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KRAS mutation and Consensus Molecular Subtypes 2 and 3 are independently associated with reduced immune infiltration and reactivity in colorectal cancer

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ABTRACT

Purpose: KRAS mutation is a common canonical mutation in CRC, found at differing frequencies in all Consensus Molecular Subtypes (CMS). The independent immunobiological impacts of RAS mutation and CMS are unknown. Thus, we explored the immunobiological effects of KRAS mutation across the CMS spectrum.

Experimental Design: Transcriptional analysis of immune genes/signatures was performed with RNA-seq using The Cancer Genome Atlas (TCGA) and the KFSYSCC data set. Multivariate analysis included *KRAS* status, CMS, tumour location, MSI status, and neoantigen load. Protein expression of STAT1, HLA-Class II, and CXCL10 was analysed by digital immunohistochemistry.

Results: The Th1-centric co-ordinated immune response cluster (CIRC) was significantly, albeit modestly, reduced in *KRAS* mutant CRC in both data sets. Cytotoxic T cells, neutrophils and the interferon gamma pathway were suppressed in *KRAS* mutant samples. The expression of STAT1 and CXCL10, were reduced at the mRNA and protein levels. In multivariate analysis *KRAS* mutation, CMS2 and CMS3 were independently predictive of reduced CIRC expression. Immune response was heterogeneous across *KRAS* mutant CRC: CMS2 *KRAS* mutant samples have the lowest CIRC expression, reduced expression of the interferon gamma pathway, *STAT1* and *CXCL10 and* reduced infiltration of cytotoxic cells and neutrophils relative to CMS1 and CMS4 and to CMS2 *KRAS* wild type samples in the TCGA. These trends held in the KFSYSCC data set.

Conclusions: KRAS mutation is associated with suppressed Th1/cytotoxic immunity in CRC, the extent of the effect being modulated by CMS subtype. These results add a novel immunobiological dimension to the biological heterogeneity of CRC.

TRANSLATIONAL RELEVANCE

Understanding how mutational and transcriptional differences mould the immune contexture in cancer is key to accurate immunobiological stratification. We analyse how KRAS mutation shapes the immune microenvironment of colorectal cancer (CRC) in the context of the Consensus Molecular Subtypes (CMS). We show that KRAS mutation is associated with modest suppression of Th1 cell and cytotoxic cell immunity independently of mismatch repair status, tumour location, neoantigen load and transcriptional subtype, but also show that the cumulative effect is dependent upon the CMS in which the mutation is found. Immunity in KRAS mutant CMS2 is more suppressed than CMS1 and CMS4 as well as in comparison with KRAS wild type CMS2. Our findings refine stratification factors for immunotherapy trial entry in CRC and suggest potential immunotherapeutic strategies to test in KRAS mutant patients. Variation in the immune status of RAS mutant CRC according to its transcriptional context might underlie part of the heterogeneity of response to molecularly stratified medicines.

INTRODUCTION

Galon and colleagues first demonstrated the positive prognostic impact of tumour infiltrating lymphocytes (TILs) in colorectal cancer (CRC) (1). The strength of T helper type 1 (Th1) adaptive immunity was shown to be a strong prognostic factor. Th1 cells have an essential role in initiating and maintaining an effective CD8+ cytotoxic T cell response (2-4), in the recruitment of CD8+ cells to the tumour bed (5) and in directly mediating immunological tumour cell death (6). Th1 cells recognize antigen in association with major histocompatibility complex class II (MHC-II) molecules. They secrete the inflammatory cytokine interferon (IFN)-γ, which provokes class II up-regulation on tumour cells. The majority of immunogenic neo-epitopes are class II restricted (7). Tumour cells evade cytotoxic immune responses by expressing the programmed death-ligand 1 (PD-L1) that activates the PD-1 negative feedback pathway (8). This checkpoint may be inhibited using an anti-PD-1 antibody that blocks interactions between the PD-1 receptor and its ligand PD-L1. However, the strategy has only been efficacious in MSI-high CRC (9), i.e., those having a high neo-antigen burden that can stimulate immune infiltration (10). Class II expression on cancer cells is clearly important in the efficacy of checkpoint blockage. Indeed, cancer-cell MHC-II-negative melanoma patients have lower response rates, PFS and OS when treated with PD-1/PD-L1 blockade relative to class II-positive patients (11). Further, *in vitro* PD-L1 blockade enhances Th1-mediated cytotoxicity only against cells that express high class II (12). Hence, an effective immune response is critically dependent on neo-antigen presentation by MHC-II molecules.

The up-regulation of MHC-II molecules via the IFNγ pathway is dependent on the STAT1 and CIITA proteins: extracellular IFNγ induces and activates STAT1, which is the key transcriptional activator of *CIITA*. CIITA is, in turn, the master transcriptional activator of MHC-II molecules. STAT1-deficient cells show no induction of *CIITA* mRNA despite IFNγ stimulation (13) and STAT1-deficient cancer cells progress rapidly due to the evasion of adaptive immunity (14). Class I-positive but class II-negative mammary adenocarcinoma cells grew rapidly in immunocompetent mice, but were rejected when these cells were transfected with CIITA. Rejection correlated with induction of class II expression and was mediated by both CD4+ and CD8+ cells. STAT1 deficiency also severely impairs the induction of CXCL10, another STAT1 target gene. CXCL10 maintains the Th1 phenotype (15) and the decreased accumulation of Th1 cells in STAT1-deficient mice is related to reduced levels of CXCL10 (16).

KRAS mutation is the commonest canonical gain of function mutation in CRC and earlier functional studies clearly demonstrated that mutant RAS reduces both STAT1 and class II expression. Using three distinct cell line models (including Hct116, clones with deleted mutant KRAS, and intestinal epithelial cells with inducible mutant RAS), Klampfer and colleagues demonstrated that mutant RAS down-regulates both constitutive and IFNγ-inducible STAT1 mRNA and protein and reduces STAT1 transcriptional activity and the expression of many IFNγ target genes including class II (17,18). Maudsley and co-workers showed that mutant KRAS resulted in loss of class II inducibility upon IFNγ treatment (without inhibiting class I expression), significantly reduced the ability of these cells to stimulate allogeneic T cells and

reduced the IFNγ secretion of the co-stimulated cells (19). They suggested that this RAS-mediated class II down-regulation interrupted an amplification loop whereby Th1 cells are stimulated to produce IFNγ that would otherwise stimulate further cancer cell class II expression.

These isolated cell line experiments suggest a role for STAT1 and its target genes in RAS mutant CRC, but fail to replicate the complexities of the intact tumoural microenvironment. Hence, guided by these pre-clinical studies, we asked whether RAS mutant CRC was associated with reduced expression of STAT1, CIITA, and CXCL10, as well as that of a number of associated signatures of immune reactivity, in human CRC tumour tissues. We have previously demonstrated using transcriptional analysis of bulk tumours that RAS mutant CRC is associated with lower expression of a Th1-centric immune metagene that we termed the Co-ordinate Immune Response Cluster (CIRC (20)). This metagene includes *STAT1*, *CXCL10*, nine separate class II genes, and the Th1 transcription factor T-bet (*TBX21*). We have also previously described a second immunological stratifier—the CRC "Consensus Molecular Subtypes" (CMS) (21). These subtypes include a "mesenchymal" group (CMS4) that is enriched for MSS tumours and yet is characterized by appreciable immune infiltration, intermediate between that of the MSI-enriched subtype (CMS1) and of the "canonical" (CMS2) and "metabolic" (CMS3) subtypes. RAS mutations occur in all of these CMS subtypes (albeit with differing proportions) and thus RAS mutations in CRC occur in different transcriptional contexts with heterogeneous biology. In particular, RAS mutations are present in both mismatch repair deficient and proficient cancers. To determine whether these two stratifiers are independent, we dissected the various innate and

adaptive immune components of the CIRC in the context of CMS and KRAS mutation status using transcriptional analysis of two large independent datasets and digital immunohistochemistry analysis of compartment-specific protein expression.

We demonstrate that CMS is more strongly associated with reduced anti-cancer immunity in CRC than RAS mutation, with both CMS2 and CMS3 being immune suppressed relative to CMS1 and CMS4. Nevertheless, we find that the modest RAS mutation association is significant and independent of expression subtype. The cumulative effect on immunity is dependent upon the CMS context of RAS mutation, with RAS mutant CMS2 being particularly immune suppressed.

MATERIALS AND METHODS

Consensus Molecular Subtype (CMS) analysis

Statistical analyses of TCGA and KFSYSCC expression data were performed in R (http://www-r.project.org). To summarize the expression of a gene set [i.e., CIRC, immune subpopulations (22), and Hallmark gene sets (23)], we condensed the expression of the multiple genes in the set into a single gene set enrichment value using Gene Set Variation Analysis (GSVA) (24). Two-tailed non-parametric Wilcoxon rank sum tests, two-tailed *t* tests, two-tailed Fisher's tests, and one-tailed *F* tests were applied, as indicated. Relative enrichments or expression between two populations is summarized by the Hodges-Lehmann estimator of the difference between those populations—e.g., the median of all pairwise differences between CIRC enrichment in a *KRAS* MT sample and a *KRAS* WT sample. 95% confidence intervals in this estimator were calculated using the method of Bauer (25). Multivariate analyses were performed using the `forestmodel` R package, with linear model `CIRC ~ KRAS + CMS + site + status + neoantigens` and where `CIRC` is the gene set enrichment for the immune signature, `site` indicates tumour location as `left`, `right`, or `rectum`, `KRAS` indicates mutation status `WT` or `MT`, `CMS` indicates subtype, `status` indicates `MSI` or `MSS`, and `neoantigens` is a continuous value indicating the (log-transformed) number of neoantigens. To assess potential synergy between the main effects corresponding to CMS subtype (`CMS`) and *KRAS* mutation status (`KRAS`), we used ANOVA to compare linear models with and without the interaction effect (`CMS:KRAS`), i.e., `CIRC ~ CMS + KRAS` versus `CIRC ~ CMS + KRAS + CMS:KRAS`. Samples that did not correspond to one of the four CMS groups (i.e., "unlabelled") were excluded from any analysis that include

CMS. Expression data sets, as well as clinical annotations, CMS labels, neoantigen predictions (obtained from The Cancer Immune Atlas (26)), and gene set definitions, are available on the Synapse data commons platform [(27) and https://www.synapse.org] under Synapse ID syn8533552. Source code to perform all genomic analyses and to generate the respective figures is available at https://github.com/Sage-Bionetworks/crc-cms-kras. Additional detail is provided in Supplemental Methods.

Immunohistochemical analysis

Samples for IHC from patients undergoing resection of primary CRC were obtained from the completed CRUK Stratified Medicine Programme One pilot study and CRC patients from the Queen Elizabeth Hospital, Birmingham. Samples were collected under ethical approval HBRC 14-205 (Sponsor: University of Birmingham). All patients had provided informed written consent for the use of their tissue, and studies were conducted in accordance with the Declaration of Helsinki. The cohort comprised 28 RAS G12D/G13D mutants (24.3%), 38 RAS non-G12D/G13D mutants (33.0%), and 49 RAS wild types (42.65) for a total of 115. Suitable formalin-fixed, paraffin-embedded (FFPE) blocks were retrieved and processed at the HBRC biobank, University of Birmingham. Microsatellite status was assessed by extracting total DNA from FFPE tumour scrolls by fragment analysis (Supplemental Methods). 7 tumours (6.09%) were MSI-H, of which 3 were RAS mutant.

IHC was performed using a Leica Bondmax autostainer. For STAT1 an antibody that had undergone robust validation was selected (Cell Signalling Technology (CST) clone D1K9Y). For Class II HLA (Abcam clone CR3/43) and CXCL10 (Novus

Biologicals clone 6D4), in-house validation was performed as described in Supplemental Methods.

Staining conditions and concentrations were iteratively optimised in conjunction with a histopathologist (PT): STAT1: 1:500, 20 minute incubation, Class II HLA: 1:100, 20 minutes, CXCL10: 1:50, 20 minutes. Slides were scanned at 40x magnification using a Leica SCN400 slide scanner and digitally analysed using Definiens Tissue Studio software. Analysis algorithms were created and optimised for each marker. Regions of interest (ROIs) were created in the tumour regions of each slide. All tumours were digitally segmented into tumour epithelium and stroma regions using trained segmentation algorithms (**Supp Figs 1 A and B**). Depending on the marker, staining was quantified on a per cell basis or on an area basis (**Supp Fig 1 C and D**). Percentages of cells or pixels with high, medium, low or no immunoreactivity were quantified in each region. This produced either histological scores for cell-based scoring, or percental scores for pixel-based scoring, which are functions of the number and intensity of immunoreactive cells or pixels in the scanned specimens respectively (1 × (% cells/pixels with low staining) + 2 × (% cells/pixels with medium staining) + 3 × (% cells/pixels with high staining) = score out of 300). Thresholds for negative/low, low/medium and medium/high were set for each antibody in conjunction with a pathologist to maximise the dynamic range of results between samples and to reduce false positive results. Haematoxylin thresholds (the staining intensities at which haematoxylin was recognised) were set individually and differed for each antibody due to differences in DAB staining. Haematoxylin thresholds were set to ensure accurate identification of individual cells. After analysis, segmentation was manually validated for each slide.

IHC results were analysed using Excel (Microsoft Corp) and Minitab (Minitab Inc). The normality of the distribution of Histological scores in each group (*RAS* mutant or *RAS* wildtype) was determined by performing the Anderson-Darling test. All data were non-parametrically distributed. Therefore, for one by one comparisons, Mann Whitney U tests were performed for significance testing. In addition, for STAT1 and CXCL10, staining for each case was grouped into low and high using H-score thresholds of both 100 and 200. For Class II HLA, cases were grouped into negative (0-5% staining), low (5-50% staining) and high (>50% staining) as described by Lovig et al (28) (**Supp Fig 2 F-H**). Chi-squared tests were performed to investigate significance between the RAS mutant and wild type groups. A p-value <0.05 was considered significant.

RESULTS

Immune subpopulations are suppressed in *KRAS* MT CRC

In our previous work we demonstrated that RAS mutant CRC had lower expression of the CIRC, a metagene that integrates 28 genes involved in innate and adaptive immunity (20). The CIRC was defined using 195 microarray CRC samples, of which 190 have also been subjected to RNA sequencing as part of an extended TCGA study. We analyzed this full data set (n=344) to validate our original findings on the orthogonal RNA-seq platform: consistent with those previous results, the analysis showed a significant reduction in the expression of the CIRC metagene in *KRAS* MT relative to WT (**Supp Fig 3A**; two-tailed Wilcoxon rank sum $p = 2.4 \times 10^{-3}$). We additionally validated these results in the independent KFSYSCC (29) data set (n=290) of fresh-frozen CRC samples (**Supp Fig 3B**; two-tailed Wilcoxon rank sum $p = 4.4 \times 10^{-3}$).

The CIRC signature was previously defined by performing an unsupervised hierarchical clustering of TCGA patients based on 61 highly-curated, immune response-related genes. The genes comprising the signature were selected based on their strong coordinated regulation across patient subgroups (20). The CIRC is enriched for Th1-associated genes, as well as genes encoding chemokines, adhesion molecules, MHC class II molecules, and immune checkpoints. Therefore, to dissect the specific immune subpopulations differentially recruited to *KRAS* MT tumours, we examined the effect of *KRAS* mutation on expression of each of seven immune cell types [neutrophils, and immature dendritic (iDC), B, T, Th1, Th2, and cytotoxic cells (22)]. Despite having few genes in common (**Supp Fig 4**), all immune

subpopulations except Th2 cells were highly correlated with the CIRC in both data sets (Pearson correlation $r \geq 0.42$; $p \leq 6.4 \times 10^{-14}$; **Supp Fig 5**). Cytotoxic ($r \geq 0.85$; $p \leq 4.3 \times 10^{-82}$), T ($r \geq 0.73$; $p \leq 2.7 \times 10^{-50}$), and, as expected, Th1 ($r \geq 0.71$; $p \leq 3.2 \times 10^{-45}$) cells were most highly correlated with the CIRC in both data sets. *KRAS* mutation is associated with reduced cytotoxic cell (**Fig 1A**; TCGA: two-tailed Wilcoxon rank sum $p = 0.04$; KFSYSCC: $p = 0.02$) and neutrophil (TCGA: $p = 9.7 \times 10^{-3}$; KFSYSCC: $p = 5.3 \times 10^{-3}$) infiltration. Th1 cells themselves consistently trend towards reduced infiltration in *KRAS* MT CRC (TCGA: $p = 0.09$; KFSYSCC: $p = 0.13$). To further characterize biological differences between KRAS MT and WT CRC we compared the differences in expression of all 50 Hallmark gene sets (23). This revealed down-regulation of multiple immune-related pathways within *KRAS* MT tumours across both data sets (**Fig 1B**). In particular, we observed suppression of the IFNγ pathway in *KRAS* MT CRC in both data sets.

STAT1 and CXCL10 are downregulated in *KRAS* MT CRC

Given the disruption of the IFNγ pathway in *KRAS* MT CRC, we hypothesized that downstream genes would also be affected in these tumours. To test this, we examined the expression of the key IFNγ response gene, STAT1, at the mRNA level and at the protein level using digital immunohistochemistry (IHC; **Supp Figs 2 A-E**). We found that *STAT1* mRNA expression was down-regulated in *KRAS* MT CRC in both data sets (**Supp Fig 6**). By performing IHC and then digitally segmenting tumours into epithelium, stromal, and background regions (**Supp Figs 1 A and B**), we found that the STAT1 protein was also down-regulated in the epithelial compartment across a series of whole mount sections taken from 115 patients with primary CRC samples (RAS G12D/G13D MT $n = 28$, RAS non-G12D/G13D MT $n =$

38, RAS WT *n* = 49): STAT1 expression was reduced by RAS mutation whether samples were analysed by H-scores (*p* = 0.016) or according to percentage of positive staining for STAT1 (χ^2 *p* = 0.033; **Table 1**).

We next asked whether STAT1 target molecules, CXCL10 and CIITA, were also dysregulated in *KRAS* MT tumours. We found that *CXCL10* was strongly down-regulated in both data sets (**Supp Fig 6**). This down-regulation was confirmed at the protein level, with significantly more MT samples having H-scores <100 (χ^2 *p* = 0.04) and significantly more WT samples having H-scores >200 (χ^2 *p* = 0.03; **Table 1**). We also found that *CIITA* was down-regulated in *KRAS* MT samples in the TCGA data set (**Supp Fig 6**). Though there was no such evidence for dysregulation of the mRNA in the KFSYSCC data set (**Supp Fig 6**), *CIITA* expression was generally low in this data set (median *CIITA* expression below the fifth percentile). At the protein level, around 50% of both RAS MT and RAS WT CRC samples were completely negative for class II expression by IHC and only 6.4% RAS MT tumours had >50% class II positive cells (**Supp Figs 1 C-D and 2 F-H**; **Table 1**). When class II protein expression was analysed in the cancer samples that had detectable expression of class II [i.e., excluding the class II negative cases in which transcriptional silencing of CIITA would prevent IFNγ inducibility via STAT1 (30,31)], we found that RAS mutation was associated with reduced class II expression on the cancer cells (RAS MT class II expressing CRC median epithelial class II H-score = 136.14, RAS WT median = 168.33, Mann-Whitney U *p* = 0.01) with no differences in stromal class II expression (RAS MT CRC stromal median = 146.96, RAS WT median = 141.56, Mann-Whitney U *p* = 0.16).

Reduced immune infiltration is independently associated with *KRAS* mutation and CMS subtype

Immune response in CRC has been reported to be suppressed in CMS2 (21). Hence, we hypothesized that the CIRC and other measures of immunity would be lowest in *KRAS* MT CMS2 tumours. We first confirmed that the CIRC was strongly suppressed in CMS2 relative to CMS1 and CMS4 in both the TCGA (**Supp Fig 7A**; CMS2 versus CMS1: two-tailed Wilcoxon rank sum p = 1.2 x 10^{-18}; CMS2 versus CMS4: p = 5.5 x 10^{-15}) and KFSYSCC (**Supp Fig 7B**; CMS2 versus CMS1: p = 1.1 x 10^{-4}; CMS2 versus CMS4: p = 9.0 x 10^{-8}) data sets. As expected, CMS2 *KRAS* MT samples had the lowest CIRC expression amongst all CMS subtype x genotype combinations in the TCGA data set (**Fig 2A**). These results were independently validated in the KFSYSCC data set (**Fig 2B**), though the consistent trends in relation to CMS3 did not always reach significance. To determine whether *KRAS* mutation status and CMS classification are significantly and independently associated with immune infiltration, we performed a multivariate analysis of CIRC expression that included as parameters *KRAS* mutation status, CMS classification, primary tumour location, and, in the TCGA data set where they were available, MSI status and neoantigen load. The analysis showed that *KRAS* MT and CMS2 (relative to CMS1 and CMS4) were independently predictive of reduced CIRC expression in the TCGA (**Fig 3A**) and KFSYSCC (**Fig 3B**) data sets. We next assessed whether *KRAS* mutation might have a CMS subtype-dependent effect. However, there was no evidence for a *KRAS* x CMS interaction in either data set (TCGA: F test p = 0.15; KFSYSCC: p = 0.67). Finally, to delineate potential differential infiltration of specific subpopulations associated with *KRAS* MT CMS2 tumours, we examined the immune subpopulations most strongly associated with *KRAS* status (**Fig 1A**) in the additional

context of molecular subtype. We found that *KRAS* MT CMS2 tumours had reduced infiltration of cytotoxic cells relative to all other patient groups in the TCGA data set (**Fig 4A**), with a similar trend in the KFSYSCC data set (**Fig 4B**). *KRAS* MT CMS2 tumours also showed reduced infiltration of neutrophils and Th1 cells in both data sets relative to CMS1 and CMS4 patients, but not necessarily to CMS2 WT or CMS3 (MT or WT) patients.

Taken together, our results indicate that there is considerable heterogeneity within CMS subtypes, even when controlling for MSI status, and that this may be further dissected using *KRAS* mutation status. Though the data could not unambiguously resolve whether *KRAS* mutation has an effect specific to CMS2, the two factors are independently significant, i.e., the level of immune infiltration and its characterization across immune cell subpopulations cannot be inferred without knowledge of both factors. The cumulative effect is such that CMS2 *KRAS* MT samples have reduced immune infiltration (of cytotoxic cells, neutrophils, and Th1 cells, as well as measured by the CIRC) relative to CMS1 or CMS4 samples harboring either MT or WT *KRAS*.

IFNγ pathway suppression is associated with both *KRAS* mutation and CMS subtype

To determine whether immune pathways down-regulated in *KRAS* MT tumours (**Fig 1B**) were additionally suppressed in CMS2 CRC, we evaluated the expression of these signatures in the context of *KRAS* mutation status and molecular classification. In the TCGA data set, we found that *KRAS* MT CMS2 tumours exhibited reduced expression of all examined immune signatures (IFNγ, inflammatory response,

IL6/JAK/STAT3 signaling, complement, and IFNα) relative to all patient groups (though the trend did not reach significance in relation to *KRAS* WT CMS2 when examining the IFNα pathway; **Fig 4C**). These trends held in the KFSYSCC data set (**Fig 4D**). In particular, *KRAS* MT CMS2 tumours showed significantly reduced expression of the IFNγ pathway relative to all other patient groups in both data sets, except relative to KRAS WT CMS2 in the KFSYSCC data set, which nevertheless exhibited the same trend ($p = 0.05$).

Finally, we examined the downstream target of the IFNγ pathway, *STAT1*, as well as its downstream targets, *CXCL10* and *CIITA*, to determine whether the previously-observed association between the reduced expression of these three genes and *KRAS* mutation was independent of molecular subtype. First, we observed that, within CMS2, *KRAS* MT samples had lower expression of each of the genes relative to WT samples in both the TCGA ($p < 0.02$) and KFSYSCC ($p < 5.8 \times 10^{-3}$) data sets, with the exception of *CIITA* in the KFSYSCC data set, as expected from its low expression in this data set (**Supp Fig 8**). Second, we performed multivariate analyses for all three genes in both data sets, excluding *CIITA* in the KFSYSCC data set, which generally indicated that both *KRAS* mutation and CMS2 (relative to CMS1 and CMS4) were significantly and independently associated with reduced expression of the three genes. Specifically, *KRAS* mutation was significantly ($p < 1.1 \times 10^{-2}$) or marginally ($p = 0.05$ for *STAT1* in the TCGA data set) associated with reduced gene expression, while CMS2 was associated with reduced gene expression relative to CMS1 ($p < 3.1 \times 10^{-3}$) and to CMS4 ($p < 1.2 \times 10^{-3}$, except for *STAT1* in the KFSYSCC data set, where $p = 0.17$).

DISCUSSION

We have previously shown that *KRAS* mutation is associated with reduced expression of the CIRC metagene, which summarizes 28 genes associated with innate and adaptive immunity. Here, we extend those earlier findings to: (1) explicitly characterize the nature of the suppressed immune infiltration, showing that *KRAS* MT tumours have reduced infiltration of cytotoxic cells and neutrophils (**Fig 1A**); (2) demonstrate that the IFNγ pathway is suppressed in *KRAS* MT tumours (**Fig 1B**); (3) demonstrate that KRAS mutation is associated with down-regulation of STAT1 and CXCL10 at the mRNA (**Supp Fig 6**) and protein (**Table 1**) levels; (4) show that *KRAS* MT-associated immunosuppression is independent of CMS classification (**Fig 3** and **Supp Fig 8**); and (5) show that *KRAS* MT CMS2 CRC is significantly immunosuppressed relative to (*KRAS* MT or WT) CMS1 and CMS4 cancers and, based on several signatures in at least one of the two data sets, relative to *KRAS* WT CMS2 CRC as well (**Figs 2** and **4**).

The *KRAS* MT-associated down-regulation of the IFNγ pathway and reduced infiltration of cytotoxic T cells (i.e., those with properties common to CD8+ T, Tγδ, and natural killer cells) and neutrophils indicate that the immunosuppressive impact of KRAS mutation that we previously observed is robust, if modest. Recent data demonstrate the interconnectedness of CD8+ T cells and neutrophils with the IFNγ pathway (32): addition of neutrophils to CD8+ T cells (activated via sub-optimal concentrations of anti-CD3 and anti-CD28 antibodies) led to increased IFNγ release and T cell proliferation. In turn, activated CD8+ cells enhanced neutrophil viability.

Furthermore, activated neutrophils co-localize with immature DCs, leading to their maturation (33). The resulting DCs drive T cell proliferation and Th1 skewing.

Pre-clinically RAS mutation has been shown to reduce the levels of STAT1 (17,18). Consistent with these findings, we demonstrated that RAS MT cancers are associated with significantly lower STAT1 within the context of the tumour microenvironment. The pre-clinical data also showed that RAS mutation reduced STAT1-dependent transcriptional activity (17); indeed, we detected reduced expression of the STAT1 target CXCL10 at the RNA and protein levels in *KRAS* MT relative to WT samples. *KRAS* mutation may additionally down-regulate CXCL10 via its activation of MEK-ERK signalling, which we observed in both data sets using a previously published (34) five-gene MEK signature (data not shown). We observed that *KRAS* MT reduced expression of a second STAT1 target, *CIITA*, in the TCGA data set. No such trend was detected in the KFSYSCC data set. However, *CIITA* expression was suppressed in this data set, which would likely mask any *KRAS* MT-mediated STAT1 impact. Transcriptional repression of CIITA is seen in a proportion of CRC samples (30) as is the complete failure of IFNγ to induce class II expression in half of primary CRC cells (31). Both of these effects are RAS-independent. To control for *CIITA* silencing (and thus lack of class II inducibilty), we analysed the 50% of CRC samples that detectably expressed class II molecules (and in which *CIITA* must be transcribed and hence under the influence of STAT1). In these samples, we demonstrated that RAS MT cancers had significantly lower expression of class II surface makers compared with RAS WT cases. Significantly, we demonstrated that both CMS classification and *KRAS* mutation status are independently and significantly associated with dysregulation of *STAT1*, *CXCL10*, and *CIITA*. The

CMS-associated effect presumably reflects previously-reported reduced IFNγ signalling in CMS2 tumours (21), which leads to correspondingly reduced transcription of STAT1 target genes (17). Our findings and the cited literature are consistent with a cell autonomous role for *KRAS* in modulating STAT1 and its downstream targets *CXCL10* and *CIITA*. Nevertheless, we cannot formally exclude the possibility that this *KRAS* effect is attributable, in whole or in part, to the reduced immune infiltration of CMS2 CRC with corresponding reduced environmental IFNγ. However these two factors are clearly intimately related.

Suppression of the CIRC was greatest in *KRAS* MT CMS2 samples. There may be a straightforward explanation for this phenomenon. CMS2 is the most Th1 immune suppressed of the molecular sub-types with the lowest level of IFNγ signalling and thus lower levels of STAT1 and STAT1 target gene transcription. KRAS mutation shifts the IFNg/STAT1 dose response curve (17), such that for any level of IFNγ there is less STAT1 transcription in a KRAS mutated context. This effect is likely to be most biologically relevant where IFNγ levels are already limiting. The cumulative impact of low IFNγ (CMS2) and blunting of the IFNγ response (via mutant KRAS) may result in a level of STAT1-dependent promoter transcription that is insufficient to support robust and consistent expression of the critical downstream molecules. We considered the alternative explanation - that the effect of KRAS mutation in CMS2 was due to it impacting the particular biology of CMS2. This subtype is characterised by high levels of Wnt and Myc signalling (21). Activation of WNT/β-catenin signalling in melanoma reduces CD8+ T and IFNγ-producing CD4+ cells, findings which have been generalized across other cancer types including CRC (35), while MYC up-regulation has been associated with reduced CD4+ T cell tumoural accumulation

(36). *In vitro*, mutant RAS significantly enhances WNT/β-catenin signalling in a mutant APC background and enhances downstream *MYC* transcription (37). Thus we investigated whether KRAS mutation was deepening the Wnt and Myc drive in CMS2, and thus deepening immunosuppression via this mechanism. We found no robust, consistent evidence that *KRAS* mutation dysregulated the expression of the WNT or MYC signatures within the context of CMS2 ($p > 0.07$ for comparisons of KRAS MT CMS2 vs KRAS WT CMS2 for WNT/β-catenin and MYC target gene sets).

As is the case for the majority of transcriptional and immunohistochemical analyses in CRC, our analysis was performed using primary resection samples. It is important to stress that the strength of Th1 immunity and class II expression in primary tissue are highly prognostic factors and are predictive of the presence of both synchronous metastatic disease and the development of subsequent metastases (38). Thus, understanding the independent impacts on the strength of Th1 immunity in primary tissue is of value in its own right. These results pose important questions for the larger body of immunotherapy trials that are instead directed at established metastatic or, in an adjuvant context, micrometastatic disease. Longitudinal expression studies following the evolution of disease progression should be undertaken to ascertain the concordance of CMS classification between primary and metastatic disease. However, existing data already suggest that immune cell densities (CD8+, dendritic, and NK cells) are highly correlated between primary and metastatic CRC and between separate metastatic sites (39). Though it has been suggested that there is significant intra-tumoural heterogeneity of CMS, this analysis used separately macro-dissected tissue from the center of the tumour and from the

invasive front rather than bulk tumour (40). As was pointed out in the accompanying editorial, biopsy from the invasive margin will result in a large admixture of stromal cells not found in the center of the tumour thus giving a CMS4-like signature and artificially introducing heterogeneity through selective sampling (41). Regardless of whether CMS or some other molecular subtypes prove to be pertinent to metastatic CRC, our results suggest that *KRAS* mutation is likely to modulate immune response within these subtypes: these data provide proof of principle that the immune status of RAS mutant CRC is not homogenous across all CRC and that RAS mutation influences the immunobiology of molecularly-defined CRC subtypes.

In summary, our results add a novel immunological dimension to the growing appreciation of the biological heterogeneity of tumours harbouring canonical mutations in CRC. The immunobiological status of RAS mutant CRC varies according to transcriptional context and the immunobiological status of CMS2 is dependent on RAS status. *KRAS* MT CMS2 appears to be a particularly immune-neglected group that will need therapy to initially activate a microenvironmental immune response if checkpoint blockade is considered in a combinatorial approach. RAS mutation itself may be a useful immunological target in this group. Adoptive T cell transfer of RAS MT-specific T cells has recently been shown to have therapeutic efficacy in CRC (42) and the use of T cells transduced with T-cell receptors recognising RAS MT epitopes is also a potential therapy option (43). Our demonstration that a canonical mutation can be associated with widely differing expression of immune-related genes based on its transcriptional subtype may underlie some of the heterogeneity of responses seen with targeted therapies, although it is important to qualify this by acknowledging that our understanding of the

transcriptional biology of metastatic disease is limited. In animal models, the activity of BRAF inhibitors is dependent on Th1 cell-mediated provision of CD40L and IFNγ (44). Similarly, the therapeutic effect of inactivation of oncogenic MYC is dependent upon CD4+ cells (45). This suggests that the use of individual mutations as predictive biomarkers in CRC may be insufficient to predict the efficacy of targeted therapies without knowledge of the associated CMS subtype and its immune contexture. This hypothesis should be readily testable in the clinic.

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TABLES

Table 1

		Epithelium			Stroma		
		RAS MT	RAS WT	p value	RAS MT	RAS WT	p value
STAT1	Median H Score	180	238	0.016	88	122	0.086
	% H score <100	32.2	10.5	0.014	54.2	47.4	0.508
	% H score >200	40.7	60.5	0.056	13.6	23.7	0.200
CXCL10	Median H Score	93.5	108	0.080	24	24	0.858
	% H score <100	58.1	38.3	0.041	85.5	85.1	0.956
	% H score >200	8	23.4	0.025	4.8	2.1	0.558
Class II HLA	Median Percental Score	125.2	136.8	0.260	143.9	135.8	0.051
	% Negative (0-5%)	50.8	51.2		11.3	20.9	
	% Positive (5-50%)	42.9	37.2	0.590	87.1	79.1	0.300
	% Strong (>50%)	6.4	11.6		1.6	0	

Table 1: Immunohistochemistry analysis. Median Histological scores or Percental scores in epithelial and stromal regions. STAT1 and PD-L1 reactivity are represented by histological scores. Class II HLA reactivity is represented by percental scores. For median H and percental scores, *p*-values are derived with Mann Whitney U test. For all other comparisons, *p*-values are derived with χ^2 test.

FIGURE LEGENDS

Fig 1: *KRAS* mutation is associated with reduced immune infiltration and downregulation of immune pathways. (A) Volcano plot showing enrichment (x axis) of immune cell subpopulations in *KRAS* MT relative to *KRAS* WT tumours, with associated *p*-values (y axis) across TCGA (red) and KFSYSCC (blue) data sets. Relative enrichment is the Hodges-Lehmann estimator of the difference between the *KRAS* MT and *KRAS* WT populations—i.e., the median of all pairwise differences between CIRC enrichment in a *KRAS* MT sample and a *KRAS* WT sample. (B) Volcano plot as in (A), but showing effect of *KRAS* mutation on Hallmark gene sets. The subset of the full set of 50 Hallmark gene sets with $p < 0.1$ are labeled.

Fig 2: CIRC expression is reduced in CMS2 *KRAS* mutant tumours. Expression of CIRC versus CMS subtype and *KRAS* mutation status in (A) TCGA (n=316) or (B) KFSYSCC (n=258) data sets. n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; MT: mutation; WT: wild type.

Fig 3: CMS subtype and *KRAS* mutation are independently predictive of CIRC expression. Multivariate analysis performed across (A) TCGA (n=310) or (B) KFSYSCC (n=258) data sets.

Fig 4: *KRAS* MT CMS2 tumours are associated with reduced immune infiltration and downregulation of immune pathways. Enrichment score (y axis) of immune populations (x axis) of indicated *KRAS* x CMS subgroup relative to *KRAS* MT CM2 subgroup in (A) TCGA and (B) KFSYSCC data sets. Relative enrichment is the Hodges-Lehmann estimator of the difference the indicated subgroup and *KRAS* WT CMS2 subgroups. Error bars represent 95% confidence intervals in estimator

calculated using the method of Bauer (25). Enrichment relative to *KRAS* MT CMS2 subgroup of Hallmark immune pathways in (C) TCGA and (D) KFSYSCC data sets.