**ARTICLE**

**Molecular Diagnostics**

**Prognostic significance of interleukin-17A-producing colorectal tumour antigen-specific T cells**

Amanda Thomson¹, Diana F. Costa Bento¹, Martin J. Scurr, Kathryn Smart¹, Michelle S. Somerville¹, Åsa V. Keita², Awen Gallimore³ and Andrew Godkin¹,³

**BACKGROUND:** The T cell cytokine profile is a key prognostic indicator of post-surgical outcome for colorectal cancer (CRC). Whilst TH1 (IFN-γ⁺) cell-mediated responses generated in CRC are well documented and are associated with improved survival, antigen-specific TH17 (IL-17A⁺) responses have not been similarly measured.

**METHODS:** We sought to determine the cytokine profile of circulating tumour antigen-(ST4/CEA) specific T cells of 34 CRC patients to address whether antigen-specific IL-17A responses were detectable and whether these were distinct to IFN-γ responses.

**RESULTS:** As with IFN-γ-producing T cells, anti-ST4/CEA TH17 responses were detectable predominantly in early stage (TNM I/II) CRC patients. Moreover, whilst IL-17A was always produced in association with IFN-γ, this release was mainly from two distinct T cell populations rather than by 'dual producing' T cells. Patients mounting both tumour-specific TH17 responses exhibited prolonged relapse-free survival.

**CONCLUSIONS:** Tumour antigen-specific TH17 responses play a beneficial role in preventing post-operative colorectal tumour recurrence.

**British Journal of Cancer** https://doi.org/10.1038/s41416-021-01283-3

---

**BACKGROUND**

The adaptive immune system plays a critical role in the control of colorectal cancer (CRC). Our previous work identified that the presence of pre-operative circulating TH1 responses specific for ST4, an oncofoetal antigen not expressed in healthy adult tissue, has a positive association with post-operative relapse-free survival,¹ in keeping with studies demonstrating that tumour infiltration of TH1-type (IFN-γ⁺) T cells associate with favourable outcome.² Unexpectedly, the presence of TH1 responses directed towards carinoembryonic antigen (CEA), an auto-antigen expressed at low levels in normal intestinal epithelium, associated with poor relapse-free survival in CRC patients.¹ Whilst both CEA and ST4 are upregulated in the majority of colorectal tumours, there is a potential that tumour-selective T cell responses impact clinical outcome differently to auto-antigen T cell responses.³

Since TH1 responses are usually associated with control of cancer progression, we postulated that CEA-specific IFN-γ-producing T cells were not the cause of the observed association with poor survival, but a surrogate marker for skewed tumour-specific T cell functionality. In particular, tumour-infiltrating TH17 cells have previously been associated with CRC progression.² IL-17A itself has been implicated in tumour progression through STAT3 activation, promoting colonic epithelial cell transformation, tumour cell proliferation and invasion.⁶ Contrarily, the presence of colorectal tumour-infiltrating TH17 cells was recently reported to correlate with improved survival,⁵ implying that IL-17A has important context and tissue-dependent roles in enacting pro- or anti-tumour immune mechanisms.⁶

This current study was designed to identify the presence of CEA-specific and ST4-specific IL-17A- and IFN-γ-producing T cells within the blood of CRC patients and healthy controls and investigate their role on post-operative outcome and colonic epithelial barrier function.

**METHODS**

**Participants**

Blood and colon samples were obtained from 34 patients undergoing colorectal cancer resection or investigative colonoscopy procedures at the University Hospital of Wales, Cardiff. Patient characteristics are summarised (Supplementary Table 1). Additional blood samples were obtained from age-matched healthy donors (age range 48–62; 5 male, 4 female). All participants gave written, informed consent personally prior to inclusion. The Wales Research Ethics Committee granted ethical approval for this study.

**Antigens**

The CEA protein was split into seventy 20mer peptides and ST4 split into forty-one 20mers, each overlapping by 10 amino acids and synthesised to >90% purity (GLBiochem, Shanghai, China). 5T4 was split into forty-one 20mers, each overlapping by 10 amino acids and synthesised to >90% purity (GLBiochem, Shanghai, China). These authors contributed equally: Amanda Thomson, Diana F. Costa Bento, Martin J. Scurr

These authors contributed equally: Awen Gallimore, Andrew Godkin

Received: 4 December 2020 Revised: 8 January 2021 Accepted: 13 January 2021

© Crown 2021 Published online: 05 March 2021
Peripheral blood mononuclear cell (PBMC) extraction and culture PBMCs were extracted from whole blood as previously described. 5 × 10⁵ PBMCs were seeded per well in a 96-well plate in 100 μl supplemented with 5% human AB serum (Welsh Blood Service, Pontyclun, UK), 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μg/ml penicillin/streptomycin (Gibco, Paisley, UK). PBMC lines were cultured for 14 days in the presence or absence of peptide pool, supplemented with 10 μl CellKine (purified T cell growth factors derived from pooled PHA-stimulated T cells; Helvetica Healthcare, Geneva, Switzerland), on day 3 and fresh media containing 40 IU/ml IL-2 on days 6 and 9.

Fluorospot assays

The human IFN-γ/IL-17A FluoroSpot kit (MabTech, Sweden) was used and performed according to the manufacturer’s instructions. Duplicate lines of cultured PBMCs were pooled together, washed and plated at 5 × 10⁴ cells/well with or without the corresponding peptide pool for direct comparison. Fluorospot plates were incubated at 37°C, 5% CO₂ for 48 h. In some instances, blocking antibodies to MHC class-I (anti-HLA-A/B/B₂7, Sigma Aldrich, UK) and MHC class-II (anti-HLA-DR (L243), anti-HLA-DQ (1A3), BioLegend) were added at a final concentration of 10 μg/ml, incubated for 30 min before addition of antigen. Cytokine-producing PBMCs were enumerated using an automated FluoroSpot reader (CTL, Germany). Dual cytokine-secreting cells were visualised and enumerated by a computerised overlay. Positive IFN-γ and IL-17A responses were identified by a minimum of 10 and 5 spot-forming cells (SFC) per well.
per $1 \times 10^5$ cultured PBMC, respectively, and a minimum 2-fold increase above background.

Intestinal permeability measurements
Electrophysiological resistance and paracellular flux in human colonic biopsies was measured using an Ussing chamber, as previously described.² Readouts of intestinal permeability were associated with the presence of IFN-γ and IL-17A CEA- and ST4-specific T cell responses, with patients differentiated based on the median magnitude of response generated to each antigen.

Statistical analysis
GraphPad Prism (v.8) was used for all statistical analyses. Dataset normality was tested using the Shapiro-Wilk test. Significance was determined using one-way ANOVA with corrections for multiple comparisons made using the Tukey test; a P-value $< 0.05$ was considered significant. For survival analyses using Kaplan–Meier curves, relapse-free survival was calculated from the date of surgery to the date of relapse, as determined by follow-up CT scan and/or endoscopy. Non-relapsing patients were censored at the time of last follow-up assessment (September 2019). Survival curves were compared using the log-rank test.

RESULTS
CEA-specific and ST4-specific IFN-γ, IL-17A and IFN-γ/IL-17A dual-secreting T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A). Whilst T cell responses from pre-operative CRC patient and age-matched healthy donor PBMC were investigated. Culturing PBMC for 14 days in the presence of a particular cytokine cocktail was necessary to expand low frequency antigen-specific T cell responses to detectable levels (Supplementary Fig. 1A).

CEA- and ST4-specific T cell responses diminished in comparison to healthy age-matched donors (Fig. 1f). No overall change in CEA-specific IL-17A production was noted between healthy donors and CRC patients (Fig. 1c). Similarly, ST4-specific T cell responses significantly declined as the colorectal tumour advanced (HD vs III, $P = 0.033$; I/II vs III, $P = 0.031$; Fig. 1d, e), validating previous findings with ST4 and other widely-expressed upregulated tumour antigens.⁹ ST4-specific T cell responses were rarely detectable in healthy donors, and only significantly increased in earlier stage CRC patients ($P = 0.036$, Fig. 1f).

The prognostic impact of pre-operative CEA and ST4-specific T cell responses was determined by associating responses with 5-year relapse-free survival. Patients were separated based on whether they mounted both an IFN-γ and IL-17A response or not to CEA (Fig. 1g) and ST4 (Fig. 1h); one patient that mounted an anti-ST4 IL-17A response only was included as a non-responder. Although the size of the groups did not allow statistical significance to be reached, it is clear that in both cases the most favourable immune profile pre-operatively for prolonged disease-free survival is the presence of both T cell responses (Fig. 1g, h).

From this we can conclude that release of IL-17A by CEA-specific T cells is not responsible for the poorer survival of patients with IFN-γ-producing CEA (but not ST4)-specific T cells. Unlike ST4, CEA is expressed at low levels in normal epithelium. A higher magnitude of IFN-γ+ CEA-specific T cell response, i.e. above the median of all anti-CEA responses tested in patients undergoing endoscopy (Supplementary Fig. 2A, B), was associated with decreased electrical resistance in ascending colonic epithelium ($P = 0.037$; Supplementary Fig. 2C) and increased paracellular passage (i.e. leak) of Lucifer yellow probe ($P = 0.11$; Supplementary Fig. 2D), implying that the integrity of the epithelial barrier is subtly compromised in patients with relatively high CEA T cell response, a finding not replicated when separating responses based on IL-17A⁺ CEA responders, nor ST4 responders from non-responders. This observation, specific to the right-sided ascending colonic epithelium, is worthy of further investigation.

DISCUSSION
Mounting pre-operative tumour antigen-specific T cell responses appears to be associated with less post-operative CRC relapse. Despite the relatively small sample size, there is a clear indication that IL-17A production by tumour antigen-specific T cells has a positive influence on anti-tumour immunity, warranting further investigation. In particular, the emergence of ST4-specific T cell response is indicative of early stage CRC and may provide diagnostic/prognostic information. This study also highlights the possibility for detrimental clinical outcomes when using IL-17A-directed chronic, subclinical enteropathy which facilitates tumorigenesis is reversed or alleviated by IL-17A production.

In conclusion, tumour antigen-specific T cell responses play a beneficial role in preventing colorectal tumour recurrence after surgical resection.

AUTHOR CONTRIBUTIONS
An.G. and A.W.G. conceived, designed and were the principal investigators of this study; A.T. and D.F.C.B. recruited the cohorts; A.T., D.F.C.B. and K.S. performed the immunological assays; A.T., K.S. and Mr. James Horwood for provision of CRC patient bloods. We thank Dr. Tom Pembroke, Dr. Gautham Appanna, Dr. Lawrence Sunder Raj, Dr. Brijesh Srivastava and Dr. Drhamaraj Dural for provision of patient blood samples and colonic epithelial biopsies from endoscopy patients.

ADDITIONAL INFORMATION
Ethics approval and consent to participate All patients and healthy donors gave their signed informed consent, and the protocol was approved by the Wales Research Ethics Committee (reference:15/WA/0291). The study was conducted in accordance with the Declaration of Helsinki.

Consent to publish Not applicable.

Data availability The datasets generated during this study are available from the corresponding author on reasonable request.

Competing interests The authors declare no competing interests.
This work was supported by Cancer Research Wales and a Cancer Research UK programme grant (C16731/A21200).

The online version contains supplementary material available at https://doi.org/10.1038/s41416-021-01283-3.

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.


