Mucosal-associated invariant T (MAIT) cells are activated in the gastrointestinal tissue of patients with combination ipilimumab and nivolumab therapy-related colitis in a pathology distinct from ulcerative colitis

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Summary

The aim of this study was to investigate the pathogenesis of combination ipilimumab and nivolumab-associated colitis (IN-COL) by measuring gut-derived and peripheral blood mononuclear cell (GMNC; PBMC) profiles. We studied GMNC and PBMC from patients with IN-COL, IN-treated with no adverse-events (IN-NAE), ulcerative colitis (UC) and healthy volunteers using flow cytometry. In the gastrointestinal-derived cells we found high levels of activated CD8^{+} T cells and mucosal-associated invariant T (MAIT) cells in IN-COL, changes that were not evident in IN-NAE or UC. UC, but not IN-C, was associated with a high proportion of regulatory T cells (T_{reg}). We sought to determine if local tissue responses could be measured in peripheral blood. Peripherally, checkpoint inhibition instigated a rise in activated memory CD4^{+} and CD8^{+} T cells, regardless of colitis. Low circulating MAIT cells at baseline was associated with IN-COL patients compared with IN-NAE in one of two cohorts. UC, but not IN-COL, was associated with high levels of circulating plasmablasts. In summary, the alterations in T cell subsets measured in IN-COL-affected tissue, characterized by high levels of activated CD8^{+} T cells and MAIT cells and a low proportion of T_{reg}, reflected a pathology distinct from UC. These tissue changes differed from the periphery, where T cell activation was a widespread on-treatment effect, and circulating MAIT cell count was low but not reliably predictive of colitis.

Keywords: checkpoint inhibitor, colitis, ipilimumab, MAIT cells, nivolumab

Introduction

Immune checkpoint inhibitors (ICI) target negative regulators of cellular activation and include cytotoxic T lymphocyte antigen 4 (CTLA-4) (inhibited by ipilimumab) and programmed cell death 1 (PD-1; inhibited by pembrolizumab and nivolumab). The resulting dysregulated T cell activation enhances anti-tumour immunity, can lead to disease regression and increased survival in patients with metastatic melanoma and a variety of other malignancies. While anti-CTLA-4 and anti-PD-1 therapies both independently improve survival in metastatic melanoma, the combination provides superior response rates and survival [1–3].

The augmented adaptive immunity induced by ICI comes at the cost of immune-related adverse events (irAE), most commonly affecting the skin, endocrine organs and gastrointestinal tract. Up to 44% of patients receiving combination anti-CTLA-4 and PD-1 inhibition develop colitis, higher than is seen with anti-PD-1 monotherapy [1,4]. ICI-related colitis typically occurs within the first few treatment cycles (3–9 weeks) as a single-episode illness [5]. If not recognized and managed promptly ICI-related colitis can cause significant morbidity, and remains the most common drug-related cause of death from combination immune checkpoint inhibition [6].

Current guidelines advise corticosteroids as first-line treatment for ICI-related colitis, followed by intravenous steroids and anti-tumour necrosis factor (TNF)-α therapy (infliximab) for steroid-refractory disease. The management of ICI-related colitis has evolved from more classical forms of inflammatory bowel disease (IBD), but it is unclear how analogous the newer disease is. It is anticipated that a greater understanding of the underlying mechanisms of ICI-related colitis may lead to more effective treatments.

The intestinal environment poses distinct immunological challenges, requiring tolerance to a diverse microbiome of commensals while mounting effective immunity to pathogens. While the pathogenesis of classical IBD is not fully elucidated, it is proposed that a breakdown of immune tolerance to bacterial species can contribute to disease development (reviewed in [7]). Propagation of intestinal inflammation is contributed to by multiple cell lineages, including epithelial cells, intestinal macrophages, dendritic cells and innate lymphoid cells [7]. A dysregulated T cell response is thought to be a key driver of the chronic inflammation of IBD, as supported by the success of T cell-directed therapies including vedolizumab (targeting the α4β7 integrin) [8,9] and interleukin (IL)-12/23 p40 pathway inhibitors [10]. A recent study of gut-isolated mononuclear cells (GMNC) by flow cytometry demonstrated that both Crohn's disease (CD) and ulcerative colitis (UC) were associated with a high proportion of CD4+ T cells and regulatory T cells (T$_{reg}$) and lower proportions of CD8+ T cells compared with tissue from healthy donors, changes that reversed during remission [11].

There are limited data on the pathogenesis of ICI-related colitis. Much of the literature relates to early use of ipilimumab monotherapy and histopathological evaluation of colon biopsies. Endoscopically, ICI-colitis is a heterogenous entity that shares features of both CD and UC [12], with the sigmoid colon and rectum usually involved. Initial reports found an increased number of CD8+ T cells in biopsies from ipilimumab-associated colitis that positively correlated with disease activity [13], while others found an increased proportion of infiltrating CD4+ T cells [14]. Up-regulation of T helper type 1 (Th1) [interferon (IFN)-γ] and Th17 (IL-17) mRNA has been reported, as well as up-regulation of regulatory factors forkhead box protein 3 (FoxP3) and IL-10 [14]. While an inflammatory cell infiltrate and cryptitis can be evident on histopathology of ipilimumab-related colitis [14], detailed phenotyping of peripheral blood mononuclear cells (PBMC) and GMNC in ICI-related colitis, including following a combination of ipilimumab and nivolumab, has not been studied to date.

Herein we characterized GMNC from patients treated with combination ipilimumab and nivolumab therapy who developed colitis (IN-COL), and compared these with those from patients who had no autoimmune adverse events (IN-NAE), patients during active UC flare and healthy volunteers. We additionally studied whether congruent change could be detected in the peripheral blood. We hypothesized that IN-COL is associated with an increased proportion of activated T cells [as defined by the well-described co-expression of *in-vivo* activation markers human leucocyte antigen D-related (HLA-DR) and CD38 [15–17] and a low proportion of T$_{reg}$ (CD4+CD25+CD127− [18] or CD4+25+FoxP3+ T cells), given the role of CTLA-4 in their function [19–22].

Mucosal-associated invariant T (MAIT) cells, innate-like T cells enriched in the mucosa [23], were also of interest. Human MAIT cells express high levels of CCR6 and CD161 and a semi-invariant T cell receptor (TCR) α-chain (Vα7·2) [24]. MAIT cells are restricted by the major histocompatibility complex class I (MR1) molecule that recognizes vitamin B metabolites (e.g. riboflavin and folate) [25,26], but can also be activated by cytokines independently of their TCR [27]. The role of MAIT cells in health and disease is still being established [28]. They are predominantly effector cells that can produce high amounts of IFN-γ, cytotoxic granules including granzyme B, IL-17 and IL-22 with a mixed Th1/Th17 phenotype [29]. There are data suggesting that MAIT cells play a regulatory role in some tissue but may be pathogenic in others (reviewed in [30]). Low circulating levels of MAIT cells have been reported in IBD [31–34],

where MAIT cell numbers accumulate in inflamed bowel and undergo activation-induced cell death [35]. MAIT cells are also depleted in the blood and gastrointestinal tissue of patients with coeliac disease [36]. MAIT cells [defined as chemokine receptor 6 (CCR6)$^+$CD161$^+$ or CD161$^+$ TCR Vα7·2$^+$] appear to play a protective role in graft-versus-host disease (GVHD; a condition that commonly affects the gastrointestinal tract), where the circulating CD8$^+$CD161$^+$ T cell count is lower in patients with acute GVHD and the circulating MAIT cell count inversely correlates to disease grade [37]. In mouse models, a higher proportion of MAIT cells protects against colonic GVHD [38].

We sought to measure what perturbations in these immune cell subtypes occurred in the gastrointestinal tissue and peripheral blood of patients with IN-COL and whether these changes were distinct from those seen in UC (Fig.1). We anticipate that a greater understanding of the cellular pathogenesis of ICI-associated colitis will eventually lead to improved and novel therapeutics.

Methods

Patients

Lymphocytes isolated from five pairs of endoscopically obtained 'pinch' colon biopsies were studied from 12 patients with melanoma treated with combination ipilimumab and nivolumab therapy in Oxford, UK (cohort 1). These included six patients with active IN-COL and six IN-NAE patients who were recruited as part of a larger longitudinal study collecting colonic biopsy from patients on ICIs at weeks 7–10, regardless of colitis status [Predicting Immunotherapy Adverse Events (PRISE) Study]. Six patients with endoscopically and histologically confirmed active UC and six healthy volunteers undergoing benign polyp surveillance (with no pathology apparent on endoscopy) were studied as comparators. All patients provided written informed consent in line with local procedure (Oxford Centre for Histopathology Research 16/A019).

Viably cryopreserved PBMC from 20 treatment-naive patients with metastatic melanoma obtained from drug licencing trials were studied (cohort 2, from the Melanoma Institute, Australia). These included patients who received combination ipilimumab and nivolumab therapy and developed colitis (IN-COL; $n = 9$), and those who received combination ipilimumab and nivolumab who did not develop colitis nor (in order to prevent confounding) any autoimmune adverse events to date according to clinical record review (IN-NAE; $n = 11$). IN-COL PBMC were studied at baseline (i.e. prior to IN treatment) and at the time of colitis. We included blood samples taken up to 7 days preceding diagnostic endoscopy. IN-NAE were studied at baseline and weeks 7–10 of treatment

(TX; a comparable time-point to time of colitis onset in the IN-COL group). These samples were compared with PBMC from patients with active UC ($n = 6$) at a single time-point, where disease activity was determined by clinical assessment in those with an established diagnosis and by endoscopy and histopathological assessment for new cases. Healthy laboratory staff volunteers ($n = 17$) were studied at a single time-point as controls. Written informed consent was obtained from each subject according to local institutional policy [Human Research Ethics Committee protocols from Royal Prince Alfred Hospital Protocol X15-0454 and HREC/11/RPAH/444, St Vincent's Hospital, Sydney HREC/13/SVH/145, Westmead Hospital HREC/14/WMEAD/324(4081), Oxford Centre for Histopathology Research (16/A019 and18/A064) and Oxford GI Biobank (REC16/YH/0247), Oxford, UK and Liverpool Hospital, Liverpool, UK (12/NW/0525)].

PBMCs from 26 additional patients with melanoma receiving combination ipilimumab and nivolumab therapy were studied at therapeutic baseline in Oxford and Liverpool (cohort 3). These included 14 patients who went on to develop endoscopically confirmed IN-COL and 12 patients in whom nil adverse events were determined, i.e. any grade of irAE through clinical record review (IN-NAE). Patients who suffered a non-colitis irAE were excluded from the study from the outset due to concerns regarding potential confounding. These patients also provided written informed consent in line with local procedure (Oxford Centre for Histopathology Research 16/A019 and18/A064, Liverpool Hospital 12/NW/0525). The baseline MAIT cell count was compared to that found in 35 community blood donors.

Isolation of mononuclear cells from gastrointestinal tissue

Endoscopically obtained pinch biopsies were collected from cohort 1 patients into Roswell Park Memorial Institute (RPMI) media containing penicillin and streptomycin and 10% fetal calf serum. Biopsies underwent enzymatic and mechanical digestion by being transferred into 5 ml of warmed RPMI with penicillin and streptomycin/10% fetal calf serum with 1 mg/ml collagenase D (Sigma-Aldrich, St Louis, MO, USA) and 100 µg/ml DNAse I (ThermoFisher, Waltham, MA, USA) and shaken at 37°C for 1 h. Biopsies were then dissociated by vigorous agitation using a gentle MACS Dissociator (Miltenyi Biotech, Bergisch Gladbach, Germany) and strained through a 70 µm strainer. Freshly isolated GMNC were analysed.

Flow cytometry

For cohort 1 GMNC and cohort 3 thawed PBMC, the cellular subset analysis and MAIT cell count was performed using a near infra-red live/dead stain (Invitrogen,

Carlsbad, CA, USA) and the following monoclonal antibodies (mAb): CD3 phycoerythrin (PE)-CF594(UCHT1), CD4-BV650(SK3), CD8-AF700(SK1), CD38-fluorescein isothiocyanate (FITC)(HIT2), CD161-PE(HP-3G10), CCR6-BV421(G034E3), FoxP3(236A) (all BD Biosciences, San Jose, CA, USA), CD45RO-BV510(UCHL1), granzyme-B-BV421(QA1828), HLA-DR-BV711(L243) (Biolegend, San Diego, CA, USA) and TCR Vα7·2-PE-VIO770(REA175) (Miltenyi Biotec). Granzyme B and FoxP3 staining was performed using an intracellular and intranuclear permeabilization kit (ThermoFisher). Isotype control stains were performed using mouse immunoglobulin (Ig)G2aκ, IgG1λ (BD Biosciences), IgG2b (Biolegend), IgG2aκ and REA control S antibody (Miltenyi Biotec).

For cohort 2, leucocyte subsets were identified on thawed PBMC using multiparameter flow cytometry, a near infra-red live/dead stain (Invitrogen) and the following mAb: CD3-peridinin chlorophyll cyanin 5.5 (PerCPCy5.5)(UCHT1), CD4-BUV496(SK3), CD8-BUV-395(G42-8), CD19-BV785 (SJ25C1), CD25-PE-Cy5(M-A251), CD27-allophycocyanin (APC)-R700(M-T271), CD27-PE-CF594(M-T271), CD38-PECy7(HIT2), CD45RA-BUV-737(HI100), CD56-APC (VNK75), CD127-BV785(HIL-7R-M21), CD161-APC(DX12), HLA-DR-BV711(G46-6), HLA-DR-FITC(G46-6), IgD-BV421(IA6-2), IgM-PE(G20-127) (BD Biosciences), IgA-APC(IS11-8E10), CD161-APC(191B8) (both Miltenyi Biotec), CD45-AF700(HI30) and CCR6-BV421(29-2L17) (Biolegend).

Flow cytometry was performed on a three-laser LSR Fortessa X-20 in the United Kingdom and a five-laser LSR Fortessa X-20 in Sydney (BD Biosciences) using FACSDIVA version 8.0.1 software (BD Biosciences). Compensation was calculated before each experiment. Approximately 150 000 lymphocytes (50 000 live T cells) were analysed per colon biopsies and 30 000 lymphocytes (10 000–15 000 live T cells) were analysed per PBMC sample. Gating strategies for both peripheral blood and gut-derived mononuclear cells are shown in Supporting information, Fig. S1. Lymphocyte populations are reported as a proportion of parent populations. Specifically, live CD3$^+$ T cells are reported as a percentage of total live lymphocytes (as defined by side-scatter *versus* forward-scatter, CD45$^+$ and exclusion of viability dye). MAIT cells are reported as a percentage of live T cells. Plasmablasts are reported as a percentage of live CD19$^+$ lymphocytes. CD4$^+$ and CD8$^+$ T cells are reported as a percentage of live T cells. Activated (HLA-DR$^+$CD38$^+$) cells are reported as percentage of memory CD45RO$^+$CD4$^+$ or CD45RO$^+$CD8$^+$ T cells. T$_{reg}$ are reported as a percentage of CD4$^+$ T cells.

MAIT cell gating strategy validation, risk factor study and MR1 tetramer staining

We validated the gating strategy of CD3$^+$CD8$^+$CD161$^+$CCR6$^+$ live lymphocytes used in cohort 2 as a comparable measurement of MAIT cells to the 'gold-standard' measurement of CD3$^+$CD8$^+$CD161$^+$TCR Vα7·2$^+$ live lymphocytes in cohort 3 patients with melanoma at baseline and 35 healthy blood donor controls.

Activation of gut-isolated MAIT cells was confirmed using human MR1/5-OP-RU tetramer provided by the US National Institute of Health Tetramer Facility (Emory University, Atlanta, GA, USA). Tetramers were generated using BV421-streptavidin (Biolegend), according to the NIH Tetramer Facility instructions. Tetramer staining was performed using a 1/200 dilution in cellular suspension at room temperature for 40 min prior to washing with phosphate-buffered saline (PBS) + 0·05% bovine serum albumin (BSA) + 1% ethylenediamine tetraacetic acid (EDTA) and proceeding with extracellular antibody staining.

Statistics

Differences between groups were determined using the unpaired non-parametric Mann–Whitney test. IN-COL samples at the point of colitis were compared with IN-COL at baseline, IN-NAE on 7–10 weeks treatment were compared with IN-NAE at baseline and UC flare was compared with healthy volunteers and IN-COL patients at the point of colitis. Categorical data were compared by the non-parametric χ^2 test. Correlation analysis was performed using the non-parametric Spearman's test. All were performed using SPSS software (IBM, Armonk, NY, USA). Medians and interquartile ranges are reported throughout. A *P*-value < 0·05 was considered statistically significant; where multiple comparisons were performed, a Bonferroni correction was made as detailed in individual figure legends.

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Patient characteristics

The clinical characteristic of cohort 1, from which gut-derived mononuclear cells were studied, are shown in Table 1). There were no significant differences between IN-COL and IN-NAE groups in regard to age, sex, melanoma stage, presence of visceral metastasis or serum LDH. Cohort 1 IN-C patients were managed with intravenous methylprednisone (IVMP; 33%), mycophenolate mofetil 500–1000 mg twice daily (17%), infliximab 5 mg/kg (33%) or vedolizumab 300 mg at weeks 0, 2 and 6 (17%). The clinical characteristics of patients with UC in cohort 1 are shown in Table 2). These patients had a median disease duration of 4 years and had been treated with mesalazine (three of six), azathioprine (one of six), infliximab (one of six) and adalimumab (one of six). The clinical, endoscopic and histopathological features

of cohort 1 IN-NAE, IN-COL and UC are shown in Table 3). Compared with the IN-COL group, UC patients were significantly younger (36 *versus* 66 years) and had a longer duration since the initial colitis diagnosis (48 *versus* 1 month). There were no significant differences between IN-COL and UC groups in disease severity score using the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) on the Nancy histological score (Table 3).

Clinical characteristics of patients in cohort 2, from which PBMC were studied, are shown in Table 4). All cohort 2 patients with colitis had high-grade disease confirmed by flexible sigmoidoscopy or colonoscopy and histopathology. The range of histopathological findings are given in Table 4). There was no significant difference between melanoma patient groups in terms of age, sex, melanoma stage or serum lactate dehydrogenase (LDH) level. The IN-COL colitis sample time-point did not differ significantly in terms of time (days) from the IN-NAE weeks 7–10 TX sample time-point. IVMP 1 mg/kg for 1–3 days was used to manage IN-COL in 78% of patients and infliximab (IFX) 5 mg/kg given once was used in

78% of IN-COL patients. The tumour response to ICIs was comparable between groups (IN-COL: progressive disease = 4, stable disease = 0; partial response = 1; complete response = 4 and IN-NAE: progressive disease = 3; stable disease = 0; partial response = 3; complete response = 5). The clinical characteristics of patients with UC in cohort 2 are shown in Table 5). These patients had a disease duration of 5 years and had been treated with mesalazine (five of six), azathioprine (five of six), prednisone (four of six) and/or infliximab (three of six). All patients with UC were in a state of disease flare at the time of blood sampling as determined by clinical features and/or endoscopy and histopathology.

The clinical characteristics of patients in cohort 3 are shown in Table 6. Patients in the IN-COL and IN-NAE groups had no significant differences in age, sex, melanoma stage, proportion with visceral metastasis or serum LDH. There were no differences in IN-COL group patient factors between cohorts 2 and 3. With regard to the IN-NAE patients, cohort 3 had earlier stage disease compared with cohort 2 ($P < 0.05$).
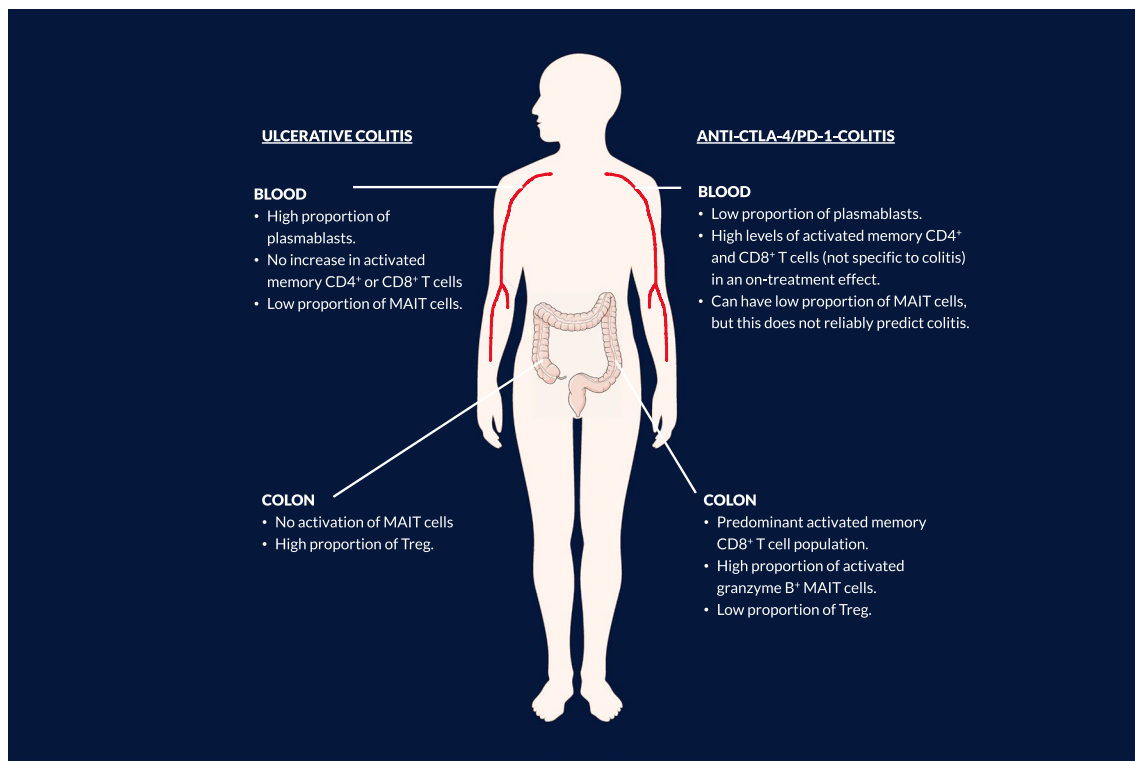

Fig. 1. Visual abstract. Peripheral blood and gut-infiltrating lymphocyte profiles are distinct between patients with anti-cytotoxic T lymphocyte antigen 4/programmed cell death 1 (CTLA-4/PD-1)-associated colitis [ipilimumab and nivolumab associated colitis (IN-COL)] and ulcerative colitis (UC). In gut-isolated lymphocytes, IN-COL is characterized by high proportions of T cells that are predominantly CD8^{+} and high levels of activated granzyme B^{+} mucosal-associated invariant T (MAIT) cells and a low proportion of regulatory T cells (T_{reg}). This is distinct from UC, where there is a lower level of CD8^{+} T cell activation, no activation of MAIT cells and a high proportion of T_{reg}. In the peripheral blood IN-COL is associated with widespread activation of CD4^{+} and CD8^{+} T cells in an 'on-treatment' effect not seen in UC. Both UC and IN-COL are associated with low proportions of MAIT cells; however, this was not a reliable predictor of IN-COL in a second cohort. UC, but not IN-COL, is associated with high levels of circulating plasmablasts.

Table 1. Clinical characteristics of cohort 1 IN-COL and IN-NAE groups

Cohort 1	Combination ipilimumab and nivolumab-related colitis (IN-COL)	Combination ipilimumab and nivolumab treated with nil adverse events (IN-NAE)	*P*-value
Number (*n*)	6	6	n.a.
Age, median years (interquartile range)	66 (56–70)	56 (41–63)	0·22
Sex (*n*, % male)	4 (67)	2 (33)	0·25
Melanoma stage (*n*)			0·51
IIIB	1	2	
IIIC	1	0	
IV	4	4	
Visceral metastases (*n*, %)	5 (83)	5 (83)	> 0·99
Serum lactate dehydrogenase (LDH) median U/l (interquartile range)	284 (214–346)	235 (203–443)	0·70
Duration of IN-C (months)	1 (1-2)	n.a.	n.a.
Colitis treatment (*n*)		n.a.	n.a.
i.v. Methylprednisone	2		
Mycophenolate mofetil	1		
Infliximab	2		
Vedolizumab	1		
Colectomy	0		

Table 2. Clinical characteristics of cohort 1 healthy volunteers and UC groups

Cohort 1	Healthy volunteers	Ulcerative colitis	*P*-value
Number (*n*)	6	6	n.a.
Age, median years (interquartile range)	67 (50–78)	36 (25–46)	0·09
Sex (*n*, % male)	2 (33)	3 (50)	0.56
Duration of ulcerative colitis (median years and interquartile range)	n.a.	4 (3–7)	n.a.
Treatment to date (*n*)	n.a.		n.a.
Mesalazine		3	
Azathioprine		1	
Methotrexate		0	
Infliximab		1	
Adalimumab		1	

A high proportion of activated memory CD8$^+$ T cells and paucity of T$_{reg}$ characterize the gastrointestinal tissue of patients with IN-COL

We first sought to understand the pathogenesis of IN-COL by direct study of the affected tissue. Patients with IN-COL had a T cell lymphocytosis (94% of lymphocytes) compared with those in the IN-NAE group [63%; $P < 0·01$; Fig. 2a(i)]. This could not be explained simply by a rise in total CD4$^+$ or CD8$^+$ T cells [Fig. 2a(iii) and (v)]. IN-treated patients had an inverted CD4 : CD8 ratio in the gut tissue (i.e. a predominance of CD8$^+$ T cells), which was distinct from both healthy volunteer gut and tissue from patients with UC [Fig. 2a(iii) and (v)]. However, perturbations in the CD4 : CD8 ratio did not differentiate between the IN-COL and IN-NAE groups. The proportion of activated memory CD4$^+$ T cells was significantly elevated in GMNC of patients with IN-COL (22% of CD4$^+$ T cells) compared with the IN-NAE group [1%; $P < 0·01$; Fig. 2a(iv)]. The proportion of activated memory CD8$^+$ T cells was significantly elevated in GMNC of patients

with IN-COL (52% of CD8$^+$ T cells) compared with the IN-NAE group [3%; $P < 0·01$; Fig. 2a(vi)] and also with the UC group [10%; $P < 0·01$; Fig. 2a(vii)]. The proportion of activated memory CD8$^+$ T cells was numerically greater than the proportion of activated memory CD4$^+$ T cells. The proportion of activated memory CD8$^+$ T cells was elevated in the GMNC of patients with UC (10% of CD8$^+$ T cells) compared with healthy volunteers [1%; $P < 0·01$; Fig. 2a(vi)]. Patients with UC had an elevated proportion of T$_{reg}$ (CD4$^+$25$^+$FoxP3$^+$) compared with healthy volunteers [6% of CD4$^+$ T cells compared with 1% $P < 0·01$; Fig. 2a(ii) and b] and with IN-COL patients [3% $P < 0·01$; Fig. 2a(ii) and 2b]. Patients with IN-COL did not have an elevated proportion of T$_{reg}$ compared with IN-NAE [3% compared to 1%; Fig. 2a(ii)].

High levels of MAIT cell activation are evident in the gastrointestinal tissue of patients with IN-COL

There was no significant difference between IN-COL and IN-NAE groups with regard to the MAIT cell count in

Table 3. Comparison of cohort 3 IN-NAE, IN-COL and UC clinical, endoscopic and histopathological findings

Cohort 1	Combination ipilimumab and nivolumab with nil adverse events (IN-NAE)	Combination ipilimumab and nivolumab-related colitis (IN-COL)	Ulcerative colitis (UC)
Number (*n*)	6	6	6
Age, median years (interquartile range)	55 (46–59)	66 (56–70)	36 (25–46)**
Sex (*n*, % male)	1 (17)	4 (67)	3 (50)
Duration of colitis (months)	n.a.	1 (1–2)	48 (36–84)**
Endoscopic findings (UCEIS score)			
0	6	0	
1	0	2	0
2	0	0	1
3	0	1	0
4	0	1	1
5	0	2	1
6	0	0	3
7	0	0	0
8	0	0	0
Histological findings			
Normal mucosa	6	0	0
Diffuse mild colitis	0	0	1
Lymphocytic colitis	0	1	0
Mild to moderate active UC with ulcers	0	0	1
Mild active chronic proctocolitis	0	0	2
Mild immunotherapy-related colitis	0	2	0
Moderate immunotherapy-related colitis	0	2	0
Moderate active chronic colitis	0	1	1
Severe active UC	0	0	1
Nancy score			
0	6	0	0
1	0	2	0
2	0	1	2
3	0	3	2
4	0	0	2

**P < 0·01 compared with IN-COL. UCEIS = Ulcerative Colitis Endoscopic Index of Severity.

GMNC [Fig. 2c(i)]. IN-COL was associated with a significantly higher proportion of HLA-DR$^+$CD38$^+$ activated MAIT cells (20% of MAIT cells) compared with the IN-NAE group [2%; $P > 0.01$; Fig. 2c(ii)]. IN-COL was also associated with a greater proportion of granzyme B$^+$ MAIT cells [19 *versus* 0% of MAIT cells; $P < 0.05$; Fig. 2c(iii)]. These changes did not occur in UC. We further confirmed activation of MAIT cells in the tissue using fluorescently conjugated tetramers to the MAIT TCR ligand (MR1-OP5) and confirmed that CD161$^+$tetramer$^+$ T cells were activated in IN-COL (19% HLA-DR$^+$CD38$^+$), but not IN-NAE (0·5%; Fig. 2d).

Finally, we confirmed that MAIT cell activation was greater in the gastrointestinal tract than in the peripheral blood by studying paired GMNC and PBMC in IN-COL ($n = 3$) and IN-NAE ($n = 3$). While patients with IN-COL had marginally higher MAIT cell activation measured in PBMCs compared with the IN-NAE group, this difference was more marked in the GMNC samples of two-thirds of patients (Fig. 2e).

Combination ipilimumab and nivolumab therapy induces a rise in circulating activated memory CD8$^+$ and CD4$^+$ T cells and activated memory gut-homing CD8$^+$ T cells

We sought to determine if changes in the PBMC compartment could be used as biomarkers for the changes seen in gastrointestinal tissue-derived lymphocytes in IN-COL. Treatment with combination ipilimumab and nivolumab therapy was associated with a rise in activated memory (CD45RA$^-$HLA-DR$^+$CD38$^+$) CD8$^+$T cells, regardless of the presence of colitis. In the IN-COL group the proportion of these cells were significantly elevated at the point of colitis (11% of memory CD8$^+$ T cells) compared with baseline (0%; $P < 0.01$; Fig. 3a,b). There was also a significant rise in the proportion of activated memory CD8$^+$ T cells in the IN-NAE group following therapy (8% of memory CD8$^+$ T cells at weeks 7–10 compared with 1% at baseline; $P < 0.01$; Fig. 3a). The proportion of these cells did not differ between patients with active UC and healthy volunteers.

Table 4. Clinical characteristics of cohort 2 IN-COL and IN-NAE groups

Cohort 2	Combination ipilimumab and nivolumab-related colitis (IN-COL)	Combination ipilimumab and nivolumab treated with nil adverse events (IN-NAE)	*P*-value
Number (*n*)	9	11	N.A.
Age, median years (interquartile range)	64 (53–67)	62 (55–70)	0·42
Sex (*n*, % male)	6(67)	6 (55)	0·69
Melanoma stage (AJCC version 8; *n*)			0·53
M1a	1	0	
M1b	3	2	
M1c	3	5	
M1d	2	4	
Visceral metastases (%)	5(56)	10 (91)	0·07
Serum lactate dehydrogenase (LDH) median U/l (interquartile range)	227 (231–327)	251 (160–426)	1·00
IN-C 'colitis' time-point or IN-NAE TX time-point days, median (interquartile range)	37 (19–46)	42 (21–70)	0·17
Colitis grade (*n*)			
1	0		
2	0		
3	8		
4	1	n.a.	n.a.
Colitis treatment (*n*, %)		n.a.	n.a.
i.v. Methylprednisone	7 (78)		
Infliximab	7 (78)		
Vedolizumab	0		
Colectomy	0		
Histopathological features		n.a.	n.a.
Diffuse active colitis	1		
Granulomas present	2		
Mild inflammation	1		
Mild non-specific acute colitis	1		
Mild to moderate acute colitis	1		
Moderate to marked acute colitis	1		
Severe acute proctocolitis	1		
Mixed acute and chronic colitis	1		
Chronic colitis	1		

AJCC = American Joint Committee on Cancer; TX = treatment.

Table 5. Clinical characteristics of cohort 2 UC group

Cohort 2	Ulcerative colitis (UC)
Number (*n*)	6
Age, median years (interquartile range)	46 (25–53)
Sex (*n*, % male)	2 (33)
Duration of ulcerative colitis (years)	5 (2–8)
Treatment to date (*n*)	
Mesalazine	5
Azathioprine	5
Methotrexate	1
Prednisone	4
Infliximab	3
Active disease at time of blood sample (*n*, %)*	6 (100)

*According to clinical (six of six), endoscopic (three of six) and/or histopathological (three of six) criteria.

Similarly, combination ipilimumab and nivolumab therapy was also associated with a rise in activated memory (CD45RA−HLA-DR+CD38+) CD4+ T cells, regardless of the presence of colitis. The IN-COL group had 4% of activated memory CD4+ T cells compared with 0% at baseline ($P < 0.01$; Fig. 3c,d). Patients in the IN-NAE group also displayed an increased proportion of activated memory CD4+ T cells following therapy (5% at weeks 7–10 compared with 1% at baseline; $P < 0.01$; Fig. 3c). The proportion of these cells did not differ between patients with active UC and healthy volunteers.

Absence of non-specific background staining for HLA-DR and CD38 was demonstrated using isotype control antibodies (Supporting information, Fig. S2a).

Table 6. Clinical characteristics of cohort 3 IN-COL and IN-NAE groups

Cohort 3	Combination ipilimumab and nivolumab -related colitis (IN-COL)	Combination ipilimumab and nivolumab treated with nil adverse events (IN-NAE)	P-value
Number (n)	14	12	n.a.
Age, median years (inter-quartile range)	62 (47–70)	59 (57–69)	0·59
Sex (n, % male)	11 (71)	4 (44)	0·38
Melanoma stage (AJCC version 8; n)		*	0·11
M1a	4	5	
M1b	2	3	
M1c	8	1	
M1d	0	0	
(Data not recorded)	(0)	(3)	
Visceral metastases (%)	75	50	0·33
Serum lactate dehydrogenase (LDH) median U/l	283 (209–618)	329 (272–351)	0·77
(interquartile range)			
Colitis treatment (n, %)		n.a.	n.a.
i.v. Methylprednisone	7 (50)		
Infliximab	6 (43)		
Vedolizumab	0		
Colectomy	0		
Histopathological features		n.a.	n.a.
Mild inflammation	4		
Focal acute cryptitis	2		
Moderate colitis	1		
Moderate to severe inflammation	1		
Focal active proctitis	1		
Chronic colitis	1		
(Data not available)	(4)		

AJCC = American Joint Committee on Cancer.

Cohort 2 IN-COL patients had lower circulating MAIT cells at baseline compared with IN-NAE

Prior to therapy, cohort 2 patients who subsequently developed IN-COL had significantly lower circulating MAIT cells at baseline (0·0% of T cells) than those in the IN-NAE group (1·4%, $P < 0·02$; Fig. 4a,b). This was also the case while on treatment, as IN-COL at the time of colitis (0·0%) had lower counts than those in the IN-NAE group at weeks 7–10 of TX (0·7%; see Supporting information, Table S1). Patients with active UC had lower proportions of circulating MAIT cells (0·3%) compared with healthy volunteers (1·0%; $P < 0·02$; Fig. 4a). Absence of non-specific background staining for CD161 and CCR6 was demonstrated using isotype control antibodies (see Supporting information, Fig. S2bi).

There were no differences in the proportion of circulating CD25^{high}CD127^{low} T$_{reg}$ or CD45RA$^-$ memory T$_{reg}$ between those with IN-COL and IN-NAE, nor those with UC or healthy volunteers (see Supporting information, Table S1).

The PBMC compartment in patients with IN-COL is distinct from that of patients with active UC

We further explored if changes in the PBMC compartment were similar between patients with IN-COL at the time of colitis and those with active UC. Compared with healthy volunteers, patients with active UC had a high proportion of CD19^{dim}CD27$^+$38^{high} circulating plasmablasts (19% of B cells compared with 0%; $P < 0·01$) that was not evident in patients with IN-COL (Fig. 5a,b); 55% of the circulating plasmablasts in patients with UC flare were IgA$^+$ compared with 25% in healthy volunteers ($P = 0·06$). Additional differences in the PBMC compartment between patients with active UC and healthy volunteers are shown in Supporting information, Table S1.

Baseline circulating MAIT cell count and colitis in cohort 3

We sought to determine if baseline MAIT cell proportion could be used as a peripheral biomarker to predict IN-COL in a second distinct cohort in a blinded manner. Our validation of gating analysis using cohort 3 found a high correlation between the two results ($R^2 = 0·989$; $P = 1·3 \times 10^{-72}$; Supporting information, Fig. S3a,b). Absence of non-specific background staining for CD161 and TCR Vα7·2 was demonstrated in additional experiments using isotype control antibodies (see Supporting information, Fig. S2bii). We confirmed in cohort 3 that melanoma patients who develop IN-COL ($n = 14$) have a lower proportion of circulating MAIT cells compared with healthy volunteers (Fig. 4c). In contrast to cohort 2, where baseline MAIT cells was a useful discriminator
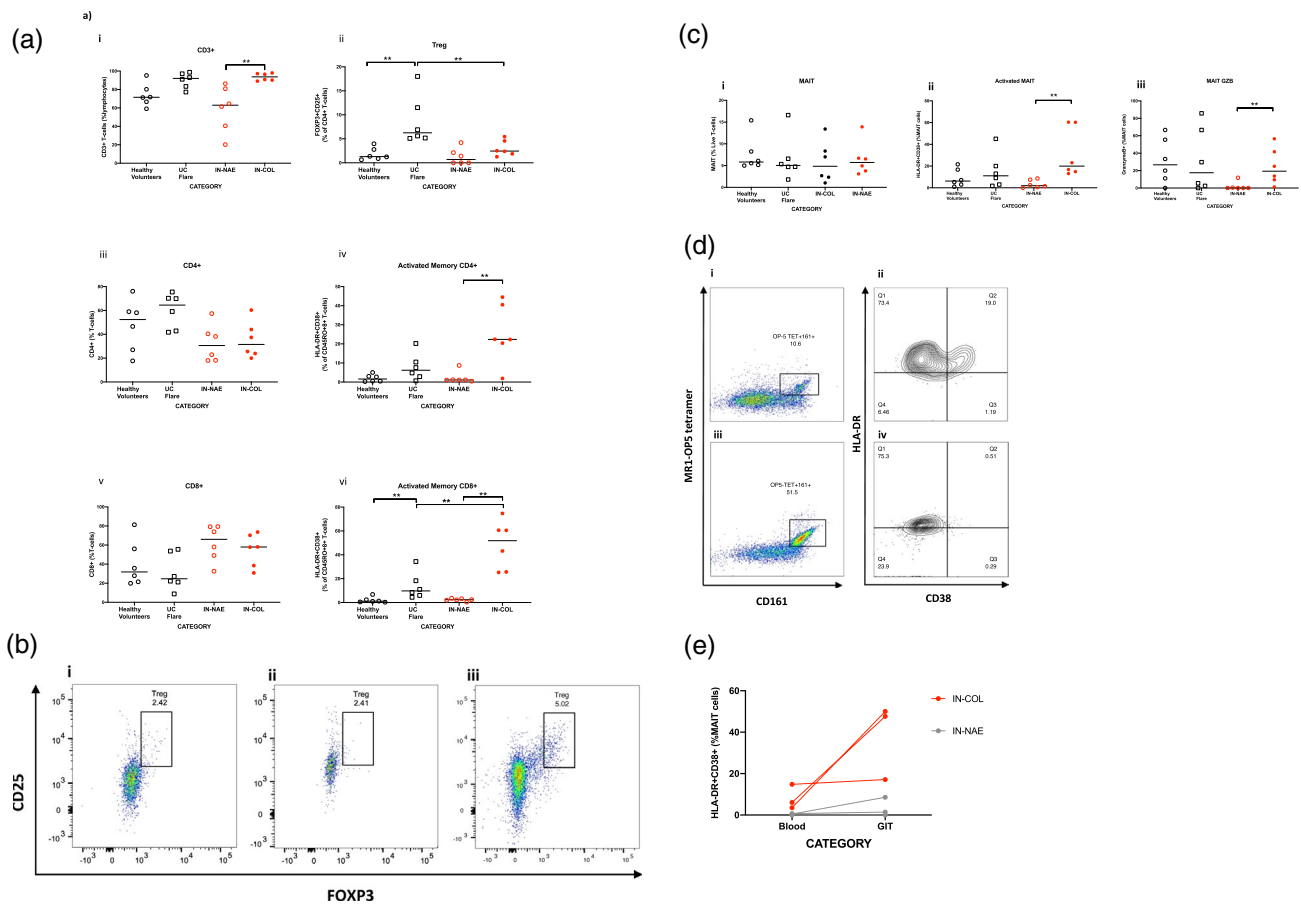
Fig. 2. High levels of activated memory CD8$^+$ T cells and mucosal-associated invariant (MAIT) T cells and low proportions of regulatory T cells (T$_{reg}$) characterize the gastrointestinal tissue of cohort 1 patients with ipilimumab and nivolumab therapy-related colitis (IN-COL). Data from freshly isolated gut mononuclear cells from healthy volunteers (*n* = 6) and patients with active ulcerative colitis (UC; *n* = 6), those who received combination ipilimumab and nivolumab therapy with nil autoimmune adverse events (IN-NAE; *n* = 6) and those with combination ipilimumab and nivolumab therapy-associated colitis (IN-COL; *n* = 6), is shown. Median and interquartile range are displayed. TX = treatment: *P < 0·02 and **P < 0·01 by Mann–Whitney test. *P*-values < 0·02 were considered significant due to Bonferroni correction for multiple comparisons. (a) Compared with IN-NAE, patients in the IN-COL group had (i) a T cell lymphocytosis that was not explained by perturbations in the total (iii) CD4$^+$ or (v) CD8$^+$ subset. Patients with IN-COL had elevated (iv) activated memory CD4$^+$ T cells and a greater proportion of (vi) activated memory CD8$^+$ T cells compared with IN-NAE. The proportion of activated memory CD8$^+$ T cells was greater than was seen in UC. (b) Compared with (i) healthy volunteers, patients with (iii) UC had a high proportion of forkhead box protein 3 (FoxP3)$^+$CD25$^+$ regulatory T cells (T$_{reg}$) (gated) while a low proportion of T$_{reg}$ were seen in (ii) IN-COL. (c) Compared with the IN-NAE groups, patients with IN-COL had (i) no change in total MAIT cell proportion but (ii) a rise in activated and (iii) granzyme B$^+$ MAIT cells. These changes were not seen in active UC. (d) Activation of MAIT cells in the gastrointestinal tissue was confirmed using a cell-specific MR1-OP5 tetramer that binds specifically to the MAIT T cell receptor (TCR). Data from thawed frozen gut-derived mononuclear cells are shown from a patient with IN-COL (i,ii) and IN-NAE (iii,iv) and gated on live CD3$^+$ single cells. MR1-OP5 tetramer$^+$CD161$^+$ T cells in ii IN-COL displayed higher levels of activation compared with those in (iv) IN-NAE. (e) Paired data from thawed peripheral blood mononuclear cells (PBMC) and freshly isolated gut mononuclear cells from patients with combination ipilimumab and nivolumab therapy-associated colitis (IN-COL in red; *n* = 3) and those who received combination ipilimumab and nivolumab therapy with nil autoimmune adverse events (IN-NAE in grey; *n* = 3) is shown. Higher levels of MAIT cell activation was found in the gut tissue compared with PBMC in two-thirds of IN-COL patients.

between the IN-COL and IN-NAE groups, in cohort 3 the IN-NAE patients also had a lower proportion of circulating MAIT cells at baseline compared to healthy volunteers, and there was no significant difference between IN-COL and IN-NAE MAIT cell proportions (Fig. 4c).

Discussion

The pathogeneses of ICI toxicities are poorly understood, with few known risk factors, biomarkers [4,39,40] or tailored therapies. As a result, clinicians cannot identify those at greatest risk or intervene to alter therapy before morbidity occurs.

For ICI-related colitis, diagnostic and treatment algorithms are being derived from classical forms of IBD and have not been completely validated in this new clinical setting.

Here we applied multiparameter flow cytometry to the study of gut-infiltrating and circulating lymphocytes from melanoma patients treated with combination ipilimumab and nivolumab therapy in the hope of finding novel insights into disease pathogenesis.

We began by directly investigating the profile of gut-infiltrating lymphocytes in affected patients (cohort 1). Here,

(a)
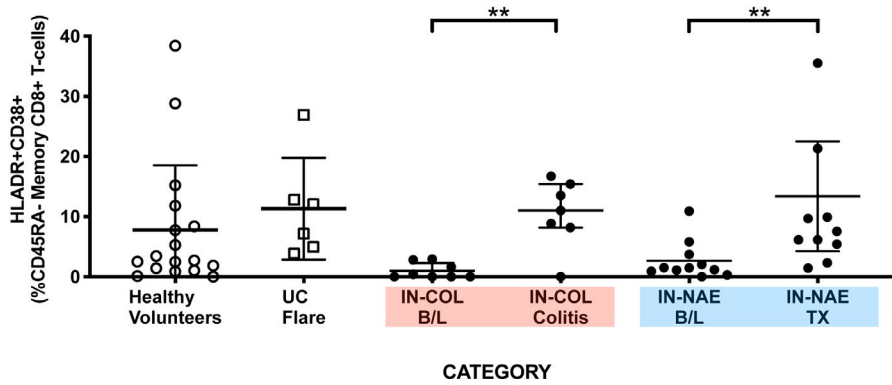
(b)
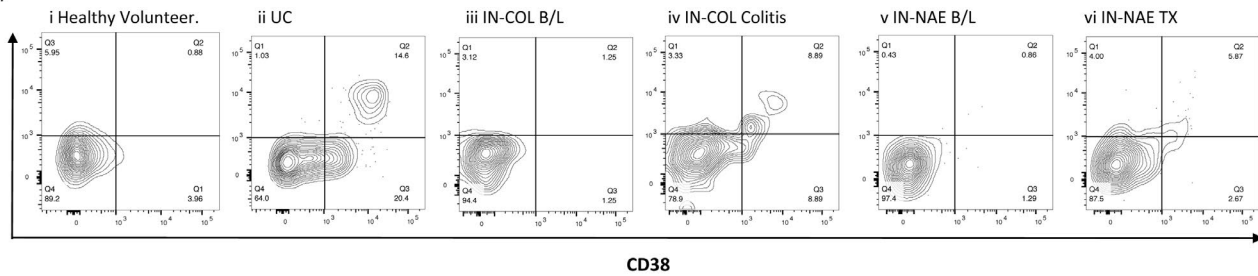
(c)
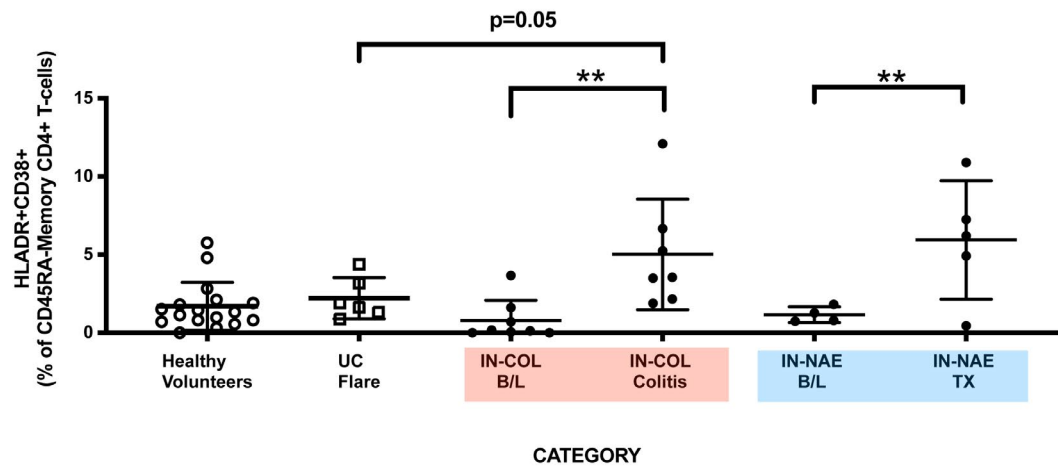
(d)
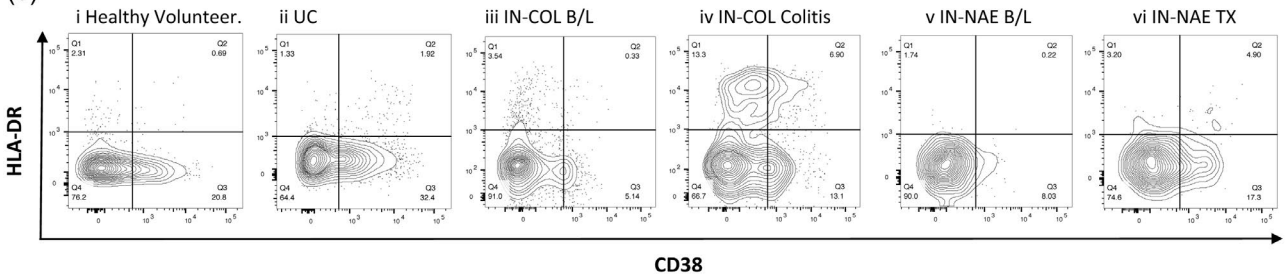
Fig. 3. In cohort 2, treatment with combination ipilimumab and nivolumab therapy resulted in high levels of activated memory CD4$^+$ and CD8$^+$ T cells, independent of the presence of colitis. Data from thawed peripheral blood mononuclear cells (PBMC) from healthy volunteers ($n = 17$) and patients with combination ipilimumab and nivolumab therapy-associated colitis (IN-COL shaded red on graphs; $n = 9$), active ulcerative colitis, (UC $n = 6$) and those who received combination ipilimumab and nivolumab therapy with nil autoimmune adverse events (IN-NAE shaded blue on graphs; $n = 11$) is shown. Median and interquartile range are displayed. B/L = baseline, TX = on treatment for 7–10 weeks. **$P < 0.01$ by Mann–Whitney test. *P*-values < 0.02 were considered significant due to Bonferroni correction for multiple comparisons. (a) Patients in the IN-COL group had high levels of circulating activated memory CD45RA$^-$human leucocyte antigen D-related (HLA-DR)$^+$CD38$^+$ CD8$^+$ T cells at the point of active colitis compared with baseline. A significant rise in this subset was also seen in the IN-NAE groups following 7–10 weeks of therapy. (b) Gating strategy showing live, single lymphocytes that are CD3$^+$8$^+$CD45-RA$^-$. (i) Healthy volunteers; (ii) patient with active UC; (iii) patient in the IN-COL group at baseline; (iv) results from the same IN-COL patient at the time of active colitis; (v) patient in the IN-NAE group at baseline; (vi) results from the same IN-NAE patient following treatment. (c) At the point of colitis IN-COL patients had high proportions of circulating activated memory CD45RA$^-$HLA-DR$^+$CD38$^+$CD4$^+$ T cells. The percentage of these cells was elevated compared with baseline values. A rise in the percentage of these cells is also seen in the IN-NAE group following 7–10 weeks of treatment. (d) Gating strategy showing live, single lymphocytes that are CD45RA$^-$CD3$^+$4$^+$. (i) Healthy volunteers; (ii) patient with active UC; (iii) patient in the IN-COL group at baseline; (iv) results from the same IN-COL patient at the time of active colitis; (v) patient in the IN-NAE group at baseline; (vi) results from the same IN-NAE patient following treatment.

we found that IN-COL was characterized by a predominantly CD8$^+$ T cell lymphocytosis, with the proportion of activated memory CD8$^+$ T cells greater than observed in active UC, and supports earlier reports suggesting T cell checkpoint inhibitor-related colitis is dominated by CD8$^+$ T cells [13]. The high proportion of T$_{reg}$ evident in the gastrointestinal tissue of patients with UC was not evident in IN-COL, and we propose that this is due to T$_{reg}$ regulatory mechanisms involving a high expression of CTLA-4 [41–43]. IN-COL was associated with a higher proportion of activated and granzyme B$^+$ MAIT cells compared with IN-NAE, changes that were not found in active UC, again highlighting the distinct pathology of IN-COL. We confirmed higher rates of MAIT cell activation in IN-COL using MAIT TCR-specific tetramer staining and paired peripheral blood and gut-isolated mononuclear cell samples from the same patients.

The comparison of IN-COL and UC patients in cohort 1 (Table 3) is important. The IN-COL patients were older than UC patients and the time since first diagnosis of colitis was much shorter (1 *versus* 48 months). Despite this, the IN-C and UC cohorts scored comparably on both the UCEIS endoscopic and Nancy histopathological grading systems, two scales commonly used to score severity of UC. The higher levels of CD4$^+$ and CD8$^+$ T cell and MAIT cell activation measurable by flow cytometry in IN-COL did not correspond with higher UCEIS or Nancy scores. Therefore, the significant cellular changes found in IN-COL does not appear to be simply a function of colitis severity; however, this will require confirmation in larger studies.

We sought to understand if changes concordant with those seen in local tissue could be detected in the peripheral blood. We found therapy results in a widespread 'on-treatment' activation of memory T cells; however, these were not predictive of colitis. We additionally determined that patients with IN-COL could also have pre-existing lower circulating MAIT cells than those in the IN-NAE group, raising the possibility that a pre-existing reduction of peripheral MAIT cells might be a risk factor for IN-COL, as similarly described in other autoimmune diseases of the gastrointestinal tract [31–34,36,37]. However, this proved an unreliable predictor in cohort 3, where the IN-NAE group also had low levels of circulating MAIT cells. The differences between the IN-NAE groups in cohorts 2 and 3 could not be explained by significant differences in age, sex or serum LDH. Endoscopic evaluation was used in both cohorts to establish the diagnosis of colitis. There is a potential confounder, in that the IN-NAE patients in cohort 2 were at a higher melanoma stage compared with cohort 3.

IN-COL was not associated with high levels of circulating plasmablasts, a point of distinction from UC, possibly suggesting that there is less of a role of pathogenic B cells in IN-COL. Analysis of gut-derived mononuclear cells demonstrated that IN-COL was associated with high rates of predominantly activated memory CD8$^+$ T cells and granzyme B$^+$ MAIT cells, with a low proportion of T$_{reg}$ in a cellular pathology that was distinct from both IN-NAE and UC groups.

Our work provides further evidence that CD8$^+$ T cells are the predominant cell type in IN-COL. Given that cytotoxic CD8$^+$ T cells are thought to be the major target of ICI, it appears that their activation in IN-COL leads to off-target collateral damage. Important questions remain, including: why IN-COL occurs in some patients and not others; if there is any mechanism to predict this toxicity prior to treatment; and why steroid and infliximab-refractory cases occur. Our finding that both CD8$^+$ T cells and MAIT cells are activated in IN-COL appears to provide important information regarding the initiating antigen, and suggests an interaction with the intestinal microbiome.

MAIT cells provide an important link between microbes and cellular immunity, being directly activated by vitamin B2 metabolites generated from bacteria or indirectly from cytokines (e.g. IFN-α and TNF-α). While we are unable

to confirm that low circulating MAIT cell counts definitively correlate with subsequent risk of IN-COL, activated MAIT cells were only seen in the gut-isolated mononuclear cells of IN-COL and not IN-NAE patients. This raises important questions that centre around whether or not specific microbes are involved in MAIT cell activation in the setting of IN-COL and how MAIT cell trafficking is regulated, particularly between the blood and gut. There is literature suggesting a host's microbiome can impact upon the efficacy of ICI therapy [44–46] and the development of associated colitis [45] that is relevant to this field and that we are exploring in a prospective trial.
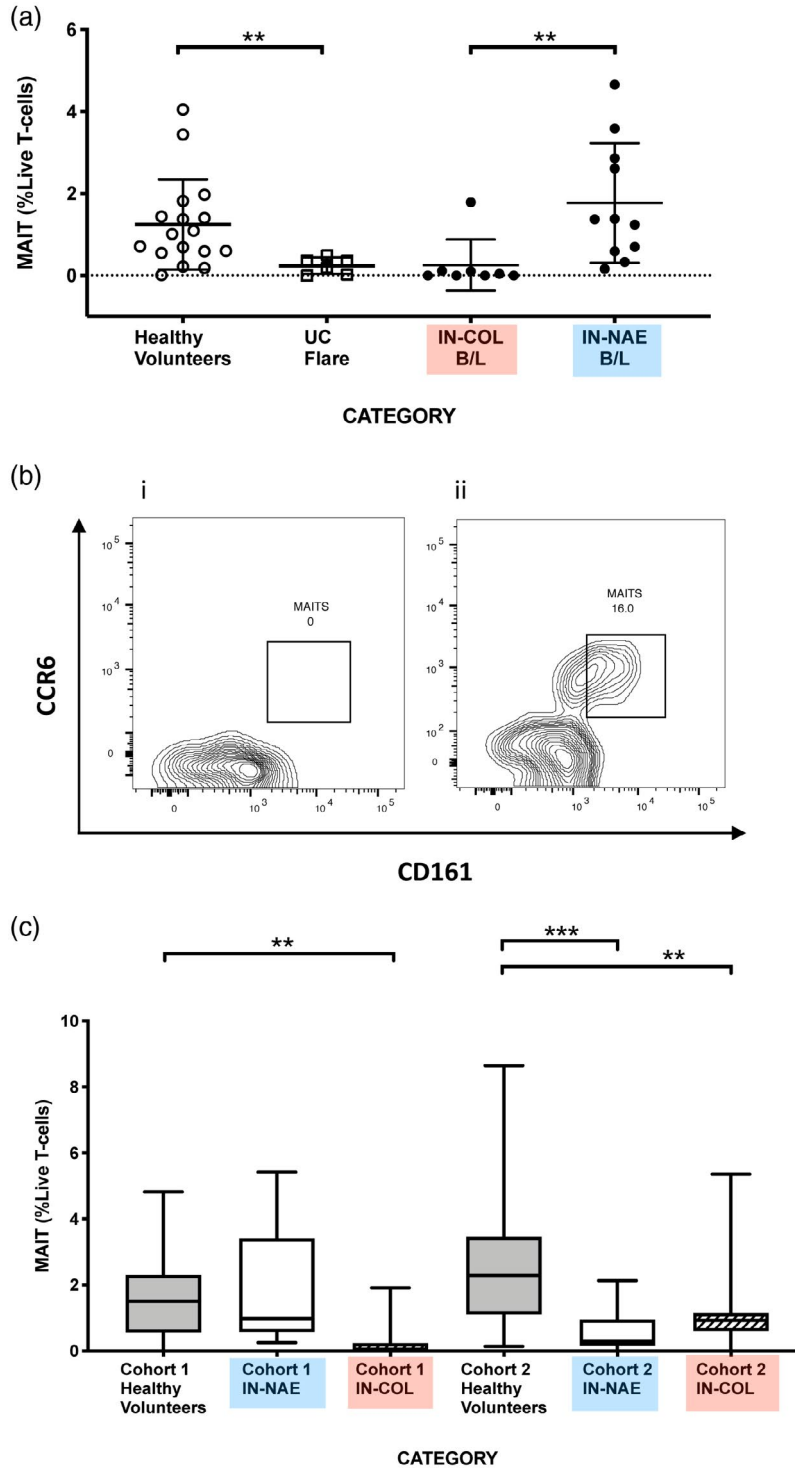
Fig. 4. Low levels of circulating mucosal-associated invariant T (MAIT) cells at baseline in cohort 2 patients who developed combination ipilimumab and nivolumab therapy-related colitis. Data from thawed peripheral blood mononuclear cells (PBMC) from healthy volunteers ($n = 17$) and patients with combination ipilimumab and nivolumab therapy-associated colitis (IN-COL shaded red; $n = 9$), active ulcerative colitis (UC, $n = 6$) and those who received combination ipilimumab and nivolumab with nil autoimmune adverse events (IN-NAE shaded blue; $n = 11$). Medians and interquartile range are shown. B/L = baseline. **$P < 0.02$ by Mann–Whitney test. *P*-values < 0.02 were considered significant due to Bonferroni correction for multiple comparisons. (a) Cohort 2 patients in the IN-COL group at baseline had a paucity of MAIT cells compared with those in the IN-NAE group at baseline. Patients with active UC flare had lower circulating MAIT cell counts compared to healthy volunteers. (b) MAIT cell gating strategy showing live, single lymphocytes that are CD38+. (i) Patient in IN-COL group at baseline; (ii) patient in IN-NAE group at baseline; (c) low circulating MAIT cells at baseline was not predictive of IN-COL in cohort 3. In cohort 2 patients with melanoma who subsequently developed dual checkpoint colitis (IN-COL shaded red; $n = 9$) had significantly lower MAIT cell proportion at baseline compared to healthy volunteers ($n = 17$). Patients with melanoma treated with ipilimumab and nivolumab who did not develop any autoimmune side effects did not display reduction in MAIT cells (IN-NAE shaded blue; $n = 11$). In cohort 3 the median baseline MAIT cell count of both melanoma patient groups IN-COL (shaded red; $n = 15$) and IN-NAE (shaded blue; $n = 9$) was significantly lower than that of healthy volunteers ($n = 35$); however, there were no further differences between the patient groups. **$P < 0.01$ and ***$P < 0.001$ by Mann–Whitney test. *P*-values < 0.01 were considered significant due to Bonferroni correction for multiple comparisons.
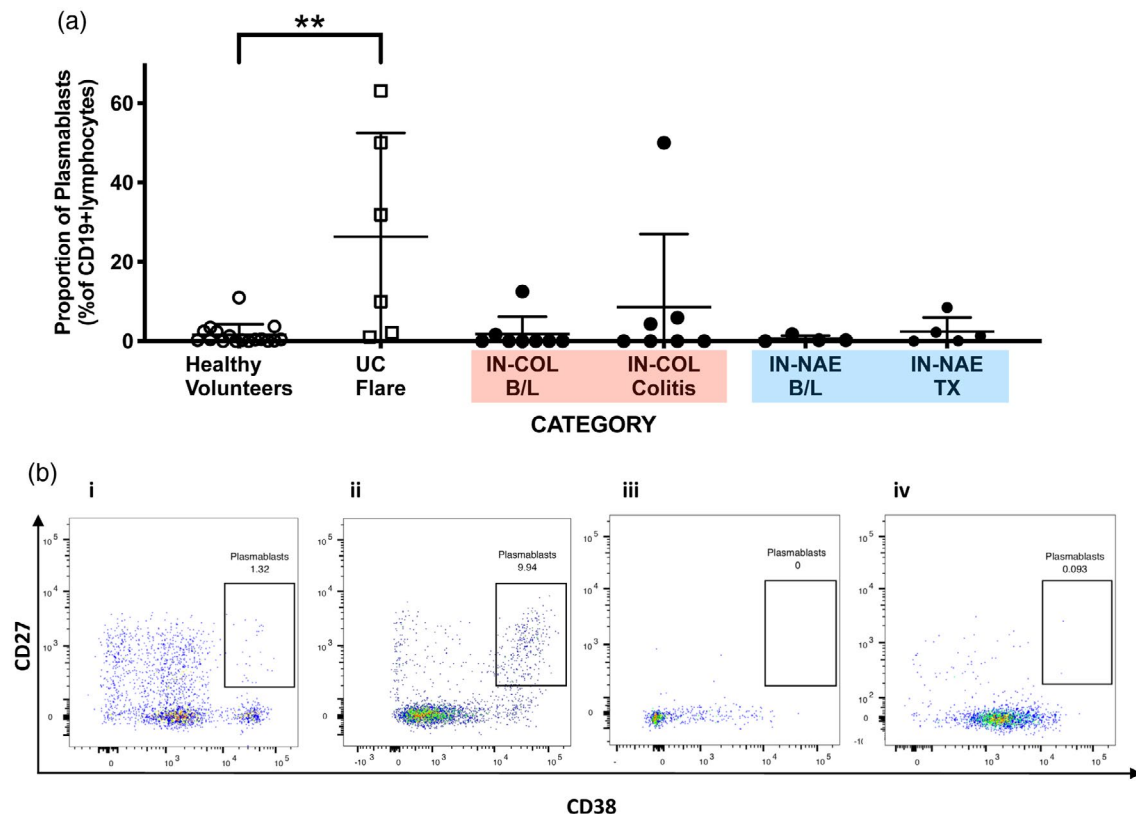

Fig. 5. Active ulcerative colitis (UC) but not combination ipilimumab and nivolumab therapy-related colitis (IN-COL) is associated with high circulating plasmablasts in cohort 2. Data from thawed peripheral blood mononuclear cells (PBMC) from healthy volunteers ($n = 17$) and patients with combination ipilimumab and nivolumab therapy-associated colitis (IN-COL shaded red; $n = 9$) active ulcerative colitis (UC, $n = 6$) and those who received combination ipilimumab and nivolumab with nil autoimmune adverse events (IN-NAE shaded blue; $n = 11$). Median and interquartile range shown. B/L = baseline, TX = on treatment for 7–10 weeks. **$P < 0.01$ by Mann–Whitney test. *P*-values < 0.02 were considered significant due to Bonferroni correction for multiple comparisons. (a) Patients with active UC had significantly elevated circulating CD19+27+38+ plasmablasts compared with healthy volunteers. This change was not seen in patients treated with combination ipilimumab and nivolumab therapy, including those with IN-COL. (b) Plasmablast gating strategy showing live, single CD19+ lymphocytes. (i) Healthy volunteers; (ii) active UC; (iii) IN-COL at time of colitis; (iv) IN-NAE at weeks 7–10.

Additionally, there are data supporting the efficacy of faecal microbiota transplant for ICI-colitis [47], suggesting that replacement of microbiome species can ameliorate disease; one mechanism of this might be through reducing the provision of species-specific bacterial-derived MAIT cell ligands.

The presence of the riboflavin operon in bacteria identifies species that can activate MAIT cells through their TCR. The activation of MAIT cells in this setting raises a compelling link between host microbiome, MAIT cells and IN-COL that warrants further investigation, especially in regard to whether these cells contribute to tissue damage. A report investigating colitis in the setting of ipilimumab monotherapy found higher levels of riboflavin (vitamin B2) in the microbiome was associated with protection from colitis [44], providing a possible link to MAIT cells (that are activated by riboflavin metabolites) as protective in ICI-associated colitis.

MAIT cells can be activated by their TCR or cytokines (IL-12, IL-15, IL-18, IFN-α/β, TNF-α) or synergistically by both. We require further work to determine which pathways are leading to the activation of MAIT cells in IN-COL and whether this activity contributes to tissue damage [48–50].

This work provides an important first report on changes in gut-derived and peripheral-blood mononuclear cells that occur in ICI-associated colitis, but our approach has several limitations. This work constitutes retrospective and cross-sectional analyses conducted in relatively small numbers of patients that requires confirmation in larger studies. We also cannot comment at this stage on whether these changes occur in anti-PD-1 monotherapy-associated colitis.

We are currently recruiting to a prospective single-centre study of oncology patients who have PBMC and GMNC collection, flexible sigmoidoscopy with gastrointestinal biopsy and microbiome analysis prior to commencement of ICI therapy, and at the time of subsequent development of related colitis. We aim to determine the mechanisms by which MAIT cells become activated in the gastrointestinal tract. Here we demonstrate that the levels of activated MAIT cells in the circulation are not a good indicator of the levels in the colon, and further investigation into how circulating MAIT cells relate to tissue-based populations would be of value. It is also unclear at this stage whether MAIT cell activation contributes to tissue damage or is part of a homeostatic repair mechanism. It is hoped that further understanding of the mechanisms that activate memory CD8+ T cells and MAIT cells in the gastrointestinal tract of patients with ICI-related colitis will lead to the development of improved and novel targeted therapies.

Conclusions

The gastrointestinal tissue of patients with IN-COL is characterized by high levels of activated memory CD8+ T cells and MAIT cells. These changes were distinct from the peripheral blood compartment, where CD8+ T cell activation reflected an on-treatment effect and where the circulating MAIT cell count was significantly lower than in health, but neither could predict the onset of colitis

(Fig. 1). Further work is required to determine the pathway by which CD8+ T cells and MAIT cells become activated in the gut of patients with ICI-associated colitis and whether MAIT cells contribute to tissue damage or repair. Advancements in this area have the potential to inform novel treatment strategies for this new and distinct inflammatory bowel disease.

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Disclosures

Author contributions

S. C. S. conceived the study, designed and performed experimental work, analysed the data, completed the literature review and wrote the manuscript. J. J. Z. designed the experimental work, provided expertise and supervision in flow cytometry and critically reviewed the manuscript. K. N. reviewed and collated clinical data and facilitated retrieval of stored specimens. C. M. L. M. performed the experimental work. B. P. F. cared for patients, oversaw translational research and collated clinical data. S. D. facilitated archiving of clinical samples. A. O. B. cared for patients and oversaw translational research. C. J. facilitated archiving and retrieval of clinical samples. S. A. R. facilitated

archiving and retrieval of clinical samples. G. A. cared for patients and oversaw translational research. U. P. supervised translational research. R. A. S. is the Director of the Melanoma Institute Australia Biobank and provided critical review of the manuscript. M. S. C. conceived and facilitated the collaboration between multiple sites and assisted in study design. M. J. P. cared for patients and oversaw clinical recruitment. V. T. F. C. cared for patients, facilitated translational research studies and collated clinical data. T. G. facilitated translational research studies. P. K. facilitated collaborations between groups, supervised translational research and critically reviewed the manuscript. G. V. L. cared for patients, supervised translational research and provided critical review of the manuscript. O. B. cared for patients and is the lead investigator of the gastrointestinal tissue studies. A. M. M. cared for patients, designed the study, supervised translational research and wrote the manuscript. A. D. K. designed the study, funded the experimental work, provided infrastructural support and provided critical review of the manuscript. All authors reviewed and approved the final manuscript.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Supporting Information

Additional supporting information may be found in the on-line version of this article at the publisher's web site:

Fig S1. Peripheral blood and gut-derived mononuclear cell (PBMC; GMNC) flow cytometric gating strategies. Data from a) thawed PBMC from a healthy volunteer is shown. Gating strategy for i) lymphocytes ii) singlets iii) Live T cells iv) CD4$^+$ and CD8$^+$ T cells v) CD4$^+$ naïve (Q2), effector memory (Q4), central memory (Q1) and T cells re-expressing CD45RA (Q4); Box includes all CD45RA$^-$ memory cells vi) Highly activated CD4$^+$CD45RA$^-$ cells in Q2 vii) CD8$^+$ naïve (Q2), effector memory (Q4), central memory (Q1) and T cells re-expressing CD45RA (Q4); Box includes all CD45RA$^-$ memory cells viii) Highly activated CD8$^+$CD45RA$^-$ cells in Q2. Data from b) Freshly isolated GMNC from a healthy volunteer is shown. The approach is different for GMNC given the very low proportion of CD45RA$^+$ and naïve T cells in the gut. Gating strategy for i) lymphocytes ii) singlets iii) Live T cells iv) CD4$^+$ and CD8$^+$ T cells v) CD4$^+$CD45RO$^+$vi) Highly activated CD4$^+$CD45RO$^+$ cells in Q2 vii) CD8$^+$CD45RO$^+$vi) Highly activated CD8$^+$CD45RO$^+$ cells in Q2.

Fig S2. Peripheral blood mononuclear cells (PBMC) stained with monoclonal isotype control antibodies. Data from thawed PBMC from a single healthy volunteer is shown gated on live, single T cells. (a) Isotype control histograms for HLA-DR versus CD38 in (i) CD8$^+$ and (ii) CD4$^+$ T cells. Cells are also stained for CD161, CCR6, CD45RO, CD25 as a comparator for Fig. 4b). (b) Isotype control histograms for CD161 versus (i) CCR6 and (ii) TCRVα7.2. Cells are also stained for CD4, CD25, CD45RO to act as a comparator for Fig. 2d).

Fig S3. (a) Validation of CD161$^+$CCR6$^+$ CD8$^+$ T cells as a corelate to CD161$^+$Vα7.2$^+$ CD8$^+$ mucosal associated invariant T (MAIT) cells in Cohort 2. Thawed peripheral blood mononuclear cells (PBMC) from healthy volunteers (N = 35; open circles) and patients with melanoma prior to treatment with ipilimumab and nivolumab (N = 24) were stained with markers for MAIT cells in a single flow-cytometric assay. Of the melanoma patients some subsequently developed T cell checkpoint-inhibitor-related colitis (N = 15; closed triangles) while others did not (N = 9; open triangles); p = 3.88x10^{-9} by Spearman's correlation. (b) Alternative flow cytometric gating strategies for MAIT cells. Data from thawed PBMC from single healthy volunteer is shown. As depicted, lymphocytes were gated on, live T cells, single cells, CD8$^+$ and then, gated on (i) CCR6 versus CD161 or (ii) T cell receptor chain Vα7.2. SSC = side scatter, FSC = forward scatter, NIR = near infra-red viability stain, H = height, A = area, TCR = T cell receptor.

Table S1. Characterisation of the peripheral blood mononuclear cell (PBMC) compartment in patients with combination ipilimumab and nivolumab-associated colitis. *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ compared with healthy volunteers; $^\dagger P < 0.05$ $^{\dagger\dagger}P < 0.01$ for combination ipilimumab and nivolumab-associated colitis (IN-COL) compared to baseline; $^\P P < 0.05$ $^{\P\P}P < 0.01$ for combination ipilimumab and nivolumab-treated patients with nil adverse events (IN-NAE) compared to baseline. All refer to Mann-Whitney tests with no correction.