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**Published article:**

El-Jawhari, JJ, El-Sherbiny, YM, Scott, GB, Morgan, RS, Prestwich, R, Bowles, PA, Blair, GE, Tanaka, T, Rabbitts, TH, Meade, JL and Cook, GP (2014)  
*Blocking oncogenic RAS enhances tumour cell surface MHC class I expression but does not alter susceptibility to cytotoxic lymphocytes.* *Molecular Immunology*, 58 (2). 160 - 168. ISSN 0161-5890

<http://dx.doi.org/10.1016/j.molimm.2013.11.020>

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**Blocking oncogenic RAS enhances tumour cell surface MHC class I expression  
but does not alter susceptibility to cytotoxic lymphocytes**

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**Short title:** RAS modulation of antigen presentation pathways

## **Abstract**

Mutations in the RAS family of oncogenes are highly prevalent in human cancer and, amongst its manifold effects, oncogenic RAS impairs the expression of components of the antigen presentation pathway. This allows evasion of cytotoxic T lymphocytes (CTL). CTL and natural killer (NK) cells are reciprocally regulated by MHC class I molecules and any gain in CTL recognition obtained by therapeutic inactivation of oncogenic RAS may be offset by reduced NK cell activation. We have investigated the consequences of targeted inactivation of oncogenic RAS on the recognition by both CTL and NK cells. Inactivation of oncogenic RAS, either by genetic deletion or inactivation with an inducible intracellular domain antibody (iDAb), increased MHC class I expression in human colorectal cell lines. The common RAS mutations, at codons 12, 13 and 61, all inhibited antigen presentation. Although MHC class I modulates the activity of both CTL and NK cells, the enhanced MHC class I expression resulting from inactivation of mutant KRAS did not significantly affect the *in vitro* recognition of these cell lines by either class of cytotoxic lymphocyte. These results show that oncogenic RAS and its downstream signalling pathways modulate the antigen presentation pathway and that this inhibition is reversible. However, the magnitude of these effects was not sufficient to alter the *in vitro* recognition of tumour cell lines by either CTL or NK cells.

**Keywords:** Tumour Immunology, antigen presentation, MHC class I, tumour immune evasion, Natural killer cells, RAS oncogene

## **Highlights**

- Genetic deletion of mutant KRAS enhances the antigen presentation pathway
- Intracellular antibody targeting of mutant KRAS increases MHC class I expression
- Mutant KRAS alters expression of the NKG2D ligand ULBP2
- Mutant KRAS does not alter susceptibility to NK cells

## **1. Introduction**

The evasion of immunity is now recognised as a hallmark of cancer alongside well established characteristics, such as loss of growth control, resistance to apoptosis and the ability to invade and metastasise [1]. Cancer development is initiated by mutations in cellular oncogenes and tumour suppressor genes. The first cellular oncogenes to be discovered were those belonging to the RAS family and mutant RAS molecules are found in 20-25% of human cancers [2, 3]. RAS proteins are small GTP-binding proteins that couple growth factor receptors to intracellular signalling pathways [2, 3]. Mutations in codons 12, 13 and 61 are the commonest in human cancer and all generate constitutively active RAS molecules [3]. The RAS pathway is directly linked to several key features of malignant development, such as cell cycle progression and survival. In addition, RAS signalling regulates several features associated with invasiveness and the development of the tumour microenvironment, including tumour angiogenesis [2, 3].

Cytotoxic lymphocytes, namely natural killer (NK) cells and cytotoxic T cells (CTL), play a key role in the elimination of tumour cells [4]. The activity of both of these cell types is regulated by target cell MHC class I molecules [5, 6]. The T cell receptor (TCR) complex on CTL can recognise tumour-associated antigens (TAA) presented by MHC class I molecules and this interaction delivers a potent activating signal to the cognate CTL. In contrast, NK cells are negatively regulated by MHC class I molecules via the interaction with inhibitory killer cell immunoglobulin-like receptors (KIRs). Thus, whilst reduction of MHC class I expression on tumours allows evasion of CTL, it favours NK cell activation, providing the host with a powerful immune surveillance system. In addition, malignant cells frequently express cell surface ligands of the NKG2D and DNAM-1 molecules found on both NK cells

and CTL. In NK cells, these deliver signals that cause NK cell activation in the absence of inhibitory signalling from KIR molecules [6, 7]. Once activated by tumour cells, CTL and NK cells exocytose cytotoxic granules containing granzymes and perforin molecules, inducing apoptosis in the tumour. In addition, both NK cells and CTL produce interferon (IFN)- $\gamma$  which enhances antigen presentation and favours the development of cell mediated immunity [8-11].

Tumours frequently downregulate the expression of MHC class I molecules from the cell surface, allowing them to evade CTL recognition [12]. This can occur as a result of mutations in genes encoding critical components of the antigen presentation machinery, such as  $\beta$ 2-microglobulin ( $\beta$ 2M) [13]. The  $\beta$ 2M molecule is required for the stable expression of the MHC class I/peptide complex at the cell surface and hence  $\beta$ 2M mutations allow irreversible evasion of CTL [14]. However, reduction in tumour cell MHC class I expression can also occur by a second, reversible pathway [12, 15]. In this case, reduced expression of antigen presentation pathway components restricts the flow of peptide loaded MHC class I molecules to the cell surface [15]. Expression of oncogenic RAS has previously been linked with the reduced expression of antigen presentation pathway components in mouse cells and in human tumour tissue [16-18] (and reviewed in [3]). These components include the transporter of antigen processing (TAP) and the TAP-associated molecule, tapasin, which are required for efficient delivery of antigenic peptides into the endoplasmic reticulum (ER) [14]. This suggests that targeted inactivation of oncogenic RAS may restore expression of MHC class I molecules to the cell surface and help to boost T cell recognition. However, there are conflicting reports on the association between RAS mutations and antigen presentation in human tumours [18-20], indicating that functional studies are required. We have analysed the role of

mutant RAS oncogenes in regulating the expression of MHC class I molecules and in determining the recognition of RAS mutant tumours by cytotoxic lymphocytes. Our results reveal that oncogenic RAS inhibits the antigen processing pathway in human tumour cells and that these RAS-mediated effects are reversible. However, enhancing the antigen processing pathway by targeting oncogenic RAS in these tumour cells did not alter their susceptibility to NK cells or CTL *in vitro*.

## **2. Materials and Methods**

### *2.1. Culture of tumour cell lines*

The inactivation of mutant KRAS in HCT116 (termed H<sup>Mu</sup> here) and in DLD-1 (D<sup>Mu</sup>) generating HKe3 (termed H<sup>WT</sup>) and DKO4 (D<sup>WT</sup>) respectively, has been previously described [21]. Mutant and wild-type cells were cultured in DMEM supplemented with 10% foetal calf serum (FCS). The HCT116, SW480 and HT-1080 cells stably transfected with the anti-RAS iDAb were previously generated [22, 23]; these cells were cultured in DMEM+10% FCS supplemented with 1mg/ml G418, 1µg/ml puromycin and 0.3mg/ml hygromycin B to maintain transfected constructs. For induction of iDAb expression, medium was supplemented with 50µg/ml doxycycline for 48 hours prior to analysis by flow cytometry or immunoblotting.

### *2.2. Protein and mRNA analysis*

The following antibodies were used for flow cytometry (antigen, clone-fluorochrome, supplier); MHC class I, W6/32-PE, Dako; HLA-A2, BB7.2-PE, Serotec; ULBP1, 170818-PE, R&D systems; ULBP2, 165903-PE, R&D systems; MICA/B, 6D4-PE, BD Biosciences; PVR, TX21-FITC, MBL International; Nectin-2, R2.525-PE, BD Biosciences; CD8, SK7-PerCP, BD Biosciences; IFN-γ, 4S-B3-FITC, BD Biosciences; CD107a, H4A3-FITC (or PE), BD Biosciences. The purity of NK cell preparations was determined using the following antibodies; CD56, AF127H3-PE, Miltenyi Biotec; CD3, UCHT1-FITC, BD Biosciences; NKp46, 9E2-APC, BD Biosciences. The MART-1 peptide loaded HLA-A2 pentamer (APC labelled) was purchased from ProImmune, together with an HLA-A2 control pentamer to allow for accurate gating of MART-1 specific T cells. Flow cytometry was performed using a FACS Calibur or LSRII flow cytometer (BD Biosciences) and analyzed using FACS



Diva or Cellquest Pro (both from BD Biosciences) or FlowJo software (from Treestar). For Western blotting, we used an anti-calnexin polyclonal sera from Cell Signaling Technology and monoclonal antibodies against Tapasin and TAP-1 (from Paul Lehner, University of Cambridge), actin and the FLAG epitope from Sigma-Aldrich. Quantitative RT-PCR analysis of gene expression was performed as in [24] using Taqman probe/primer sets from Applied Biosystems/Life Technologies.

### *2.3. Preparation and functional analysis of NK cells and MART-1 specific T cells*

Human NK cells were prepared from blood samples using indirect immunomagnetic separation, using a kit from Miltenyi Biotec, as previously described [24]. NK cell purity, as judged by either the CD56<sup>+</sup>CD3<sup>neg</sup> or NKp46<sup>+</sup> cell surface phenotype was routinely >90%. For IL-2 stimulation, NK cells were cultured for 5-7 days in 50 units/ml IL-2 (R&D systems). NK cell mediated killing of tumour cells and granule exocytosis assays were performed as we have described previously [24-26], including after siRNA transfection of target cells [26, 27]. T cells restricted to the HLA-A2 restricted MART-1 epitope were generated *in vitro* and assayed as described [28]. The tumour target cells were pulsed with 10ng/ml of either MART-1 peptide (ELAGIGILTV) or the control HER2/neu peptide (ILHNGAYSL) for 30 minutes prior to co-culture with the CTL. Discarded blood donations (from the Leeds NHS Blood and Transplant Service) were used as a source of HLA-A2<sup>+</sup> lymphocytes.

### 3. Results

#### 3.1. Mutant RAS decreases cell surface expression of MHC class I molecules

The human colorectal cancer cell line HCT116 contains a wild type KRAS allele and a second mutant allele with the oncogenic G13D mutation. We compared the cell surface expression of MHC class I molecules on HCT116 and a derivative (HKe3) in which the mutant KRAS allele has been deleted by homologous recombination [21]; this cell line retains the wild-type KRAS allele and differs from the parental HCT116 cells only by the absence of mutant KRAS. For simplicity, we refer to this pair of cell lines as H<sup>Mu</sup> (for HCT116 with mutant KRAS) and H<sup>WT</sup> (HCT116 with wild-type KRAS). Loss of the oncogenic KRAS allele impairs (but does not halt) growth of these cells both *in vitro* and *in vivo* [21]. Flow cytometry using a pan-reactive anti-MHC class I antibody (W6/32) indicated that the H<sup>Mu</sup> cells expressed lower levels of cell surface MHC class I than H<sup>WT</sup> in which the KRAS G13D allele had been deleted (Fig. 1A). Antibodies specific for HLA-A2 molecules revealed that cell surface expression of this MHC class I allotype was downregulated in the presence of oncogenic KRAS (Fig. 1A). Stable expression of MHC class I molecules at the cell surface requires translocation of antigenic peptide from the cytosol into the ER, loading of this peptide into the MHC class I molecule and association of the MHC class I heavy-chain polypeptide with  $\beta$ 2M [14]. The H<sup>Mu</sup> cells had reduced expression of the TAP-1, tapasin and  $\beta$ 2M (Fig. 1B), indicating that mutant KRAS was inhibiting expression of key antigen processing pathway components, as previously suggested in immunohistochemical studies of human tumours [18]. Furthermore, the steady state levels of  $\beta$ 2M mRNA were reduced by a factor of two in H<sup>Mu</sup> compared to H<sup>WT</sup>, in close agreement with the difference in cell surface expression of MHC class I molecules in these cell lines (data not shown). Expression

of the ER chaperone calnexin was unaffected by mutant KRAS (Fig. 1B); this protein facilitates the folding of MHC class I, but is dispensable for antigen presentation [14]. These results indicate that mutant KRAS decreases cell surface expression of MHC class I molecules in human tumour cells by reducing expression of multiple components in the antigen processing and presentation pathway. Cell surface expression of MHC class I could be enhanced in both H<sup>WT</sup> and H<sup>Mu</sup> by IFN- $\gamma$  stimulation, although H<sup>Mu</sup> cells had an impaired response compared to H<sup>WT</sup> (Fig. 1C). Thus, mutant KRAS inhibits the antigen presentation pathway but the pathway remains intact, suggesting that it could be enhanced by therapeutic targeting of mutant RAS.

### *3.2. A RAS-targeting strategy increases MHC class I expression in tumour cells*

We verified the role of mutant RAS in inhibition of MHC class I expression using a separate approach. The selective targeting of mutant RAS is a major goal of cancer therapy. Strategies have been developed that allow the selection of antibody-like fragments (termed intracellular domain antibodies or iDABs) that possess antigen-specificity within the intracellular environment [29]. This approach was used to develop an iDAB that selectively binds to the active conformation of RAS [23, 30]. Inducible expression of this iDAB is coupled to a reduction in RAS signalling and reduced tumourigenicity *in vivo* [22, 23]. We analysed the effect of inducing anti-RAS iDAB expression in three cell lines, the colorectal cell lines HCT116 and SW480 containing mutant KRAS (G13D and G12V respectively), and HT-1080, a fibrosarcoma cell line with a mutant NRAS (Q61K). Induction of the iDAB resulted in increased expression of cell surface MHC class I in all three cell lines (Fig. 2). The iDAB open reading frame is linked to GFP via an internal ribosome entry site [23];

more pronounced MHC class I expression was observed in the cells exhibiting higher levels of GFP expression (Fig. 2). Importantly, these results confirmed the data obtained using the H<sup>Mu</sup>/H<sup>WT</sup> isogenic pair (Fig. 1) and showed that inactivation of all three common RAS mutants restored cell surface MHC class I expression to these tumour cell lines. Collectively, these results suggest that therapeutic blockade of oncogenic RAS might alter the recognition of these targeted cells by cytotoxic lymphocytes.

### 3.3. Mutant RAS and recognition by CTL

We first investigated whether the oncogenic RAS-mediated inhibition of MHC class I expression affected antigen-specific recognition by CTL. Use of established human tumour cell lines has the disadvantage that it is not possible to obtain autologous T cells. However, the HCT116 cell line expresses the HLA-A2 molecule (Figure 1A) and it is possible to use *in vitro* priming to generate CD8<sup>+</sup> T cell populations from healthy HLA-A2<sup>+</sup> donors that are reactive against the HLA-A2 restricted melanoma antigen, MART-1 (Fig. 3A). We pulsed H<sup>Mu</sup> and H<sup>WT</sup> cells with MART-1 peptide (or a control peptide derived from the HER2/Neu molecule) and co-cultured these antigen-pulsed tumour cells with T cells enriched for MART-1 specificity. Antigen-specific T cell activation was assessed by intracellular IFN- $\gamma$  production by the MART-1 reactive cells (Fig. 3B and C). Interferon- $\gamma$  production by the MART-1 reactive T cells was induced by the MART-1 peptide pulsed cells, but not the control peptide, indicating antigen-specificity of the assay. Comparison of IFN- $\gamma$  production in response to MART-1 pulsed H<sup>WT</sup> and H<sup>Mu</sup> cells suggested a diminished CTL response in the presence of mutant RAS, as expected with reduced MHC class I expression on H<sup>Mu</sup> cells. However, this data did not reach statistical significance (Fig.

3C).

#### *3.4. Mutant RAS does not alter NK cell recognition*

According to the missing-self model, the reduction in cell surface MHC class I molecules mediated by mutant KRAS should increase the susceptibility of these cells to NK cell attack. We compared the ability of unstimulated and IL-2 activated NK cells to kill H<sup>WT</sup> and H<sup>Mu</sup> cells; IL-2 enhanced the killing of both cell lines but, contrary to the predictions of the missing-self hypothesis, there was no difference between them in their susceptibility to NK cells over a range of effector:target ratios (Fig. 4A). However, the ability of an NK cell to kill a tumour target is dependent upon NK cell recognition of the target and the susceptibility of the tumour to NK cell-mediated apoptosis induction. Mutant RAS can modulate apoptotic functions [2, 3], suggesting that any increased NK cell activation resulting from RAS-mediated reduction in MHC class I expression on H<sup>Mu</sup> cells might be balanced by a decreased susceptibility of the tumour cells to NK cell-mediated apoptosis. To test this, we analysed NK cell activation by H<sup>Mu</sup> and H<sup>WT</sup> using the granule exocytosis assay [25, 31]. This assay dissociates the NK cell response from the susceptibility of the tumour target cells to granule-induced apoptosis. Again, IL-2 stimulated NK cells demonstrated increased response to both H<sup>Mu</sup> and H<sup>WT</sup>, but there was no difference in the ability of these two cell lines to induce NK cell activation (Fig. 4B).

The colorectal cell line DLD1 also has a KRAS G13D mutation and this allele has been deleted to generate the DKO4 cell line [21] (we denote these cells as D<sup>Mu</sup> and D<sup>WT</sup> respectively). Interestingly, DLD1 does not express any cell surface MHC class I molecules because it lacks functional  $\beta$ 2M [13] (Fig. 4C). We used D<sup>Mu</sup> and D<sup>WT</sup> to analyse the effect of mutant KRAS on NK cell recognition in the absence of

MHC class I expression. As found in the HCT116 background, IL-2 treatment enhanced the NK cell degranulation and killing of this isogenic pair of cell lines, but there was no difference in their recognition or susceptibility to killing in the presence or absence of oncogenic RAS (Fig. 4D and E).

### *3.5. Expression of NK cell activation ligands in the presence of oncogenic RAS*

NK cell activation is not solely determined by target cell MHC class I expression, but by the balance of signals delivered from inhibitory receptors (including the KIRs) and activating receptors such as NKG2D and DNAM-1 [6, 7]. The human NKG2D molecule binds to a series of cell surface ligands, comprising members of the cytomegalovirus UL16 binding protein (ULBP) family and MHC Class I polypeptide related sequence (MIC)A and MICB [32]. Two ligands of DNAM-1 have been identified, PVR and Nectin-2 [6, 7]. We analysed the expression of ULBP1, ULBP2, MICA/B, PVR and Nectin-2 in the HCT116 and DLD-1 derived isogenic pairs. Amongst these molecules, only ULBP2 showed consistent alteration in expression in the two backgrounds, with cell surface expression being reduced in the presence of oncogenic RAS (Fig. 5). Furthermore, this reduction in ULBP2 expression in the presence of oncogenic RAS was mirrored at the mRNA level (data not shown). These results, together with those in Figure 1, indicated that mutant KRAS reduces the cell surface expression of molecules that inhibit NK cells (MHC class I) as well as those that activate (ULBP2). However, this modulation did not affect the activation of NK cells or their ability to kill these tumour targets.

## **4. Discussion**

These results using the H<sup>Mu</sup>/H<sup>WT</sup> isogenic cell lines and the anti-RAS iDAb system

clearly demonstrate a role for oncogenic RAS activity in the inhibition of MHC class I expression in human tumour cells. The concept that oncogenes inhibit expression of MHC class I molecules and reduce the host immune response to tumours stems largely from observations showing that the adenovirus 12 (Ad12) E1A molecule downregulates MHC class I and allows Ad12-transformed rodent cells to evade T cell mediated immunity [33, 34]. These findings prompted a series of studies investigating the ability of different oncogenes to modulate MHC class I expression [35-38]. Subsequently, oncogenic RAS was shown to reduce cell surface expression of mouse MHC class I molecules [16, 17] and this was associated with reduced CTL recognition [17]. The reduced expression of MHC class I molecules in human tumours is well documented [12]. Early immunohistochemical studies showed that there were three phenotypes of colorectal cancer; absence of MHC class I expression was observed in approximately one third of samples, whereas two-thirds of samples had reduced MHC class I expression. Tumours with apparently normal levels of MHC class I expression were a very small minority, accounting for less than one in twenty cases [39]. RAS mutations have been associated with reduced expression of antigen processing pathway components in human tumour samples [18], although earlier studies did not reach this conclusion [19, 20], possibly due to technical differences between the studies. Our results reveal a direct role for oncogenic RAS in mediating these effects in human tumours. Treatment of tumour cells with IFN- $\gamma$  enhances cell surface expression of MHC class I and restores presentation of endogenous antigen to CTL [15]. Using the H<sup>WT</sup>/H<sup>Mu</sup> isogenic pair, Klampfer *et al* showed that oncogenic KRAS inhibited steady-state levels of the STAT1 and STAT2 transcription factors, resulting in reduced expression of genes encoding  $\beta$ 2M and MHC class I, amongst others [40]. We found that H<sup>Mu</sup> cells retain IFN- $\gamma$

responsiveness, as assayed by cell surface expression of MHC class I molecules. However, the response to IFN- $\gamma$  was impaired in H<sup>Mu</sup> compared to the isogenic H<sup>WT</sup> cells. Our results demonstrate that oncogenic RAS inhibits antigen presentation by targeting expression of multiple components of the pathway, with the net result of reducing MHC class I molecules at the cell surface. Our data using HLA-A2+ restricted MART-1 reactive T cells and MART-1 peptide pulsed tumour target cells was suggestive of reduced CTL responses against H<sup>Mu</sup>, but these results did not reach statistical significance. Overall, mutant KRAS inhibited the expression of cell surface HLA-A2 by less than two-fold and this difference did not significantly alter T cell recognition. However, the use of exogenous antigenic peptide in our assays may underestimate the effect of oncogenic RAS inhibition on antigen-specific CTL recognition since the inhibition of peptide transport into the ER will be negated. Furthermore, antigen presentation by HLA-A2 is not dependent upon TAP activity [41, 42], suggesting that CTL responses to TAP-dependent HLA molecules (and their associated peptides) may be more severely affected by oncogenic RAS activity than observed in our *in vitro* assays.

According to the missing self model, reductions in MHC class I expression are expected to result in increased susceptibility to NK cells. However, H<sup>WT</sup> and H<sup>Mu</sup> showed no differences in either their susceptibility to NK cell mediated killing or their ability to induce NK cell granule exocytosis. The possibility that MHC class I molecules expressed by the HCT116 background do not engage the KIR molecules of the NK cells used in these experiments is unlikely since HCT116 expresses both HLA-C1 and HLA-C2 epitopes (HLA-Cw7 and Cw5 respectively) and will thus engage KIR2DL1, 2DL2 and 2DL3, which are common amongst KIR haplotypes [43, 44]. Similar to the situation with CTL, it is likely that the relatively small inhibition



in cell surface MHC class I expression is insufficient to alter NK cell recognition. The existence of a threshold of NK cell activation/inhibition regulated by MHC class I levels [26, 45, 46] means that once MHC class I expression is below the threshold required for NK cell inhibition, any further reduction in its expression does not alter NK cell activation [26]. We envisage that the H<sup>WT</sup> cells, despite having more MHC class I at the cell surface than H<sup>Mu</sup>, still express MHC class I at a sub-inhibitory threshold level. In support of this, further reduction of cell surface MHC class I expression by H<sup>Mu</sup> (using a  $\beta$ 2M-specific siRNA) did not enhance killing (data not shown).

In colorectal cancer, patients whose tumours expressed high levels of MHC class I showed similar survival times to those whose tumours had lost MHC class I [47]. However, patients with reduced MHC class I expression had the worst prognosis of these three groups; the authors of this study suggest that reduced MHC class I levels on these tumours might be insufficient to mediate CTL recognition but are nevertheless still capable of inhibiting NK cell activity, rendering the tumour resistant to both classes of cytotoxic lymphocytes [47]. By inhibiting expression of multiple antigen presentation pathway components, mutant RAS molecules reduce total MHC class I expression levels at the cell surface. Expression of the major inhibitory ligands of NK cells, HLA-B and -C, is highly dependent upon TAP, tapasin and  $\beta$ 2M, all of which are downregulated by oncogenic RAS. Further investigation is required to determine the effects of oncogenic RAS on individual HLA molecules and how these might alter tumour cell recognition by both NK cells and CTL. Indeed, the differential regulation of HLA molecules is exploited by HIV, allowing infected cells to minimise their recognition by both CTL and NK cells [48] and, in melanoma, HLA expression patterns are believed to be shaped by immune

selection [49].

The mechanisms that underlie the expression of NKG2D ligands are beginning to be uncovered [50]. Oncogenic transformation itself is insufficient to induce NKG2D ligand expression [51]. However, a variety of stress signals (such as DNA damage and intracellular infection) result in expression of these ligands [32, 51, 52]. The existence of multiple NKG2D ligands presumably allows coupling of different stress signals to the expression of different ligands, providing the host with an efficient response system to eliminate damaged cells [32, 52]. Oncogenic RAS inhibited the expression of the NKG2D ligand ULBP2 in both the HCT116 and DLD1 backgrounds, yet in neither case did this alter their susceptibility to NK cells. This is most likely because NK cells express a large repertoire of activating and inhibitory receptors whose signals intersect to control activation [6, 7]. Either the change in ULBP2 expression was too small to manifest itself as altered susceptibility, or reduction of ULBP2 expression by oncogenic RAS was compensated for by reduced expression of an inhibitory ligand expressed in both the HCT116 and DLD1 backgrounds (i.e. a ligand other than MHC class I). The coupling of the RAS pathway to ULBP2 expression has been observed previously [53]; RAS activation leads to MAPK and MEK activation and MEK inhibition, like RAS inactivation, resulted in increased cell surface expression of MHC class I and ULBP2. Similarly, MEK inhibition resulted in increased recognition by CTL without altering susceptibility to NK cells. Furthermore, expression of STAT1 and ULBP2 was enhanced in MEK inhibitor treated HCT116 cells [53]. Interestingly, nuclear STAT1, MHC class I expression and the presence of infiltrating T cells are markers of good prognosis in colorectal cancer [54].

However, the inhibition of ULBP2 expression by the RAS/MEK pathway is

not universal; Liu *et al* demonstrated that oncogenic HRAS (G12V) transfection induced ULBP2 expression [55]. This difference may reflect cell type-specific, or potentially RAS gene-specific differences. For example, we studied the effect of oncogenic KRAS in the colorectal tumour cell lines HCT116 and DLD1, whereas Liu *et al* transfected oncogenic HRAS gene into MCF7 (breast cancer-derived cells) and adenovirus immortalised 911 and 293T cells. In addition to its KRAS G13D mutation, the Catalogue of Somatic Mutations in Cancer (COSMIC; [56, 57]) lists HCT116 as harbouring an oncogenic mutation in  $\beta$ -catenin (CTNNB1), whereas MCF7 has wild type KRAS and  $\beta$ -catenin alleles. Interestingly, both cell lines harbour somatic mutations in the catalytic subunit of phosphatidylinositol 3 kinase (PIK3CA) and activation of this pathway has also been linked to NKG2D ligand expression in infection and cancer [58]. The diversity of the mutational landscape in human cancer and the consequent dysregulation of numerous pathways will no doubt make overarching rules governing the expression of NKG2D ligands in cancer difficult to define. Less is known regarding the regulation of expression of DNAM-1 ligands [59]. Expression of the mouse *Pvr/Cd155* gene (encoding the ligand for mouse DNAM-1) is upregulated by oncogenic RAS via a MEK dependent pathway [60]. However, we did not find consistent changes in the expression of PVR (or the other DNAM-1 ligand, Nectin-2) in the HCT116 and DLD-1 based isogenic pairs.

The ability to delete oncogenic RAS from HCT116 and DLD1 reveals that this oncogene plays a non-essential role in the *in vitro* growth of tumour cell lines. More compelling is the *in vivo* use of the anti-Ras iDAb; induction prevents tumour initiation or halts the ongoing growth of the tumour, but does not cause regression of established tumours [22]. Hence, the concept of oncogene addiction does not apply in this instance [61]. This suggests that agents targeting RAS will need to be combined

with other strategies for effective therapy. Direct inhibition of RAS activity has not met with great success [62], but targeting of downstream signalling molecules (such as RAF and MEK) holds promise [63]. In addition, synthetic lethality screens have identified potential targets selective to RAS mutant cells [64]. We did not find statistically significant differences in the CTL recognition of H<sup>Mu</sup> and H<sup>WT</sup> cells. However, our results show that inhibition of oncogenic KRAS does lead to enhanced antigen presentation and this offers encouragement that future therapeutic strategies might enhance the anti-tumour activity of CTL *in vivo*, especially if targeted against TAP-dependent HLA/peptide combinations and/or if used in combination with inhibitors of other components of the RAS signalling pathway. Importantly, the interaction between the anti-RAS iDAb and oncogenic RAS disrupts the binding of PI3K to the GTP bound form of RAS and identifies this structural interface as a potential site for anti-RAS drug design [23].

**Acknowledgments:** This work was supported by Yorkshire Cancer Research, Cancer Research UK (via the Leeds CRUK Centre) and the UK Medical Research Council. JJE-J received a postgraduate scholarship from the Egyptian Government and JLM was supported by a Researcher Award from Yorkshire Cancer Research. We are grateful to those colleagues who provided cell lines and reagents for this study and for their comments on the manuscript.

## Figure Legends

**Figure 1.** Cell surface MHC class I expression in the presence or absence of oncogenic RAS.

(A) Total cell surface MHC class I (left panel) and HLA-A2 (right panel) expression by  $H^{Mu}$  and  $H^{WT}$  cells (as labelled). The ratio of expression (based on the geometric mean of fluorescence) of  $H^{WT}/H^{Mu}$  is indicated in brackets. The grey/black areas are unstained and isotype control stained cells. The data shown are representative of experiments repeated five times for MHC class I and twice for HLA-A2.

(B) Expression of antigen processing pathway components in  $H^{Mu}$  and  $H^{WT}$  cells determined by immunoblotting, CANX; calnexin, TAPBP; tapasin, TAP1; transporter of antigen processing-1 and B2M; Beta-2-microglobulin. Actin (ACT) is shown as a loading control in each case. Quantitative RT-PCR analysis demonstrated a two-fold reduction in  $\beta$ 2M mRNA levels in  $H^{Mu}$  compared to  $H^{WT}$  (data not shown).

(C) Relative expression of cell surface MHC class I (determined by flow cytometry) in response to IFN- $\gamma$  stimulation, with expression in unstimulated  $H^{Mu}$  cells assigned an arbitrary value of 1 relative unit (RU). Expression was inducible in  $H^{Mu}$  (dark line) but did not reach the levels achieved following mutant KRAS inactivation in  $H^{WT}$  (grey line). Standard deviation from the mean is indicated (based on triplicates) and the data shown is one of two independent experiments.

**Figure 2.** Cell surface MHC class I expression following anti-RAS iDAb induction.

Expression of GFP and MHC class I in cell lines stably transfected with the iDAb construct in the absence (-T) or presence (+T) of the inducer tetracycline. (A) Shows HCT116 cells harbouring the KRAS G13D (B) HT-1080 fibrosarcoma cells harbouring the NRAS Q61K mutation and (C) SW480 cells harbouring the KRAS

G12V mutation. In each case, the top panels show expression in the presence (+T, grey line) or absence (-T, dark line) of tetracycline induction in the total population of cells. In the bottom panels, expression was determined by gating on the cells expressing the highest (GFP<sup>hi</sup>) and lowest (GFP<sup>lo</sup>) amounts of GFP (as labelled), thereby analysing non-responding and responding cells separately within the population. The ratio of expression (based on the geometric mean of fluorescence) of GFP<sup>hi</sup>/GFP<sup>lo</sup> is indicated in brackets. For HCT116 cells (A), expression of total cell surface HLA class I and HLA-A2 was analysed. The data shown in Figure 2 are representative of three independent experiments.

**Figure 3.** CTL recognition of H<sup>Mu</sup> and H<sup>WT</sup> cells.

(A) *In vitro* primed T cells, enriched for reactivity against the MART-1 peptide prepared as described in the materials and methods. The plot shows the primed T cells co-stained with an HLA-A2 pentamer loaded with the MART-1 peptide and with an anti-CD8 antibody. The boxed area indicates the CD8<sup>+</sup> T cells with the strongest binding to the MART-1 peptide loaded HLA-A2 tetramer. This gate was set in conjunction with staining of the MART-1 primed T cells with a control HLA-A2 pentamer (not shown).

(B) For CTL responses, H<sup>WT</sup> or H<sup>Mu</sup> cells (HLA-A2<sup>+</sup>) were pulsed with either MART-1 or HER2/neu peptides as indicated and co-cultured with the T cells enriched for reactivity to MART-1. CTL responses were determined by analysis of intracellular IFN- $\gamma$  production by the MART-1 reactive T cells. The percentage of MART-1 reactive T cells producing IFN- $\gamma$  are indicated. We also analysed IFN- $\gamma$  production by the CD8<sup>+</sup>, HLA-A2/MART-1<sup>neg</sup> population of cells; no more than 2.1% of this population produced IFN- $\gamma$  in response to H<sup>WT</sup> or H<sup>Mu</sup> (data not shown),

indicating antigen-specificity in these assays.

(C) IFN- $\gamma$  production by MART-1 reactive CTL responding to MART-1 peptide loaded H<sup>WT</sup> or H<sup>Mu</sup> as indicated. This data was collected from four separate experiments. The error bars show standard deviation. An unpaired Student's T test revealed the differences to be non-significant.

**Figure 4.** NK cell recognition of H<sup>Mu</sup> and H<sup>WT</sup> cells.

(A) NK cell mediated killing of H<sup>WT</sup> and H<sup>Mu</sup> cells at differing E:T ratios (as indicated). The left hand panel shows killing by unstimulated NK cells, the right hand panel by IL-2 stimulated NK cells (with standard deviation based on triplicates).

(B) NK cell granule exocytosis (CD107+ cells), in response to H<sup>WT</sup> or H<sup>Mu</sup> cells using unstimulated or IL-2 treated NK cells .

(C) Cell surface MHC class I staining (using W6/32) of D<sup>Mu</sup> and D<sup>WT</sup> cells as indicated. The grey/black area is isotype control/unstained cells. These cells are in the DLD-1 background and are MHC class I negative due to a  $\beta$ 2M mutation [13].

(D) NK cell granule exocytosis (CD107+ cells), in response to D<sup>WT</sup> or D<sup>Mu</sup> using unstimulated or IL-2 treated NK cells at an E:T ratio of 1:1.

(E) NK cell mediated killing of D<sup>Mu</sup> and D<sup>WT</sup> at differing E:T ratios (as indicated). The left hand panel shows killing by unstimulated NK cells, the right hand panel by IL-2 stimulated NK cells. A), B), D) and E), show the standard deviation from the mean of three independent experiments, each performed in triplicate.

**Figure 5.** Cell surface expression of NKG2D and DNAM-1 ligands in the presence or absence of oncogenic RAS. The ligands and their respective receptor are indicated. The analysis was performed in the HCT116 (H<sup>WT</sup> and H<sup>Mu</sup>) and DLD1 (D<sup>WT</sup> and D<sup>Mu</sup>) backgrounds and is representative of two separate experiments. The unstained/isotype control stained cells are also shown.

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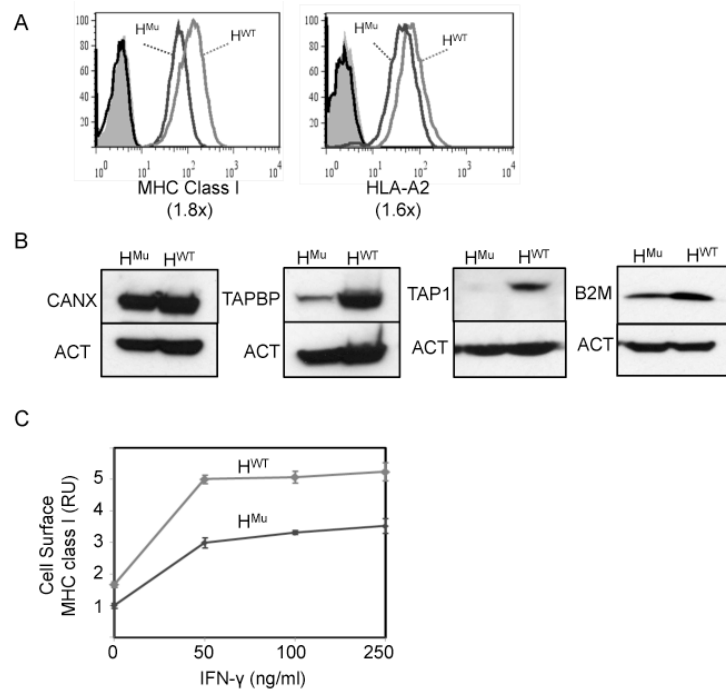
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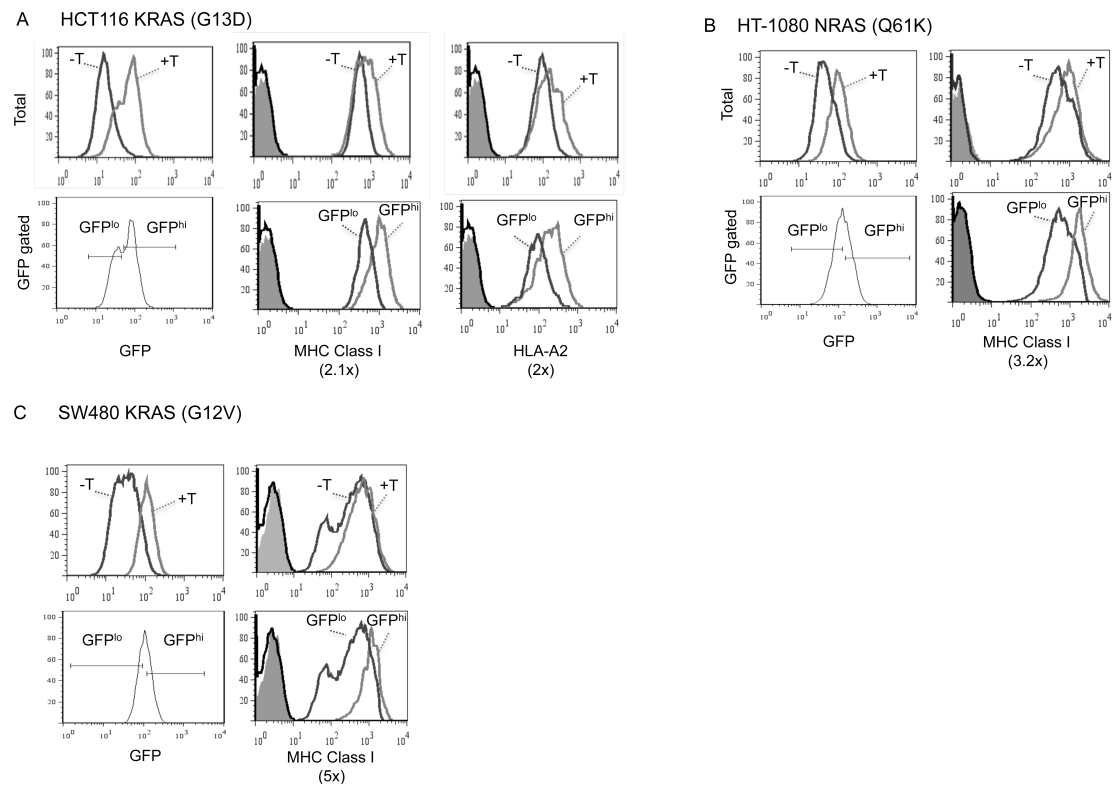
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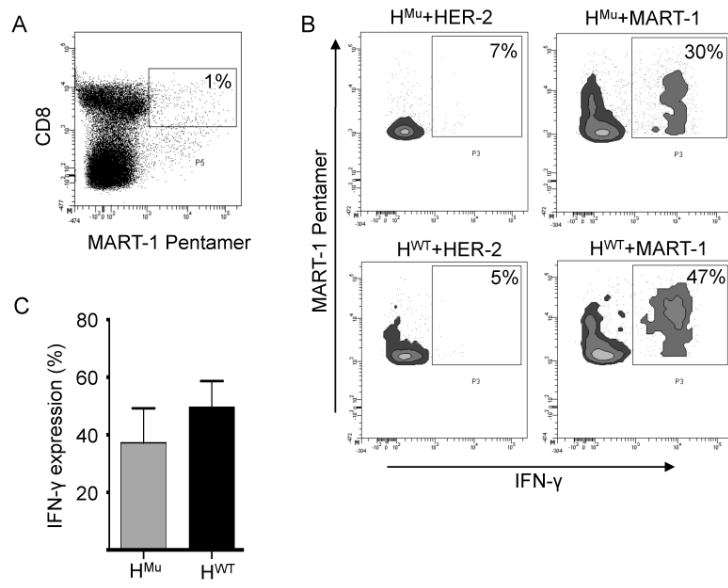
**Figure 1**



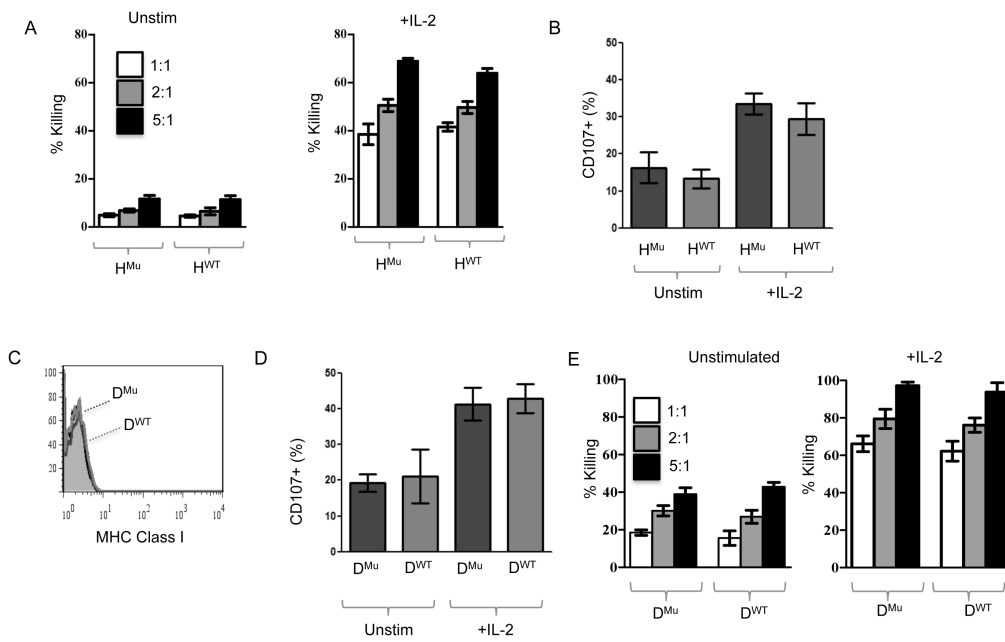
**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**

