Azathioprine therapy selectively ablates human Vδ2+ T cells in Crohn’s disease

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Introduction

Tumor cells and bacteria produce nonpeptide metabolites known as phosphoantigens (PAg), which are uniquely recognized by a population of unconventional lymphocytes that express a Vγ9Vδ2 T cell receptor (Vδ2 T cells). Unusually among lymphocytes, Vδ2 T cells are found only in humans and higher primates, where they mediate host protection against a wide range of microbial infections, lymphoproliferative disorders, and solid cancers (1, 2). Although numerous constituents of the gut microbiota are thought to be obligate producers of PAg (1), the absence of Vδ2 T cells in rodent models has so far prevented detailed investigation of their role in mucosal inflammation.

Nonpeptide products of the gut microbiota have been shown to influence the balance of pro- and antiinflammatory lymphocytes in the intestine (3), and studies in macaques have demonstrated that injection of nonpeptide PAg stimulates circulating Vδ2 T cells to proliferate and accumulate in mucosal tissues (4). PAg are produced by a wide range of bacteria that can colonize the gut (1) and can also accumulate in host cells due to dysregulation of the mevalonate kinase metabolic pathway during malignant transformation or microbial infection (5, 6). Intriguingly, human patients with mutations in the mevalonate kinase gene exhibit a severe neonatal colitis that can be successfully treated with bisphosphonate drugs, which modulate PAg synthesis and alter Vδ2 T cell function in vivo (7–10). We recently reported that PAg exposure stimulates human blood Vδ2 T cells to upregulate the gut-homing integrin α4β7, and we identified Vδ2 T cells in human colonic biopsies that produced proinflammatory cytokines and enhanced IFNγ synthesis by intestinal CD4+ T cells (11). These data indicate a potential role for Vδ2 T cells in the pathogenesis of Crohn’s disease (CD), which is characterized by enhanced effector function of CD4+ T cells directed against components of the gut microbiota. In addition to our own detection of Vδ2 T cells in human colonic lamina propria in situ (11), these cells have also been observed in gastrointestinal lymphoid tissues (12) and were previously identified in the gut in a small number of CD patients (13, 14), but the role played by these cells in mucosal inflammation in CD is currently unknown.

The early pathogenesis of CD is thought to involve increased intestinal permeability and altered innate responses to bacterial products that cross the gut barrier, leading to the establishment of a disease-permissive environment in the intestine (15–17). In healthy humans, activation of intestinal Vδ2 T cells by bacterial PAg is likely to be restricted by the gut barrier, but increased intestinal permeability and/or dysbiosis of the gut microbiota in CD could permit increased
Results

Enhanced gut-tropism and depletion of circulating CD27⁺ Vδ2 T cells in patients with CD. Vδ2 T cells are unconventional blood lymphocytes that are uniquely able to recognize PAg produced by tumor cells and microbes (1, 5, 6). We recently demonstrated that PAg exposure stimulates human blood Vδ2 T cells to upregulate the gut-homing integrin α4β7 and increase binding to the intestinal addressin MAdCAM-1 (11). To test the hypothesis that human Vδ2 T cells contribute to mucosal inflammation in CD, we first used flow-cytometry to assess the gut-homing potential of blood Vδ2 T cells in a cohort of CD patients who had moderately active disease but who were not receiving immunosuppressive therapy. We observed that the overall proportion of β7⁺ Vδ2 T cells in blood was comparable with adult CD patients (NS, not shown), but the CD45RA CD27⁺ subset was selectively depleted compared with sex/age/ethnicity-matched IBS controls (*P < 0.001; n = 8 per group), while the subset distribution of conventional αβ T cells as defined by these markers was unaltered in CD (data not shown). The CD27⁺ population of circulating Vδ2 T cells expressed significantly higher levels of β7 integrin than did any other Vδ2 T cell subset (*P < 0.05; n = 8), consistent with increased trafficking of these cells to the gut and a corresponding depletion from the blood in CD (11, 18). We therefore investigated whether human Vδ2 T cells contribute to mucosal inflammation in CD by assessing Vδ2 T cell phenotype, frequency, gut-homing potential, and cytokine production in peripheral blood and colonic biopsy tissue from CD patients and healthy controls. We observed that Vδ2 T cells from CD patients exhibited increased expression of the gut-homing integrin β7 in blood together with a selective depletion of CD27⁺ “Th1-committed” cells from the circulation, while also displaying a corresponding population of CD27⁺ Vδ2 T cells in colonic biopsy tissue that produced elevated levels of TNFα relative to healthy controls. Furthermore, manipulation of Vδ2 T cell function by inhibition of retinoic acid receptor-α (RARα) signaling or exposure to the thiopurine drug azathioprine (AZA) exerted potent effects on Vδ2 T cell frequency and cytokine production both in vitro and in vivo. These data could have substantial implications for the management of malignancy risk in patients with CD and other chronic inflammatory disorders, and may lead to the development of therapies that target Vδ2 T cells to reduce inflammation in the human intestine.

Figure 1. Enhanced gut-homing potential and depletion of circulating CD27⁺ Vδ2 T cells in CD. (A) In patients with moderately active CD about to receive de novo AZA therapy (n = 12), flow-cytometric analysis of blood lymphocytes revealed a significant increase in the proportion of β7⁺ gut-homing cells within the CD3⁺ Vδ2 T cell population when compared with healthy controls (n = 26). (B) In a separate analysis of pediatric CD patients (mean age 13 years), we observed that the overall proportion of β7⁺ Vδ2 T cells in blood was comparable with adult CD patients (NS, not shown), but the CD45RA CD27⁺ subset was selectively depleted compared with sex/age/ethnicity-matched IBS controls (*P < 0.001; n = 8 per group), while the subset distribution of conventional αβ T cells as defined by these markers was unaltered in CD (data not shown). (C) The CD27⁺ population of circulating Vδ2 T cells expressed significantly higher levels of β7 integrin than did any other Vδ2 T cell subset (*P < 0.05; n = 8), consistent with increased trafficking of these cells to the gut and a corresponding depletion from the blood in CD. (D) Example histograms showing integrin β7 expression levels in the different Vδ2 T cell subsets detected in blood from an IBS control (representative of n = 8). Significant differences between groups were determined by Mann-Whitney rank-sum test (A), or repeated-measures ANOVA (B and C).
CD45RA+CD27+), central-memory (CD45RA−CD27+), effector cells are commonly divided into subsets of putative naive δ-terminated to a specific subpopulation of these cells. Human Vδ2 T cells in CD patients included both CD103+ and CD103- subsets in proportions comparable to those present in the healthy intestine. Shown is an example flow-cytometry analysis of colonic biopsy tissue after extensive removal of the epithelium and showing positive identification of Vδ2 T cells (thus excluding Vα1Vδ1 T cells) among the egressed leukocytes in an 18-year-old patient with active CD (B, representative of n = 11 CD patients), as well as grouped data showing Vδ2 T cell subset balance in multiple individuals (C: n = 11 CD patients, n = 9 healthy controls; comparison by t test).

We next sought to determine whether the enhanced gut-tropic phenotype of Vδ2 T cells in CD patients could be attributed to a specific subpopulation of these cells. Human Vδ2 T cells are commonly divided into subsets of putative naive (CD45RA-CD27+), central-memory (CD45RA-CD27+), effector-memory (CD45RA-CD27-) and terminally differentiated CD103+ subsets in proportions comparable to those present in the healthy intestine (ref. 11, 12). NS indicates no significant difference.

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tissue were relatively poor cytokine producers (11). Given the limited amount of intestinal tissue available at endoscopy, we instead used unstimulated biopsy cultures in the current report, enabling us to compare Vδ2 T cell cytokine responses to endogenous factors between CD patients and controls rather than risk abolishing differences between groups by adding a potent PAg stimulus throughout. First, we assessed whether CD103− Vδ2 T cells were enriched in the colonic mucosa in CD, but instead we observed no significant difference in subset balance when compared with healthy controls (Figure 2C). However, when we assessed cytokine production profile, mucosal Vδ2 T cells from CD patients produced significantly higher levels of TNFα and IL-17A than those from healthy volunteers (Figure 3A). There was a similar trend for IFNγ production, but this did not reach statistical significance. We further observed that mucosal Vδ2 T cells from CD patients displayed high levels of cytokine synthesis irrespective of CD103 expression (Figure 3B), suggesting that both major Vδ2 T cell subsets in the CD mucosa are strongly activated by endogenous factors. Thus, unlike healthy biopsy tissue (11), the relative frequencies of CD103− and CD103+ cells in CD colon did not influence cytokine production by conventional colonic T cells (data not shown).

We next sought to identify factors in the human intestinal environment that might increase proinflammatory cytokine production by Vδ2 T cells in CD patients. Intestinal antigen-presenting cells (APCs) metabolize dietary vitamin A into retinoic acid (RA), which exerts potent effects on T cell differentiation, effector function, gut tropism, and migration to inflamed tissues (29–31). Having recently reported that human intestinal APC display increased capacity for RA synthesis in inflammatory bowel disease (IBD) (32), and that human Vδ2 T cell function can be modified by exposure to RA in vitro (11), we hypothesized that RA signaling might influence Vδ2 T cell production of proinflammatory mediators in the CD intestine in vivo. To test this hypothesis, we collected biopsies of inflamed colonic mucosa from patients with new diagnoses of CD and assessed
cytokine production by intestinal Vδ2 T cells after 3d culture with either exogenous RA or with the specific RARα antagonist Ro41-5253. Supplementation with 2nM all-trans RA appeared to induce a marginal increase in TNF production by Vδ2 T cells in CD biopsy tissue (the small effect likely due to endogenous RA in the biopsy cultures; compare CM with RA in Figure 3, C and D; $P = 0.357$ and $P = 0.209$, respectively), but inhibition of RARα signaling with Ro41-5253 led to a substantial reduction in TNF synthesis by these cells ($P < 0.001$; Figure 3, C–E). Taken together with our previous finding that RA can increase the gut-homing potential of Vδ2 T cells in human peripheral blood (11), these data indicate that RA signaling in the local microenvironment influences Vδ2 T cell function and may alter cytokine production by these cells in the inflamed human intestine.

**AZA selectively and reversibly ablates Vδ2 T cells in patients with CD.** Having observed that exposure to pharmacological agents can substantially reduce TNF synthesis by intestinal Vδ2 T cells in CD patients, we next sought to determine whether the therapies routinely used to treat CD could similarly impair Vδ2 T cell function. Indeed, stimulation with microbial PAg induced rapid expansion of blood Vδ2 T cells over 5d culture in vitro, but even subtherapeutic concentrations of the thiopurine drug AZA (33) were sufficient to disrupt this response, while comparable effects on autologous conventional αβ T cells were only achieved at 100-fold higher doses (Figure 4). Staining with the viability dyes annexin V and 7-AAD confirmed that Vδ2 T cells and αβ T cells displayed comparable rates of drug-induced cell death in these assays, suggesting that AZA can selectively impair Vδ2 T cell proliferation (not shown). Accordingly, while Vδ2 T cells made up approximately 2% of total circulating T cells in both healthy volunteers and AZA-naïve CD patients, these cells were essentially absent from the blood of CD patients established on AZA therapy (Figure 5A). There was a selective loss of circulating Vδ2 T cells in AZA-treated CD patients, whether numbers were assessed as a proportion of the total T cell pool (Figure 5B) or by calculating the absolute number of these cells per unit volume of blood (Figure 5C). In order to confirm the selective effects of AZA therapy on Vδ2 T cells, we next generated new data using a previously published cohort (16) of AZA-treated CD patients ($n = 5$) and healthy controls ($n = 8$), and determined the extent to which AZA depletes different populations of conventional blood T cells (CD4⁺, CD4⁺ memory, CD8⁺, CD8⁺ naive, CD8⁺ memory) this analysis confirmed that no major subset of αβ T cells in blood was depleted by more than 25% in AZA-treated CD, whereas circulating Vδ2 T cell numbers in the same individuals were reduced by $>95%$ (Supplemental Figure 2). Indeed, an independent analysis of the blood γδ T cell compartment in newly recruited cohorts of AZA-treated CD patients and healthy controls (both $n = 3$), confirmed a selective loss of Vδ2⁺ cells only ($<0.1%$ of total T cells in AZA-CD, $>1%$ in healthy controls) whereas Vδ1⁺ cells were preserved ($1%$ in CD, $-1%$ in controls; data not shown), thus indicating that the drug ablates Vδ2 T cells in CD while sparing other major subsets of circulating γδ T cells. Consistent with these data, intestinal Vδ2 T cells were relatively frequent in biopsy tissue obtained from healthy controls ($n = 4$, mean 0.82% of total lamina propria T cells) or AZA-naïve CD patients ($n = 6$, 0.78%), but numbers were significantly suppressed in CD patients receiving AZA ($n = 5$, <0.2%; $P < 0.05$).
Previous data from a murine model have indicated that selective depletion of conventional memory T cells can be driven by repeated stimulation in the presence of AZA (33), which is known to disrupt purine biosynthesis, inhibit DNA replication, and induce apoptosis of highly activated T cell clones (34). Since human Vδ2 T cells are almost exclusively comprised of memory cells, we next sought evidence of ongoing Vδ2 T cell activation in CD that might drive the depletion of these cells in the presence of AZA. Indeed, the proportion of circulating Vδ2 T cells that displayed an activated CD69+ phenotype was significantly higher in CD patients than in healthy volunteers (Figure 6A). We therefore assessed whether the ablation of Vδ2 T cells was an AZA-specific phenomenon by analyzing the blood frequency of Vδ2 T cells in CD patients treated with the alternative immunosuppressant drug methotrexate (MTX), which exhibits a distinct mechanism of action from AZA but induces a similar inhibition of purine biosynthesis to restrict the proliferation of highly activated T cells (35). Using this approach, we observed evidence of ongoing Vδ2 T cell activation in CD that might drive the depletion of these cells in the presence of AZA. Indeed, the proportion of circulating Vδ2 T cells that displayed an activated CD69+ phenotype was significantly higher in CD patients than in healthy volunteers (Figure 6A). 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Discussion

This study provides evidence of enhanced gut-tropism and selective depletion of circulating CD27^+ Vδ2 T cells in patients with active CD, consistent with increased recruitment of these cells to the inflamed intestine. Accordingly, we detected a corresponding population of CD27^+ Vδ2 T cells in the lamina propria of CD patients that displayed elevated TNF production, which could be disrupted by pharmacological blockade of signaling through RARα. Furthermore, we observed that Vδ2 T cells in CD patients are highly sensitive to AZA exposure, leading to the selective ablation of these cells over about a year of continuous therapy. While impaired Vδ2 T cell function may contribute to the therapeutic effects of AZA in active CD, complete loss of this tumor surveillance population may also confer increased risk of malignancy, consistent with the increased rates of lymphoma observed in AZA-treated CD patients (41–43).

Due to ethical constraints, it is difficult to demonstrate cell recruitment to peripheral tissues in human patients in vivo, but our observation that CD27^+ Vδ2 T cells express high levels of β7 integrin in blood, are selectively depleted from the circulation in CD, and predominate in the inflamed colonic mucosa strongly suggest that these cells are activated and recruited to the intestine in patients with CD. Further investigation will now be required to identify the microbial/host-derived signals that may drive this process, although these are likely to include increased intestinal permeability (17), changes in the composition of the microbiota (1, 44), and systemic priming of innate leukocytes by bacterial products translocated from the gut (21). Indeed, it has already been reported that environmental exposures influence γδ T cell frequency in human peripheral blood (25) and that stable expansions of Vδ2 T cells can occur in the circulation of patients with IBD (45, 46). Consistent with these data, Vδ2 T cells have also been identified in the gut in a small number of CD patients (13, 14), although the role played by these cells in mucosal inflammation in CD has, until now, remained obscure.

In the current report, we observed a selective depletion of the CD27^+ subset of blood Vδ2 T cells that reportedly displays high sensitivity to PAg, exhibits substantial proliferative poten-

![Figure 6. Activation and depletion of Vδ2 T cells in AZA-treated CD.](image-url)
intestinal V62 T cells were exclusively CD45RA CD27+ and displayed a cytokine profile that was highly consistent within patient groups, it is unlikely that our cultures contained substantial numbers of blood lymphocytes. Our in vitro data also demonstrated that RARs signaling is required to mediate TNF production by these cells, suggesting that the vitamin A metabolite RA may modulate inflammatory responses in the human gut. Taken together with our report that intestinal APC exhibit increased capacity to generate RA in patients with IBD (32), these findings suggest that RA synthesis by colonic APC may regulate V62 T cell function and influence TNF production in the human intestine (31).

AZA-treated CD patients exhibit increased risk of lymphoproliferative disorders (42, 47), nonmelanoma skin cancers (48), and hepatosplenic T cell lymphoma (HSTCL) (49). Given that V62 T cells are a key component of tumor surveillance in humans (41), selective depletion of this population in AZA-treated CD would be expected to confer increased risk of malignancy. Indeed, circulating V62 T cells can suppress Epstein-Barr virus (50), have displayed therapeutic potential in a range of hematological and solid cancers (2, 51), and contain a recently identified population of skin-homing cells that may prove capable of destroying cutaneous tumors (11, 19). Cases of HSTCL in CD are known to occur primarily among young, male patients receiving long-term AZA therapy (49), and in an analysis of published reports and data collected via the MedWatch reporting system of the US Food and Drug Administration, 34 of 36 patients presenting with HSTCL were male, 90% were <35 years old, and most had been receiving thiopurine therapy for >2 years (49). It is notable therefore that the natural loss of V62 T cells observed in adults over 30 years of age is more pronounced in males and that progressive depletion of the antitumor subset of IFNγ-producing V62 T cells features only in men (24). AZA may therefore accelerate the natural decline of V62 T cell tumor surveillance in CD patients more acutely in men than in women. Indeed, lymphoma risk in AZA-treated IBD has been reported to progressively increase with therapy duration (52), and older male patients are at the greatest risk of AZA-induced malignancy (53). Furthermore, HSTCL in AZA-treated CD is frequently of γδ T cell origin (49), and anti-TNF therapy reportedly increases the proliferation of γδ T cells in young, male CD patients (46). These data suggest that the V62 T cell population itself may become dysregulated and eventually undergo malignant transformation in AZA-treated CD.

Figure 7. Post-AZA recovery of blood V62 T cells is influenced by patient age and time elapsed since drug withdrawal. (A) Cross-sectional study of CD patients previously treated with AZA for >1 year but not receiving thiopurine therapy at the time of sampling (ex-AZA; n = 10). Evidence of blood V62 T cell recovery was observed in 5 of 10 ex-AZA CD patients (recovery threshold Q1 defined as the 25th percentile of V62 T cell frequency in AZA-naive CD patients), but not in any of the CD patients on concurrent AZA therapy (AZA+; n = 17). Fisher exact test was used to assess V62 T cell recovery after AZA withdrawal. (B and C) Blood V62 T cell frequency in the ex-AZA CD patients was positively correlated with time elapsed since drug withdrawal (B; n = 9), and was inversely correlated with patient age (C; n = 13). Correlations were assessed using Pearson product-moment.
Our analyses revealed that the Vδ2 T cell population can be reconstituted in CD patients that cease AZA therapy, consistent with reports that lymphoma risk returns to normal levels after withdrawal of treatment (42). Since greater numbers of young patients are now receiving AZA for increasing lengths of time, either for CD or other types of chronic disease, new treatment strategies that spare the tumor-responsive Vδ2 T cell compartment may help to mitigate long-term risk of malignancy (42, 48). Preclinical studies have already identified methods of inducing Vδ2 T cell expansion for therapeutic benefit (40, 51), and similar approaches may also prove useful for preserving Vδ2 T cell function and reducing cancer risk in AZA-treated diseases. However, the potential benefits of these strategies will need to be weighed against the possibility of exacerbating mucosal inflammation by activating Vδ2 T cells in the intestine.

Methods

Study participants. Peripheral blood and colonic biopsies were obtained from patients undergoing colonoscopy as part of routine clinical care to assess CD activity, or for colorectal cancer screening or investigation of rectal bleeding but with no endoscopic abnormalities (controls). Additional samples of mucosal tissue were obtained from patients undergoing surgical resection for colorectal cancer, noninflammatory intestinal motility disorders, or severe CD. The CD cohort was heterogeneous; distinct subgroups of patients were recruited to address specific experimental questions (details in the corresponding results sections). The overall characteristics of the CD cohort were as follows: 41% male; mean age, 35 years (range 7–79); age at diagnosis, 21 years (range 4–51); disease duration, 14 years (range 1–60); C-reactive protein (CRP), 36 mg/l (range <5–318). Montreal classification: A1, 40.7%; A2, 51.9%; A3, 7.4%; L1, 25.9%; L2, 18.5%; L3, 55.6%; L4, 29.6%; B1, 42.1%; B2, 31.6%; B3, 26.3%; P, 7.4% (54). To assess the effects of thiopurine treatment, some CD patients were recruited immediately prior to commencing AZA therapy, and longitudinal blood sampling was conducted over a followup period of 6 months. The analyses of Vδ2 T cell subset distribution in peripheral blood were conducted in a subgroup of pediatric CD patients (mean age, 13 years [range 7–19]); 50% male; CRP, mean 12.5 mg/l [range 5–39 mg/l]) and sex/ethnicity-matched controls (mean 12.5 mg/l [range 5–39 mg/l]) in order to limit the effects of demography, historical pathology, and sex/age/ethnicity-matched controls (mean 12.5 mg/l [range 5–39 mg/l]).

Peripheral blood cells. Human whole blood was directly labeled with monoclonal antibodies for 15 minutes at room temperature, and the red blood cells were lysed by addition of Optilyse C (Beckman Coulter). Labeled cell suspensions were washed twice in cold FACS buffer (PBS containing 2% fetal calf serum, 0.02% sodium azide, 1 mM EDTA) and fixed in 1% paraformaldehyde.

Lamina propria mononuclear cells. Intestinal biopsies were washed in 1 mmol/l dithiothreitol (Sigma-Aldrich), and incubated in 1 mmol/l EDTA for 1 hour to thoroughly detach the epithelium and remove epithelial lymphocytes prior to biopsy culture in complete medium (Dutch-modified RPMI-1640 medium, 10% FCS, 2 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml gentamicin, 30 μl recombinant human IL-2) in 24-well plates in the presence or absence of 2 nM all-trans RA (Sigma-Aldrich), or 1 μM RARα-selective antagonist Ro41-5253 (Enzo Life Sciences Inc.), for 3–5 days at 37°C, 5% CO₂. Lamina propria mononuclear cells that migrated out of the biopsies were labeled with mAb for flow-cytometry or were reactivated with PMA (10 ng/ml) and ionomycin (2 μM) in the presence of monensin (3 μM) for 4 hours at 37°C, 5% CO₂ prior to surface labelling. The reactivated cells were then permeabilized using Leucopener reagents (AbD Serotec), labeled with anti-cytokine mAb, and fixed in 1% paraformaldehyde for analysis by flow cytometry.

Flow-cytometry. Monoclonal antibodies were: PerCP-Cy5.5-conjugated CD3 (clone HIT3a); FITC-conjugated TCR Vδ2 (B6); Alexa Fluor 647-conjugated CD27 (O323) and CD103 (Ber-Act7); PE/Cy7-conjugated CD45RA (H100) and IFNγ (4S.B3); PE-conjugated integrin β7 (FIB504), CD69 (FN50), TNFα (Mab11), IL-17A (BL168), and IL-10 (JES3-9D7) from BioLegend UK; and FITC-conjugated TCR Vβ1 (REA173) from Miltenyi Biotec. Labeled cells were acquired on a FACSCanto II flow-cytometer using FACSDiva 6.1.2 software (BD Biosciences), and data were analyzed using WinList 6.0 (Verity Software House). Absolute cell numbers were determined using Flow-Count Fluorospheres (Beckman Coulter).

Statistics. Statistical analyses were performed using SigmaStat 3.5 (SYSTAT). Normal data were analyzed by 2-tailed t test or 1-way ANOVA as appropriate. Mann-Whitney and Wilcoxon signed-rank tests were used to evaluate non-normally distributed data. One-way repeated measures ANOVA was used to determine the effects of biopsy supplementation with RA or Ro41-5253. Two-way repeated measures ANOVA was used to test the influence of cell type and drug dose on proliferated cell number in the AZA in vitro assays. Kruskal-Wallis 1-way ANOVA on ranks was used to compare blood Vδ2 T cell frequencies between healthy controls and CD treatment groups. Correlations were assessed using Pearson product-moment and Spearman rank tests. Fisher exact test was used to assess Vδ2 T cell recovery after AZA withdrawal. Error bars in the figures indicate standard error of the mean. P < 0.05 was considered significant.

Study approval. Ethical permissions for the study were granted by the appropriate local research ethics committees (approvals 05/Q0405/71 from Harrow Research Ethics Committee; 10/H0704/74 from East London Research Ethics Committee 2, London, UK; P01/023 from East London and City Health Authority Research Ethics Committee, London, UK; and 10/H0805/46 from Bromley Local Research Ethics Committee). All volunteers gave written informed consent prior to inclusion in the study.

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