ORIGINAL ARTICLE

Absent in Melanoma 2 (AIM2) is an important mediator of interferon-dependent and -independent *HLA-DRA* and *HLA-DRB* gene expression in colorectal cancers

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Absent in Melanoma 2 (AIM2) is a member of the HIN-200 family of hematopoietic, IFN-inducible, nuclear proteins, associated with both, infection defense and tumor pathology. Recently, AIM2 was found to act as a DNA sensor in innate immunity. In addition, we and others have previously demonstrated a high frequency of AIM2-alterations in microsatellite unstable (MSI-H) tumors. To further elucidate AIM2 function in colorectal tumors, we here addressed AIM2-responsive target genes by microarray based gene expression profiling of 22 244 human genes. A total of 111 transcripts were significantly upregulated, whereas 80 transcripts turned out to be significantly downregulated in HCT116 cells, constitutively expressing AIM2, compared with AIM2-negative cells. Among the upregulated genes that were validated by quantitative PCR and western blotting we recognized several interferon-stimulated genes (*ISGs*: *IFIT1*, *IFIT2*, *IFIT3*, *IFI6*, *IRF7*, *ISG15*, *HLA-DRA*, *HLA-DRB*, *TLR3* and *CIITA*), as well as genes involved in intercellular adhesion and matrix remodeling. Expression of *ISGs* correlated with expression of *AIM2* in 10 different IFN- γ treated colorectal cancer cell lines. Moreover, small interfering RNA-mediated knock-down of *AIM2* resulted in reduced expression of *HLA-DRA*, *HLA-DRB* and *CIITA* in IFN- γ -treated cells. IFN- γ independent induction of *HLA-DR* genes and their encoded proteins was also demonstrated upon doxycyclin-regulated transient induction of AIM2. Luciferase reporter assays revealed induction of the *HLA-DR* promoter upon AIM2 transfection in different cell lines. STAT-signaling was not involved in IFN- γ independent induction of *ISGs*, arguing against participation of cytokines released in an autostimulating manner. Our data indicate that AIM2 mediates both IFN- γ dependent and independent induction of several *ISGs*, including genes encoding the major histocompatibility complex (MHC) class II antigens HLA-DR- α and - β . This

suggests a novel role of the IFN/AIM2/*ISG* cascade likewise in cancer cells.

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Introduction

Interferon-inducible Absent in Melanoma 2 (AIM2), a member of the HIN-200 family of hematopoietic, IFN-inducible, nuclear proteins, was recently found to act as a DNA sensor in innate immunity (Schroder *et al.*, 2009). By direct binding to foreign double stranded DNA in infected macrophages, AIM2 triggers the assembly of an AIM2 inflammasome resulting in caspase-1-mediated inflammatory responses and cell death (Burckstummer *et al.*, 2009; Fernandes-Alnemri *et al.*, 2009; Hornung *et al.*, 2009; Roberts *et al.*, 2009). Moreover, the AIM2 inflammasome appears to be essential for host defense against DNA viruses and cytosolic bacteria, such as *Francisella tularensis* and *Listeria monocytogenes* (Fernandes-Alnemri *et al.*, 2010; Rathinam *et al.*, 2010; Sauer *et al.*, 2010). Although this activity places AIM2 function in the context of the cell's defense against infection, the role of AIM2 in carcinogenesis is less clear.

Both, tumor suppressive and tumor promoting functions of AIM2 have been reported. Exogenous AIM2 expression was shown to reduce human breast cancer cell proliferation by inhibition of NF- κ B transcriptional activity and to suppress mammary tumor growth in a mouse model (Chen *et al.*, 2006). In addition, AIM2 is frequently affected by genetic and epigenetic alterations in different human tumor entities: In pre-neoplastic and neoplastic microsatellite unstable (MSI) lesions, a coding 10-bp polyadenine region in Exon 6 of the *AIM2* gene appears to be positively selected for frameshift mutations (Michel *et al.*, 2010a; Woerner *et al.*, 2003, 2005). Finally, we identified additional mutations and epigenetic silencing of the *AIM2* promoter, the

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latter conferring insensitivity to IFN- γ -induced AIM2 expression in MSI colorectal cancers (Woerner *et al.*, 2007). How this aberrant or missing AIM2 expression affects tumor cell fate and tumor immunity is unknown. However, recent evidence suggests that the immune response modulates the fate of human tumors. Type, density and location of immune cells within human colorectal tumors were shown to predict clinical outcome (Galon *et al.*, 2006). Furthermore, the high local lymphocyte infiltration of MSI colorectal tumors, which is associated with a low frequency of distant metastases (Buckowitz *et al.*, 2005) suggests that inflammatory mechanisms, including induction of IFN- γ -regulated target genes are involved in tumorigenesis. Thus, considering the role of AIM2 in innate immunity and intracellular DNA recognition, we hypothesized that AIM2 expression in tumor cells may bridge cytokine release from tumor-infiltrating immune cells with tumor cell fate.

By restoration of AIM2 expression in AIM2-deficient colorectal cancer cell lines, we recently demonstrated that AIM2-mediated inhibition of cell proliferation is associated with accumulation of cells at late S-phase, resulting in G2/M arrest (Patsos *et al.*, 2010). Remarkably, persistent AIM2 expression affected adhesion of colorectal cancer cells to fibronectin and stimulated invasion through extracellular matrix-coated membrane in transwell assays (Patsos *et al.*, 2010). To further elucidate the immunomodulating AIM2 function in colorectal tumors, we here addressed AIM2-regulated target genes by microarray based gene-expression profiling of colorectal cancer cells. Our data indicate that AIM2 mediates IFN- γ -induced induction of several Interferon-stimulated genes (ISGs) including genes encoding the major histocompatibility complex (MHC) II antigens HLA-DR- α and - β .

Results

Persistent AIM2 expression results in induction of interferon stimulated genes, including HLA-DRA, HLA-DRB and (class II transactivator) CIITA

To investigate the modulation of gene expression in response to constitutive AIM2 expression we used

Affymetrix microarray analysis for studying transcriptional changes in the AIM2-deficient MSI-H tumor cell line HCT116. Two recently characterized AIM2-expressing subclones (D1 and B8; Patsos *et al.*, 2010) were chosen for comparison with MOCK-transfected HCT116 cells (subclone D3). Cluster analysis of the data revealed that D1 and B8 subclones are similar to each other but distinct from the control ((D3), data not shown). Among the 22 244 genes tested on arrays, 111 transcripts were significantly upregulated, whereas, 80 transcripts were significantly downregulated more than 1.4-fold in both AIM2-expressing cell subclones (Table 1 and Supplementary Tables 2 and 3). As expected, AIM2 was found to be the most upregulated gene, with a slightly higher expression level in subclone B8 (Supplementary Table 2). A number of induced genes appear to be linked to the immunomodulating function of AIM2, such as the IFN-stimulated genes (ISGs) *HLA-DRA*, *HLA-DRB*, *IFIT1*, *IFIT2*, *IFIT3*, *IFI6*, *IRF7*, *ISG15* and *TLR3* (Table 1 and Supplementary Table 2). Moreover, genes involved in intercellular adhesion and matrix remodeling (for example, *COL9A3*, *COL12A1*, *COL13A1*, *CSPG2*, *CTHRC1*, *ECM1*, *LOXL4*, *MCAM*, *NANOS1*, *TIMP2*, *VIM* and others) were found to be upregulated in response to constitutive AIM2 expression (Supplementary Table 2), which is in line with our previous findings of AIM2 affecting cell migration and invasion (Patsos *et al.*, 2010).

The gene expression profile in AIM2-transfected cells was validated by quantitative real-time PCR of selected genes from independent samples of subclones D1 and B8 versus the control. As shown in Figure 1a, upregulation of *VIM*, *CSPG2*, *MCAM*, *CTGF*, *FGF9*, *BMP4* and *CPA4* was confirmed in AIM2-expressing cells, whereas *ANXA10*, *GPR110*, *CDH1*, *NRIP*, *S100A14*, *GPR87*, *HMGCS1* and *CK20* were confirmed to be downregulated (Figure 1b and Supplementary Table 3). Moreover, increased expression of the ISGs was demonstrated by both, real-time PCR (Figure 1c) and by immunoblotting (Figure 1d) in AIM2-positive cells.

As transcription of genes encoding MHC class II antigens HLA-DR- α and - β is known to be tightly controlled by the CIITA and the essential RFX complex comprising RFX5, RFXAP and RFXANK (Harton and Ting, 2000; Krawczyk and Reith, 2006; Satoh *et al.*,

Table 1 Interferon-stimulated genes (ISGs) upregulated by AIM2

Unigene ID	Gene symbol	Description	Microarray		qRT-PCR	
			Fold change D1	Fold change B8	Fold change D1	Fold change B8
Hs.520048	<i>HLA-DRA</i>	Major histocompatibility complex, class II, DR- α	1.55	2.24	1.5	3.1
Hs.523847	<i>IFI6</i>	Interferon alpha-inducible protein 6	3.01	2.20	2.7	2.8
Hs.20315	<i>IFIT1</i>	Interferon-induced protein with tetratricopeptide repeats 1	3.27	2.16	2.8	4.3
Hs.437609	<i>IFIT2</i>	Interferon-induced protein with tetratricopeptide repeats 2	1.53	1.86	2.0	2.1
Hs.654405	<i>HLA-DRB</i>	Major histocompatibility complex, class II, DR- β 1	1.53	1.86	1.4	2.4
Hs.458485	<i>ISG15</i>	Interferon stimulated gene 15, ubiquitin-like modifier	1.58	1.62	1.3	1.7
Hs.47338	<i>IFIT3</i>	Interferon-induced protein with tetratricopeptide repeats 3	1.43	1.52	ND	ND
Hs.166120	<i>IRF7</i>	Interferon regulatory factor 7	1.53	1.50	1.4	1.9
Hs.657724	<i>TLR3</i>	Toll-like receptor 3	1.52	1.45	3.3	3.1

Figure 1 Validation of the expression profiles obtained by microarray analysis. A set of eight genes from each list of upregulated (a) or downregulated (b) AIM2 target genes, respectively, was selected for expression analysis by quantitative real-time PCR. (c) Real-time PCR analysis of ISGs. (d) Immunoblot analysis of AIM2, IFIT1, IFIT2 and HLA-DR- α , - β protein expression. Flag-AIM2 was detected by using an anti-FLAG antibody as described in the Materials and methods section. (e) Analysis of *CIITA*, *RFX5*, *RFXAP* and *RFXANK* gene expression by quantitative real-time PCR. Bars represent the mean and s.d. of three experiments. Black bars: fold expression of HCT116-tet-AIM2 clone D1 versus AIM2-negative HCT116 clone D3; gray bars: fold expression of HCT116-tet-AIM2 clone B8 versus AIM2-negative HCT116 clone D3.

2004), we were interested in expression of *CIITA* and its cofactors in response to AIM2. Although no induction was detected on the microarrays (data not shown), real-time PCR revealed a 15- and 20-fold induction of *CIITA* transcripts in AIM2-positive cell subclones D1 and B8, respectively, when a primer pair that corresponds to a region shared by all *CIITA* transcripts was used (Figure 1e). In contrast, expression of *RFX5*, *RFXAP* and *RFXANK* was unchanged. This suggests that *CIITA* expression might likewise be regulated by AIM2, thereby triggering induction of HLA-DR- α and - β .

Induction of ISGs by IFN- γ correlates with AIM2 expression in different colon cancer cell lines

On the basis of our observation that AIM2 mediates upregulation of a subset of ISGs, we assumed that IFN- γ -stimulated expression of these ISGs will also correlate with AIM2 expression. To determine the optimal time frame for IFN- γ treatment we performed a time response analysis in HT-29 cells, which revealed maximum transcript expression of *AIM2* and most ISGs within 24 h of treatment (Supplementary Figure 1A). HLA-DR- α and HLA-DR- β protein expression could be detected after 15 h, the level increasing with time (Supplementary Figure 1B). Figure 2 shows fold induction of *AIM2* and ISG transcripts in 10 colorectal cancer cell lines upon treatment with IFN- γ for 48 h versus untreated cells. Consistent with previous findings (Woerner *et al.*, 2007), the absolute *AIM2* transcript level (data not shown) and the magnitude of induction

were very low in Vaco-432, SW48 and Caco-2 cells; no *AIM2* induction was observed in (parental) HCT116 and RKO cells. In fact, expression of the ISGs was induced by IFN- γ only in cell lines where *AIM2* was induced as well, whereas none of the genes was upregulated in AIM2-negative cell lines RKO and HCT116. To further elucidate whether upregulation of ISGs and *HLA-DR* genes is AIM2 dependent and does not result from a more general unresponsiveness of AIM2-null cells to IFN- γ -signaling, we analyzed IFN- γ -induction of an interferon-inducible gene, *IFI16*, that is thought to be independent from AIM2 expression. As shown in Figure 2 (last panel), *IFI16* was clearly induced by IFN- γ to a similar degree in 9 of the 10 cell lines, including the AIM2-deficient HCT116 cells. In contrast to these cell lines, *IFI16* was strongly expressed in RKO cells even in the absence of IFN- γ (data not shown), therefore no further induction was detected upon IFN- γ treatment, here. We thus conclude that IFN- γ -signaling is basically intact in AIM2-null cells. This finding is in line with our recent study (Woerner *et al.*, 2007) that revealed IFN- γ -responsiveness of AIM2-deficient HCT116 cells. Taken together, our data further support the idea that AIM2 may have a role in IFN- γ -stimulated induction of target genes.

Downregulation of AIM2 by RNA interference results in reduced IFN- γ -induced expression of HLA-DRA, HLA-DRB and CIITA

The expression of class II MHC antigens is considered a crucial step in immune response towards cancer cells

including colorectal carcinomas (Matsushita *et al.*, 1996, 2006; Oshita *et al.*, 2006). We therefore focused our further analysis on the role of AIM2 in regulation of *HLA-DRA* and *HLA-DRB* expression. To elucidate whether AIM2 is compulsory for IFN- γ -mediated induction of MHC class II transcripts, we transiently knocked down *AIM2* in IFN- γ -treated HT-29 colorectal cancer cells by RNA interference (siAIM2-6, siAIM2-249 and siAIM2-500) targeting independent sequences in the *AIM2* transcript. The efficiency of knockdown was verified by real-time RT-PCR, showing that *AIM2* expression was reduced by three AIM2-specific small interfering RNAs (siRNAs) to 35%, 25% and 47% of the original IFN- γ -induced expression level (Figure 3a). Using a mix of the three siRNAs (Mix siAIM2 A), *AIM2* expression was reduced to 19% of the original IFN- γ -induced expression level, whereas nonsense siRNA and siRNA targeting an unrelated gene (siUn-related; Figure 3e) did not affect *AIM2* expression (Figure 3a). In the consequence of *AIM2* knockdown, the expression of *HLA-DRA* and *HLA-DRB* mRNAs was specifically decreased (Figures 3b and c), indicating that AIM2 is indeed required for IFN- γ -induced expression of these *MHC class II* genes. Likewise, IFN- γ -stimulated *CIITA* induction was diminished, albeit one of the siRNAs (siAIM2-249) was ineffective, here (Figure 3d).

AIM2 can induce HLA-DR expression even in the absence of IFN- γ and CIITA

Considering the upregulation of *CIITA* in consequence of persistent AIM2 expression, we assumed that AIM2 indirectly mediates induction of *HLA-DR* genes via *CIITA*. The *CIITA* gene is controlled by several distinct promoters, two of which direct constitutive expression in immune cells, whereas promoter IV mediates IFN- γ -stimulated expression of a specific *CIITA* transcript in epithelial cells (Muhlethaler-Mottet *et al.*, 1997). Using transcript-specific primers we could demonstrate that induction of *CIITA* in IFN- γ -stimulated HT-29 indeed results from the *CIITA* type IV product (*CIITA-p4*; Figure 4a). Moreover, IFN- γ -stimulated *CIITA-p4* expression was specifically reduced on knockdown of *AIM2* transcripts via RNA interference (Figure 4b), which points to involvement of AIM2 in *CIITA-p4* induction as well. Like it was observed for *CIITA* (all transcripts) siAIM2-249 was ineffective, here.

To elucidate whether short-term expression of AIM2 can trigger *HLA-DR* transcription in the absence of IFN- γ , we used a subclone of HCT116 expressing AIM2 under the control of a tetracycline-inducible promoter (HCT116-tetON-AIM2), in which high amounts of

Figure 2 Co-expression of *AIM2* and *ISGs* in eight colorectal cancer cell lines. For analysis, half of the cells from each cell line were treated with 200 U/ml IFN- γ for 48 h and half of the cells were left untreated. Following RNA-extraction and reverse transcription, expression of *ISG* transcripts was determined by quantitative real-time PCR analysis. Bars represent fold expression of mRNA in IFN- γ -treated versus untreated cells, showing the mean and s.d. of three independent experiments.

Figure 3 Downregulation of IFN-induced *AIM2* expression in HT-29 cells by siRNA results in reduced expression of target genes. Cells were transfected with siRNAs as indicated. After 24 h, 100 U/ml IFN- γ was added for another 48 h. Following RNA extraction and reverse transcription, expression of *AIM2* (a), *HLA-DRA* (b), *HLA-DRB* (c), *CIITA* (d) and an unrelated gene (e) was determined by quantitative real-time PCR analysis. Gene expression in cells that were treated with IFN- γ without addition of siRNA was set 100%. Bars represent the mean and s.d. of three independent experiments. Mix siAIM2 A: pooled siAIM2-6, siAIM2-249, siAIM2-500. Mix siAIM2 B: commercially available AIM2 siRNA (h) as described in the Materials and methods section.

AIM2 transcripts and protein are detectable within 6–12 h after addition of doxycycline (Figures 5a and b). In contrast to the D1 and B8 subclones, which persistently express low levels of *AIM2* and *CIITA*, neither *CIITA* nor *CIITA-p4* transcripts were detectable in HCT116-tetON-AIM2 cells in response to AIM2 expression (Figure 5a and data not shown). However, *HLA-DRA* and *HLA-DRB* transcription increased 2.8 and 5.1-fold, respectively after 24 h (Figure 5a). Furthermore, HLA-DR antigen expression was clearly detectable after 48 h of doxycycline-treatment and disappeared when the amount of AIM2 decreased again (Figure 5b).

We next investigated activation of the *HLA-DRA* promoter region in response to AIM2 by luciferase reporter assays in different cell lines. 293T, HCT116 and HT-29 cells were co-transfected with a luciferase reporter that is driven by a fragment of the *HLA-DRA* promoter region (pDRA-luc) and an expression vector encoding either EGFP or an EGFP-AIM2 fusion protein. The activity of the empty vector pGL3-basic was used for comparison. In AIM2-transfected cells, the relative activation of the *HLA-DRA* promoter was 6.1-, 3.1- and 2.6-fold higher compared with EGFP-transfected cells (Figure 6), consistent with the observation that AIM2 is able to induce HLA-DR. In accordance to our previous data, the *HLA-DRA* promoter was not activated by IFN- γ in HCT116 cells, whereas it was strongly induced in HT-29 and 293T cells. Although the

magnitude of *HLA-DR* induction without *CIITA* participation is lower in AIM2-transfectants than upon IFN- γ induction, the above experiments argue for a *CIITA*-independent mechanism of AIM2-mediated HLA-DR induction.

AIM2 action does not involve STAT-signaling for induction of target genes, which argues against an autocrine production of IFNs or other cytokines

As demonstrated by our data, *CIITA* was induced in HCT116 subclones D1 and B8 that persistently express AIM2, but not upon transient AIM2 expression in the absence of IFN- γ . This suggested that persistent AIM2 expression might result in release of factors such as IFNs and other cytokines into the medium, thereby activating expression of *CIITA* and ISGs in an auto-stimulating manner. We therefore analyzed Janus kinase-signal transducers and activators of transcription (JAK-STAT)-signaling in the D1 and B8 subclones in comparison with HT-29 on treatment with IFN- γ by immunoblotting. The JAK-STAT pathway is activated in response to numerous cytokines via specific receptors resulting in a phosphorylation cascade and STAT homo/hetero dimerization (Aaronson and Horvath, 2002). STAT phosphorylation thus indicates activation of the JAK-STAT pathway by the majority of cytokines, including IFNs. As shown in Figure 7a, AIM2 expression did

Figure 4 The increased level of *CIITA* results from transcriptional activation from promoter p4. (a) Time course of *CIITA*-p4 mRNA expression in HT-29 cells upon induction with 100 U/ml IFN- γ . (b) Downregulation of IFN- γ -induced *AIM2* expression in HT-29 cells by siRNA results in reduced expression of *CIITA*-p4. Experiments were performed as described in Figure 3.

not result in phosphorylation of STATs 1, 2, 3, 5 and 6 in untreated HCT116 subclones D1 and B8. This indicates that production and autostimulating mechanisms of cytokines are not involved in AIM2-mediated activation of *CIITA* and *ISGs*. The treatment of the cells with IFN- γ resulted in a transient phosphorylation of STAT1 and STAT3 in each of the cell lines, HT-29, HCT116 parental cells and subclones D1 and B8, irrespective of AIM2 expression, with a maximum phosphorylation after 1 h of treatment (Figures 7a and b). Interestingly, the total amount of STAT1 increased upon IFN- γ treatment in each of the cell lines, reaching a peak at 24 h, whereas the total amount of STAT3 remained unaffected (Figure 7b). However, again these effects were independent from AIM2 expression. In addition, STAT phosphorylation/activation were not detectable in doxycyclin-inducible HCT116-tetON-

Figure 5 Induction of AIM2, HLA-DRA and HLA-DRB in HCT116-tetON-AIM2 in the absence of *CIITA* expression. (a) Fold induction of mRNA expression in Doxycyclin-inducible HCT116-tetON-AIM2 cells. (b) Immunoblot analysis, showing induction of AIM2 (using an antibody that detects overexpressed AIM2; anti-AIM2, B01P) and HLA-DR protein in HCT116-tetON-AIM2 cells upon induction with doxycyclin.

AIM2 cells (data not shown). In summary, this argues for an AIM2-mediated mechanism of induction that proceeds independently from cytokine-mediated induction of *ISGs*.

AIM2 does not confer activation of caspase-1 or IL-1 β in colorectal cancer cells

Considering the recently identified role of AIM2 in activation of inflammasome-mediated cell death in response to cytoplasmic DNA (Burckstummer *et al.*, 2009; Fernandes-Alnemri *et al.*, 2009; Hornung *et al.*, 2009; Roberts *et al.*, 2009), we investigated activation of caspase-1 and its substrate interleukin 1 β (IL-1 β) in response to AIM2 expression in colorectal cancer cells. Western blot analysis revealed induction of pro-Caspase-1 expression in HT-29 cells, HCT116 parental cells and AIM2-expressing subclones D1 and B8 upon IFN- γ treatment (Figure 8). In agreement with previous findings (Patsos *et al.*, 2010), pro-Caspase-1 was not detected in untreated colorectal cancer cell lines. No difference was observed between the pro-Caspase-1 expression level in parental HCT116 cells and AIM2-transfected subclones D1 and B8, indicating that this induction is not caused by AIM2, but rather results from other IFN- γ -mediated mechanisms. Moreover, no activation of caspase-1, as indicated by emergence of cleaved IL-1 β (p17), was observed, neither in response to IFN- γ nor as a result of AIM2 expression (Figure 8). In fact, IL-1 β precursor was not expressed in any of the cell lines independent from treatment or AIM2-status. We therefore conclude that AIM2 function does not

Figure 6 Luciferase reporter assays showing induction of the *HLA-DRA* promoter upon transient AIM2 expression and induction with IFN- γ in different cell lines. Cells were co-transfected with either empty vector (pEGFP) or an AIM2 cDNA (pEGFP-AIM2) in combination with either a luciferase reporter driven by a fragment of the *HLA-DRA* promoter (pDRA-luc) or an empty reporter construct (pGL3basic). 100 U/ml IFN- γ was added for 24 h as indicated. Bars represent the mean and s.d. of three experiments. Fold induction is expressed in relation to luciferase activity in MOCK-transfected, un-treated cells.

involve activation of caspase-1 or IL-1 β in colorectal cancer cells and in this regard apparently behaves differently in macrophages.

Discussion

The impact of induced AIM2-expression on colorectal tumors is largely unknown. Using a microarray expression profiling approach and subsequent functional analysis in colorectal cancer cells, we here present an evidence that AIM2 mediates induction of genes that were previously shown to be stimulated by IFN- γ . The idea that AIM2 may have a role in IFN- γ action was originally stated by De Young *et al.* who detected increased *AIM2* mRNA levels in HL-60 promyelocytic leukemia cells after IFN- γ treatment (DeYoung *et al.*, 1997). Response to IFNs and subsequent expression of *ISGs*, in particular expression of class II MHC antigens (HLA-DR, -DQ, -DP) is considered a crucial step in immune response towards colorectal carcinomas (Matsushita *et al.*, 1996, 2006; Pfizenmaier *et al.*, 1985). Accordingly, our finding that AIM2 is involved in induction of these genes adds important information to understanding the impact of inflammatory cytokines on tumor cells. Until now, AIM2 has been primarily identified to act as a DNA sensor in macrophages that induces a machinery of defense mechanisms against cytosolic bacteria and DNA viruses via innate immunity (Burckstummer *et al.*, 2009; Fernandes-Alnemri *et al.*, 2009; Hornung *et al.*, 2009; Jones *et al.*, 2010; Rathinam *et al.*, 2010; Roberts *et al.*, 2009; Tsuchiya *et al.*, 2010). For the first time we present the evidence that AIM2 may likewise have a role in epithelial cells responding to inflammatory processes during tumor progression. Most

notably, we show that AIM2 is required for IFN- γ -dependent induction of HLA-DR- α and - β on colorectal cancer cells, thus linking AIM2 function with adaptive immunity as well.

The relationship between HLA-DR antigen expression on cancer cells and prognosis has been pointed out repeatedly. Although HLA-DR antigen expression is barely detectable in normal colorectal epithelium, strong HLA-DR antigen expression on cancer cells relates to better prognosis of colorectal cancer patients (Andersen *et al.*, 1993; Diederichsen *et al.*, 2003; Lovig *et al.*, 2002; Matsushita *et al.*, 2006; Walsh *et al.*, 2009). Consequently, regulation and impairment of HLA class II antigen expression in colorectal tumors is subject of intensive investigations (Garrrity-Park *et al.*, 2009; Michel *et al.*, 2010b; Satoh *et al.*, 2004). Constitutive expression of HLA class II antigens is restricted to 'professional antigen-presenting cells', but can be induced on various tissues by IFN- γ . This tissue-specific expression pattern is mainly determined by a highly specialized molecular machinery that involves interaction between ubiquitous DNA-binding transcription factors and the non-DNA-binding coactivator CIITA. CIITA comprises three cell-type specific protein isoforms, depending on which of three alternative promoters is used for transcription (Harton and Ting, 2000). In epithelial cells, IFN- γ activates transcription of CIITA from promoter p4 through JAK/STAT signaling pathway. Upon interaction with several co-factors, including the essential RFX complex, CIITA then activates the HLA class II promoter (Krawczyk and Reith, 2006).

In accordance with this, we could demonstrate the concerted induction of *CIITA* and *HLA-DRA*, and *B* transcripts in 8 out of 10 colorectal cancer cell lines stimulated with IFN- γ , whereas neither of these genes was induced in IFN- γ -responsive, AIM2-null

Figure 7 STAT phosphorylation in IFN- γ treated and AIM2-transfected cells. Cells were treated with 100 U/ml IFN- γ for the indicated times followed by cell lysis and immunoblotting as described in the Materials and methods section. (a) AIM2 expression does not result in STAT-phosphorylation in untreated HCT116 subclones D1 and B8. (b) STAT-phosphorylation and increased STAT1 expression are induced by IFN- γ , but are independent from AIM2 expression.

cells. The IFN- γ -stimulated induction was significantly decreased when AIM2 was downregulated by RNA interference. In addition, *CIITA* and *HLA-DR* transcription appeared to be induced by AIM2 that was derived from constitutive overexpression in colorectal cancer cells in the absence of IFN- γ . AIM2 may thus be considered as a co-factor of *CIITA* for transcriptional activation of *HLA class II* genes or rather indirectly by regulating *CIITA* gene expression, which in turn activates transcription of *HLA class II* genes. Our data showing that AIM2 downregulation results in reduced IFN- γ -stimulated *CIITA* transcription argues for a role of AIM2 in transcription of the

Figure 8 Caspase-1 and IL-1 β activities in colorectal cancer cells. Cells were treated with 100 U/ml IFN- γ followed by cell lysis and immunoblotting as described in the Materials and methods section. *Control: For detection of Caspase-1, a lysate from a Morbus Crohn patient was used; For detection of IL-1 β precursor and IL-1 β p17, an extract from bacterial lipopolysaccharide (LPS)-treated THP-1 cells (New England Biolabs, Frankfurt, Germany) was used for control.

CIITA gene. On the other hand, we demonstrated a direct induction of HLA-DR- α and - β expression without participation of *CIITA*, when AIM2 was transiently induced in HCT116-tetOn-AIM2 cells. Furthermore, the *HLA-DR* promoter was activated in AIM2-transfected cells, even in the absence of IFN- γ . As the magnitude of HLA-DR induction without *CIITA* participation was much lower than after IFN- γ -induction in IFN- γ -responsive cells, we assume that full activation of HLA-DR transcription requires the presence of both, *CIITA* and AIM2.

Interestingly, *CIITA* and AIM2 both appear to be evolutionarily related to a group of receptors for the recognition of intracellular pathogens. *CIITA* shares structural features with the nucleotide-binding oligomerization domain family of proteins (Krawczyk and Reith, 2006), which function as cytosolic sensors for the induction of apoptosis, as well as for innate recognition of microorganisms and regulation of inflammatory responses in mammalian cells (Inohara and Nunez, 2003). AIM2 was recently shown to share the functions of nucleotide-binding oligomerization domain-like receptors by forming inflammasomes, which mediate caspase-1-activation in response to intracellular pathogens (Brennan and Bowie, 2010). So far it is unknown, whether *CIITA* and AIM2 likewise share some functional roles in adaptive immunity, such as activation of HLA class II-mediated antigen expression and presentation to CD4⁺ T cells. Unfortunately, the demonstration of protein-protein interaction or co-expression of endogenous AIM2 and *CIITA* is currently hindered by the lack of experimental tools, such as specific antibodies detecting either protein via immunoblots or immunohistochemistry (Supplementary Figure 2 and data not shown).

Regardless of the precise mechanism of HLA-DR induction mediated by AIM2, our data add to understanding alterations of HLA class II antigen expression in colorectal tumors. Deficiency of HLA class II antigen

expression despite the presence of HLA-class II-inducing factors, such as IFN- γ , had previously been reported to occur in about one third of primary MSI-H colorectal carcinomas and in more than half of microsatellite stable colorectal carcinomas (Lovig *et al.*, 2002; Michel *et al.*, 2010b). Moreover, reduced or absent IFN- γ -stimulated induction of HLA class II transcripts and/or protein had been demonstrated in colorectal cancer cell lines HCT116, RKO and Caco2 (Michel *et al.*, 2010b; Satoh *et al.*, 2004), which is in line with our findings. The underlying mechanisms of this deficiency, however, have been elucidated only in part. The lack of HLA class II expression in primary colon tumors has been attributed to mutations in several regulatory genes, such as *RFX5* (26.5 %) and *CIITA* (2.9%) (Michel *et al.*, 2010b). In addition, epigenetic silencing of *CIITA* by promoter VI methylation was identified in gastric cancers (Satoh *et al.*, 2004) and in cell lines HCT116, RKO and Caco2 (Michel *et al.*, 2010b; Satoh *et al.*, 2004). Here, we present the evidence for an additional mechanism interfering with HLA class II antigen expression in cancer cells, namely alterations in AIM2 expression. We have previously shown that AIM2 promoter hypermethylation conferred insensitivity to IFN- γ -induced AIM2 expression of some MSI-H colon cancer cell lines. Complete silencing of the AIM2 promoter region analyzed was observed in HCT116, whereas considerably reduced AIM2 transcript expression and partial silencing of the AIM2 promoter was found in SW48, Vaco432, RKO and Caco2 cells (Woerner *et al.*, 2007) and unpublished data). Defects in AIM2 expression, as confirmed in the present study, may thus contribute to the low *HLA-DRA* transcript expression in IFN- γ -stimulated cells. Immunohistochemical analysis may help to support these findings as a mechanism that is also relevant in primary colorectal cancer tissue, as soon as specific AIM2 antibodies will be available.

Our hypothesis that AIM2 has an important role in cytokine mediated immunomodulation of colorectal cancer cells was further supported by the observation that AIM2 mediates induction of a subset of ISGs in different colorectal cancer cell lines. *IFIT1*, *IFIT2*, *IFIT3*, *IFI6*, *IRF7*, *ISG15* and *TLR3* transcripts were upregulated upon constitutive AIM2 expression and/or in eight colorectal cancer cell lines, in which AIM2 was induced by IFN- γ . The precise mechanism of this gene induction remains to be elucidated, as we cannot conclude from our data whether the upregulation is directly induced by AIM2 or whether it involves intermediate mechanisms, such as participation of *CIITA* or other factors. Preliminary analysis of HCT116-tetON-AIM2 cells revealed a maximum induction of ISG expression at 24–48 h after doxycycline-stimulated expression of AIM2 in the absence of *CIITA* (data not shown), thereby correlating with the here reported time course of HLA-DR expression in these cells.

The physiological significance of AIM2-mediated upregulation of *IFIT1*, *IFIT2* and *IFIT3* is difficult to evaluate, as the function of these genes is poorly understood. The *IFIT1/ISG56* gene family is known to be activated in response to interferons, as well as by

a multitude of viruses through involvement of IFN-regulatory factors (IRFs), with and without functional JAK/STAT signaling and IFN- γ action (Fensterl and Sen, 2011). The latter is in good agreement with our observation that AIM2 was capable to induce ISGs in the presence or absence of IFN- γ and without using JAK/STAT signaling. The function of *IFI6*, also known as *GIP3*, is likewise largely unknown. It was reported to be overexpressed in human senescent fibroblasts and to inhibit mitochondrial-mediated apoptosis in cancer cells (Cheriyath *et al.*, 2007; Tahara *et al.*, 2005). IRF-7 belongs to a family of interferon regulatory factors, which interact with IFN-stimulated response elements in promoters of IFN-responsive target genes (reviewed in (Zhang and Pagano, 2002)). Among these target genes, there are many additional members of the *IFNA* gene family, encoding type I interferons. The *IRF7* gene has previously been shown to be activated from a basal level by endogenous or exogenous IFN- α , bacterial lipopolysaccharide and some viruses. In contrast to previous studies, in which *IRF7* gene expression was restricted to induction by IFN- α/β in lymphoid cell types (Marie *et al.*, 1998), we clearly demonstrated *IRF7* upregulation in colorectal cancer cells in response to IFN- γ and AIM2 expression, and may thus expand IRF-7 function to cancer cells. *ISG15* is known as one of the earliest ISGs induced by type I IFNs. The encoded ISG15 protein is an ubiquitin-like protein that targets many components of the antiviral innate immune response (reviewed in (Pitha-Rowe and Pitha, 2007)). Recently, ISG15 conjugation was shown to be involved in ubiquitination of β -catenin, thereby suppressing Wnt/ β -catenin signaling activity, the ‘driving force’ of tumorigenesis in colorectal cancer tissues (Lee *et al.*, 2010). In addition to being a post-translational protein modifier, ISG15 is also a cytokine that is secreted from immune cells and epithelial cancer cell lines and was shown to activate monocyte-derived dendritic cells and cytolytic activity of NK cells (D’Cunha *et al.*, 1996a, 1996b). Finally, *TLR3* encoding toll-like receptor 3, serves as an important signaling receptor for the recognition of dsRNA for the triggering of antiviral and inflammatory responses to combat viral infections. Whether these ISG functions are important in the context of AIM2 expression in cancer cells remains to be investigated.

In conclusion, our data place AIM2 in a signaling cascade, mediating the biological effects of interferons that result in expression of immunomodulating target genes. Although the AIM2-regulated ISGs and AIM2 itself were previously associated with innate immune response to pathogens, our findings suggest a novel role of the IFN-AIM2-ISG-machinery in cancer cells. Our data provide a functional evidence that AIM2 is required for induction of some ISGs, as well as *HLA-DRA*, *HLA-DRB* and the master regulator *CIITA* in colorectal tumor cells. In contrast to macrophages, AIM2 action appears to not involve Caspase-1 or IL-1 β activities, here. Further *in vivo* and *in situ* studies will help to understand the biological significance of these interactions for the susceptibility of tumor cells towards anti-tumoral immune response.

Materials and methods

Cell lines and culture conditions

Colorectal cancer cell lines LoVo, KM12, HCT116, SW48, LS174T, LS180, RKO, Vaco-432, Caco-2 and HT-29 have been described previously (Woerner *et al.*, 2001, 2007). Construction of HCT116-tet-AIM2 (subclones D1 and B8) and HCT116tTA-Hyg (subclone D3) has been described in (Patsos *et al.*, 2010). All cell lines were grown in RPMI 1640 (Invitrogen, Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin using standard conditions. INF- γ was added as indicated in the figures.

RNA extraction, Affymetrix microarray analysis, cDNA synthesis and real-time PCR analysis

RNA was extracted from the indicated cell lines using RNeasy Kit (Qiagen, Hilden, Germany). Samples of two independent HCT116-tet-AIM2 subclones (D1 and B8) and Mock-transfected, AIM2-deficient HCT116-tTA-hyg cells (subclone D3) were subjected to oligonucleotide microarray analysis using GeneChip HG-U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) according to manufacturer's recommendation. Raw data obtained from microarray were reported to GEO (<http://www.ncbi.nlm.nih.gov/geo/>), sample number GSE24155. Microarray data were annotated using a Unigene based customer CDF-file (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF7CDF_download.asp), version 10) and raw signals were quantile normalized. A mixed model of ANOVA for differential gene expression was performed with the JMP genomics (SAS) software (SAS, Cary, NC, USA).

For cDNA synthesis, 1–2 μ g of total RNA was reverse transcribed using oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen, Life Technologies, Karlsruhe, Germany) following the manufacturer's instructions. Power-SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany) was added to appropriate cDNA samples and primers (Supplementary Table 1). Samples were loaded onto 96-well PCR plates and analyzed in an ABI Prism 7300 thermo-cycler (Applied Biosystems, Darmstadt, Germany) with the following cycling program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Quantitative analysis of gene expression was performed relative to expression of 18S RNA in corresponding samples by using individual standard amplification curves of each transcript. For undetectable levels of transcripts, we used a baseline of 40 cycles for calculation.

Western blotting

Cells were harvested on ice in cell lysis buffer (25 mM Tris-PO₄ pH 8.0; 2 mM DTT; 2 mM CDTA; 10% Glycerin; 1% Triton-X-100) supplemented with protease inhibitor cocktail (Complete EDTA-free, Roche, Mannheim, Germany) and 1 mM Na₃VO₄. Cell lysis was performed by two freeze-thaw cycles. For immunoblotting, equal amounts of cell lysates were separated on a 10–12.5% SDS-polyacrylamide gel and blotted on nitrocellulose by semi-dry electroblotting. Equal loading was verified by staining with Ponceau S solution, and blots were blocked for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween-20 (TBST); 5% non-fat dry milk) before primary antibodies were added in blocking buffer (containing 5% non-fat milk for mouse antibodies and 5% BSA for rabbit-antibodies) for overnight incubation at 4 °C. After four times washing in TBST, appropriate peroxidase-coupled secondary goat or rabbit antibodies (Dianova, Hamburg, Germany) were added in blocking buffer for one hour. After six times washing

in TBST, signals were detected by chemoluminescence (Western Lightning Plus ECL, PerkinElmer LAS, Rodgau, Germany).

Antibodies

Anti-FLAG (mouse monoclonal, clone M2, Sigma-Aldrich, Taufkirchen, Germany); Anti-AIM2 (purified MaxPAb polyclonal antibody B01P, Abnova, Biozol, Eching, Germany), dilution 1:500. This antibody can detect overexpressed AIM2 but not endogenous AIM2 (Fernandes-Alnemri *et al.*, 2009) and our own unpublished observations). Anti-IFIT1 (mouse polyclonal, Abcam, Cambridge, UK), dilution 1:1000; Anti-IFIT2 (MaxPab mouse polyclonal antibody, Abnova, Biozol Eching, Germany), dilution 1:1000; Anti-actin (mouse monoclonal antibody, clone C4; MP Biomedicals, Aurora, OH, USA), dilution 1:5000. Anti-HLA class II (monoclonal antibody LGII-612.14) was kindly provided by Soldano Ferrone, Department of Microbiology and Immunology, New York Medical College, NY, USA (Temponi *et al.*, 1993). The Phospho-Stat Antibody sampler kit (Cell Signaling Technology, NEB, Frankfurt, Germany) was used for detection of Phospho Stat1 (Tyr701), Phospho Stat2 (Tyr690), Phospho Stat3 (Tyr705), Phospho Stat3 (ser727), Phospho Stat5 (Tyr694) and Phospho Stat6 (Tyr641) as recommended by the manufacturer. Anti-STAT1, anti-STAT3 and anti-IL-1 β , dilution 1:1000, were purchased from Cell Signaling Technology, NEB, Frankfurt, Germany; Anti-caspase-1, dilution 1:500, was derived from Merck Biosciences (Darmstadt, Germany).

RNA interference

HT-29 cells were plated at 1.5×10^5 cells in 24-well plates and immediately transfected with siRNA duplexes targeting AIM2 as indicated in the figures at a final concentration of 100 nM using 9 μ l/well HiPerFect transfection reagent (Qiagen, Hilden Germany). siRNAs were (sense strand) siAIM2-6: 5'-CCCG AAGAUCAACACGCUUCA-3', siAIM2-249: 5'-GAGAGU AAAUACAAGGAGA-3', si-AIM2-500: 5'-GGAGAAAGU UGAUAAGCAA-3'. AllStars Negative Control ('nonsense') siRNA was derived from Qiagen, Hilden Germany; AIM2 siRNA (Santa Cruz Biotechnology, Heidelberg, Germany), AIM2 siRNA (h), representing a pool of three target-specific 19–25 nt siRNA duplexes (Santa Cruz Biotechnology, Heidelberg, Germany) was used as a positive control. In addition, siRNA targeting an irrelevant gene (siUnrelated) was used for control. After 24-h transfection, cells were stimulated for 48 h with 100 U/ml IFN- γ .

Generation of HCT11-tetON-AIM2 cells

A multistep cloning strategy was pursued to generate a doxycycline-inducible flag-tagged AIM2 expression construct (pTRE-Tight-BI-DsRedExpress-FH-AIM2(wt)). The AIM2 cDNA was amplified by PCR using primers that harbor NheI or NotI restriction sites (sense primer AIM2-NheI: 5'-CCCC CGCTAGCATGGAGAGTAAATACAAGGAGAT-3'; antisense primer AIM2-NotI: 5'-CCCCCGCGCCGCGAGAG GAGCCTGTGAACTGC-3'). The NheI/NotI digested AIM2 cDNA amplicon was subsequently cloned into NheI/NotI digested plasmid pFH-IRESneo (Robert Roeder, The Rockefeller University, New York, NY, USA), thereby generating plasmid pFH-AIM2-IRESneo that contains the Flag-HA-AIM2 expression cassette. Bacterial clones that harbor recombinant plasmids were identified by colony PCR and sequence analysis of the expression cassette. After KpnI/NotI digestion of pFH-AIM2-IRESneo, the 1257 bp Flag-HA-AIM2 expression cassette was cloned into KpnI/NotI digested pTRE-Tight-BI-DsRed-Express plasmid (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). Recombinant bacterial clones were identified by colony

PCR and sequencing across the insert cloning sites. For construction of double-stable cell lines inducibly expressing the above construct, the Tet-On colorectal cancer cell line HCT116-AWE17 (Welman *et al.*, 2005) was used as a background. 10^7 cells were transfected with $10\ \mu\text{g}$ of pTRE-Tight-BI-DsRed-FH-AIM2 expression plasmid using electroporation (AMXA Nucleofector I, solution V). Cells were seeded onto 10-cm dishes and subjected to G418 selection (PAA Laboratories, $600\ \mu\text{g}/\text{ml}$) for 3 weeks. Single-cell clones were obtained by limiting dilution of pooled G418-resistant clones from each plate. Double stable DsRed-positive clones were identified upon doxycycline induction ($1\ \mu\text{g}/\text{ml}$; 24 h) by fluorescence microscopy.

Analysis of HLA-DRA promoter activity by luciferase reporter assays

Cells were grown at a density of 2×10^5 cells in 24-well plates for 24 h. For reporter assays, cells were transfected with equal amounts ($300\ \text{ng}/\text{well}$) of pGL3-basic or pDRA-luc, respectively, using Eugene HD transfection reagent (ROCHE Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. pDRA-luc containing a 6.1 kb fragment of the HLA-DRA-promoter region driving a luciferase gene (Osborne *et al.*, 2001), was kindly provided by George Blanck, University of South Florida, Tampa, FL, USA. Expression plasmids encoding EGFP or EGFP-AIM2, respectively, (Patsos *et al.*, 2010) were added as indicated in the figures. For normali-

zation of transfection efficiency, 60 ng of a beta-galactosidase-encoding reporter (pRSV-lacZ) was included in each sample. Non-coding DNA was used to fill all transfection samples to a total of 600 ng per well. $100\ \text{U}/\text{ml}$ of IFN- γ was added as indicated in the figures. Transfected cells were incubated for 24 h, lysed and collected for reporter assays as described previously (Dihlmann *et al.*, 1999) with the following modification: for beta-galactosidase reporter assays, $15\ \mu\text{l}$ of each lysate was added to $100\ \mu\text{l}$ of assay buffer ($60\ \text{mM}\ \text{Na}_2\text{HPO}_4$, $40\ \text{mM}\ \text{NaH}_2\text{PO}_4$, $10\ \text{mM}\ \text{KCl}$, $1\ \text{mM}\ \text{MgSO}_4$, $2.5\ \text{mM}\ \text{EDTA}$ and $3\ \mu\text{l}/\text{ml}$ beta-Mercaptoethanol) and $25\ \mu\text{l}$ of Chlorophenol Red- β -D-galactoside (CPRG, Sigma-Aldrich, Taufkirchen, Germany) Colorimetric detection was performed at 570 nm using a photometer. For comparison of promoter activity, basic induction of pGL3-Basic was used as a reference and set as 1.0.

Conflict of interest

The authors declare no conflict of interest

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