-73.7)	61.7 (26.5-77.7)	nd	nd	51.6 (21.3-84.3)	53.1 (7.1-69.5)	48.7 (26.5-91.1)	45.8 (41.6-99)	0.73	0.11
NKG2C	6.9 (0.12-47.1)	11.3 (0.9-30.2)	15.3 (2.0-34.5)	11.7 (2.8-15.3)	nd	nd	12.3 (3.0-24.3)	11.8 (2.4-22.2)	7.9 (2.0-60.1)	5.2 (0.1-19.1)	0.28	0.73
NKG2D	84.9 (47-95.5)	57.0 (23.0-82.1)	70.2 (48.1-95.8)	45.1 (36.0-70.6)	nd	nd	72.5 (43.2-78.0)	19.7 (19.4-73.0)	nd	nd	0.84	0.41
NKp30	16.4 (0.8-38.5)	21.5 (0.6-64.9)	14.7 (2.1-23.0)	35.9 (26.9-44.9)	7.7 (2.1-13.3)	30.6 (9.9-51.2)	3.3 * (0.7-12.9)	22.1 (8.2-48.2)	14.0 (1.8-76.0)	11.1 (5.5-91.8)	0.13	0.70
NKp44	1.3 (0.1-3.8)	48.8* (33.3-71.7)	0.3 (0.1-1.5)	27.6 * (25.5-29.6)	4.3 (2.4-6.2)	63.3* (45.5-81)	0.6 (0.1-5.4)	43.6* (21.2-57.5)	1.6 (0.2-6.8)	51.8* (22.2-77.7)	0.19	0.13
NKp46	66.8 (21.8-73.4)	76.9 (63.3-97.0)	61.9 (22.0-66.7)	95.9 (92.7-99.0)	39.2 (6.0-72.3)	77.6 (63.4-91.7)	40.7 (17.7-68.4)	73.8 (62.3-93.7)	68.9 (17.1-95.0)	73.9 (34.2-98.2)	0.20	0.15

* Significant differences are observed in the proportions of NKp44⁺ cells between blood and gut within all groups and are maintained with Azathioprine treatment. (Wilcoxon signed rank test). Significant p values comparing Healthy control and Crohn's disease and ulcerative colitis groups without Azathioprine treatment are depicted in bold.