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Chapter 7: Chemical and genetic control of IFN γ -induced MHCII expression

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Under review

Abstract

The cytokine Interferon- γ (IFN γ) can induce expression of MHC class II (MHCII) on non-hematopoietic cells, leading to antigen presentation to CD4⁺ T-cells and immune activation, which has been linked to anti-tumour immunity and graft-versus-host disease. The extent of MHCII upregulation by IFN γ is highly variable between cell types and under extensive control by epigenetic regulators and signalling pathways. Here, we identified novel determinants in the control of MHCII expression. Loss of the oxidative stress sensor Keap1 and autophagy adaptor p62/SQSTM1 impaired IFN γ -mediated MHCII expression, by decreasing the levels of histone acetylation. A similar phenotype was observed for arsenite, an oxidative stressor, and these phenotypes could be reversed by inhibition or depletion of histone deacetylase 1/2, suggesting that oxidative stress conditions are translated into epigenetic alterations that affect MHCII expression. This is further illustrated by dimethylfumarate, an antioxidant used for the treatment of several autoimmune diseases, which impairs the IFN γ response. Using an integrated chemical genetics approach, we identified novel pathways and associated drugs controlling IFN γ -mediated MHCII expression, which provides a molecular basis for the treatment of MHCII associated auto-immune diseases.

Introduction

Antigen presentation by Major Histocompatibility Complex class II (MHCII) molecules is critical for the initiation of an adaptive CD4⁺ helper T-cell response (1). Many MHCII alleles are correlated to specific autoimmune disorders, for example HLA-DRB1*1501 to multiple sclerosis (2, 3), and it is anticipated that MHCII antigen presentation sensitizes to particular autoimmune diseases. Expression of MHCII is limited to professional antigen presenting immune cells such as dendritic cells and B-cells. However, normal tissue cells can also express MHCII, usually under inflammatory condition following release of various cytokines, predominantly interferon gamma (IFN γ), thus resulting in antigen presentation by non-hematopoietic cells (4). The relevance of non-immune cell mediated antigen presentation is highlighted by its important role in the onset of graft-versus-host disease (GVHD)(5, 6), transplant rejection (7, 8), some autoimmune diseases (9), as well as its potential for T-cell priming on tumour cells (10, 11).

Transcription of MHCII, as well as the associated invariant chain (Ii), which aides MHCII trafficking and occupies the peptide binding groove before antigen loading, is governed by transcriptional master regulator CIITA (12, 13). CIITA does not bind the MHCII promoter directly, but rather assembles a complex of transcription factors at the MHCII promoter, which includes RFX5, CREB and NF- γ (14). Furthermore, CIITA alters the chromatin environment by recruiting chromatin remodelling factors such as BRG-1, histone acetyltransferases (HATs) and deacetylases (HDACs)(15-17), as well as via its intrinsic HAT activity (18). In its turn, CIITA is transcribed from different promoters in different cell types, with its IFN γ -induced isoform being initiated by transcription factor IRF-1 (19).

Besides upregulating MHC class II antigen presentation, IFN γ induces a pro-inflammatory gene signature in both immune and non-immune cells and is important for clearance of viral and bacterial infections (20). Furthermore, recent findings have

demonstrated that cancer cells promote resistance to immunotherapy by altering their IFN γ signalling pathway (21, 22), illustrating the importance of an intact IFN γ response for immune recognition. As for MHCII, sustained IFN γ signalling can lead to uncontrolled activation of the immune system, causing MHCII-dependent transplant rejection (23) as well as autoimmunity, albeit its importance in the disease pathology for different autoimmune diseases is ambiguous (24-27). Molecularly, engagement of the IFN γ receptor by IFN γ leads to the activation of JAK kinases, which phosphorylate and stimulate nuclear translocation of transcription factor STAT1 (28). STAT1 subsequently induces transcription of IRF-1, which stimulates expression of many pro-inflammatory genes, along with CIITA (29). While the central pathway leading to transcription of IRF-1, CIITA and MHCII is conserved in most cells, many IFN γ -induced genes are expressed cell-type specifically upon activation (15, 30-32), suggesting major regulation by epigenetic modifiers and additional signalling pathways to steer the response. This is in agreement with the notion that HDAC inhibitors increase the expression of IFN γ -target genes in different tumours, hereby sensitizing tumour cells to immune checkpoint inhibition (33, 34). Understanding the factors regulating the IFN γ -response could thus provide novel means of interfering with this important signalling pathway.

Here, we identified two novel regulators of IFN γ -mediated expression of MHCII, the oxidative stress sensor Keap1 and autophagy adaptor p62. Both proteins positively control histone acetylation, and their loss can be compensated by inhibition of HDACs. Furthermore, immunotoxic agent arsenite, as well as anti-autoimmunity drug dimethylfumarate, impinge on the MHCII pathway, providing an additional mechanism of action for these compounds.

Results

Keap1 regulates IFN γ -induced expression of MHCII

Previously, we identified OTUD1 as a novel regulator of MHCII transcription (Chapter 4). To gain insight into the molecular basis for this, a secondary siRNA screen was performed for potential interactors of OTUD1, as previously identified in a mass-spec interaction screen (35). The siRNA screen identified Keap1 as a novel regulator of IFN γ -induced MHCII expression (Fig. 1a and 1b), however, no effect was observed on constitutive MHCII expression in MeJuSo cells (data not shown). Keap1 is involved in many processes and best studied for its inhibitory role in the oxidative stress response by facilitating Cullin3 dependent ubiquitination and degradation of Nrf2, a transcription factor for antioxidant genes (36). In addition, Keap1 regulates NF κ B signalling (37, 38), autophagy (39), DNA repair (40), drug resistance (41), and cell migration (42), via binding to a variety of substrates. However, a role for Keap1 in IFN γ -induced MHCII expression has not been reported.

We continued by testing how Keap1 controls MHCII expression. qPCR analyses of different genes in HeLa cells stimulated with IFN γ for 24 hours indicated that Keap1 silencing inhibited the expression of HLA-DR α , as well as *li*, but not *CIITA* (Fig. 1c), suggesting a different mechanism of regulation from OTUD1. To assess whether Keap1 controls the activity of CIITA or the associated transcription factors, a luciferase construct under control of the MHCII promoter was utilized. In contrast to the control knockdown of STAT1, depletion of Keap1 had no effect on MHCII pro-

motor activity (Fig. 1d), indicating Keap1 does not control any of the factors involved in promoter activation. Furthermore, Keap1 did not affect the stability of HLA-DR α transcripts, since a pulse chase for mRNA degradation using Actinomycin D did not yield any difference in degradation rates (Fig. 1e). Thus, Keap1 is a novel regulator of MHCII transcription, independently of promoter activation or mRNA stability.

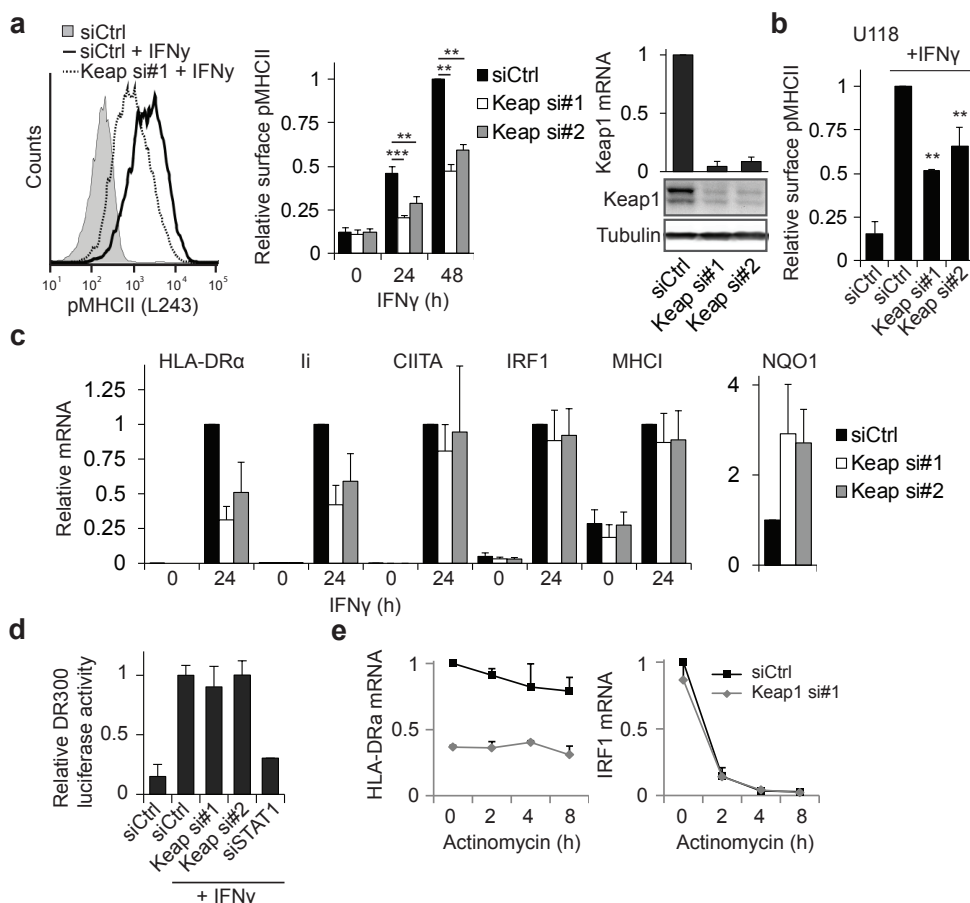


Figure 1: Keap1 positively regulates IFN γ mediated MHCII expression. (A) HeLa cells were transfected with siCtrl or siRNAs targeting Keap1 and stained 72h later for peptide loaded MHCII (L243-cy5), stimulated or not with 100ng/ml IFN γ for the indicated time. Representative histogram and quantification are shown. Right: Keap1 silencing was determined by Western blot analysis (bottom) and qRT-PCR (top, normalized to GAPDH). (B) U118 cells were analysed for pMHCII levels according to the same protocol as in (A). (C) HeLa cells transfected with siCtrl or siKeap1 were stimulated or not with IFN γ for 24h and mRNA expression of the indicated genes was analysed using qRT-PCR. (D) MHCII promoter activity was analysed using a Luciferase under control of the MHCII promoter (DR300) in cells transfected with the indicated siRNAs and treated with IFN γ when indicated. siSTAT1 was used as a positive control and signals were normalized to a Renilla control plasmid. (E) Cells transfected with siCtrl or siKeap1 were treated with IFN γ for 24h and lysed, or Actinomycin D (2uM) was added and cells were lysed 2, 4 or 8h later. mRNA expression level of HLA-DR α and IRF1 was analysed using qRT-PCR, IRF1 was used as a control for effectivity of Actinomycin D. Experiments shown represent mean $\pm$ SD of at least three independent experiments (except D, n=2). Statistical significance was calculated as compared to control cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Keap1 regulates MHCII expression epigenetically via HDAC1/2

The discrepancy between the effect of Keap1 on endogenous MHCII transcription versus exogenous MHCII promoter activity proposes a role for epigenetic regulation controlled by Keap1 to influence IFN γ -induced MHCII transcription. MHCII expression is controlled by various epigenetic markers, including H3K27me₃, DNA methylation, and histone deacetylation (17, 43, 44). Treatment of cells with inhibitors of EZH2, which prevent H3K27me₃ modifications (45, 46), as well as HDAC inhibitors, indeed induced MHCII expression in HeLa cells (Fig. 2a), whereas inhibition of DNA methylation using Decitabine had no effect (data not shown). Whereas the relative effect of Keap1 depletion remained intact upon treatment with EZH2-inhibitors, all three HDAC inhibitors corrected the inhibition of IFN γ -induced MHCII expression following Keap1 silencing, implying that Keap1 controls MHCII via histone acetylation/deacetylation.

SAHA is a pan-HDAC inhibitor, MGCD0103 specifically targets HDAC1/2 and MS-

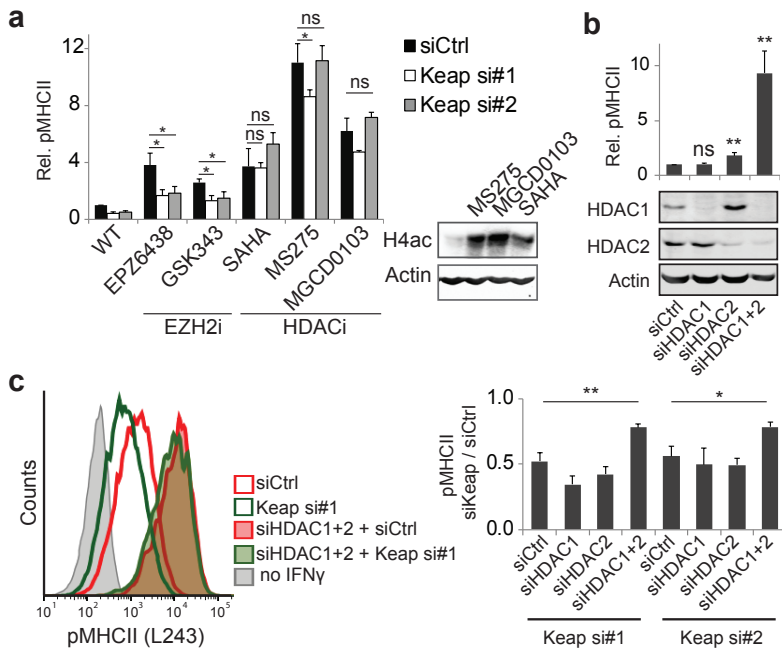


Figure 2: Keap1 regulates MHCII expression via HDACs. (A) HeLa cells transfected with the indicated siRNAs were treated for 48h with IFN γ and inhibitors and expression of pMHCII was analysed by flow cytometry. Maximum non-toxic doses of the inhibitors were used: EPZ6438 (2 μ M), GSK343 (10 μ M), SAHA (5 μ M), MS-275 (0.1 μ M) and MGCD0103 (1 μ M). Right: Western blot for H4ac, to test effectivity of the HDAC inhibitors. (B) Cells transfected with siCtrl (75nM), siHDAC1 (37.5nM siHDAC1 + 37.5nM siCtrl), siHDAC2 (37.5nM siHDAC2 + 37.5nM siCtrl), or siHDAC1+2 (37.5nM siHDAC1 + 37.5nM siHDAC2) were stimulated for 48h with IFN γ and expression of pMHCII was analysed by flow cytometry. Bottom: silencing efficiency was evaluated using Western blot. (C) Cells transfected with the indicated siRNAs (37.5nM HDAC1, 37.5nM HDAC2 and 37.5nM Keap1 or siCtrl) were stimulated for 48h with IFN γ and expression of pMHCII was analysed by flow cytometry. Signal for siKeap1 was normalized to the respective siCtrl. All experiments shown represent mean $\pm$ SD of at least three independent experiments. Statistical significance was calculated compared to control cells (* $p < 0.05$, ** $p < 0.01$).

275 inhibits HDAC1 and to a minor extent HDAC2 (47), arguing that HDAC1 or HDAC2 is the primary regulator of MHCII expression, which is in line with data that overexpression of HDAC1 or HDAC2 represses MHCII expression (17). However, silencing of HDAC1 did not upregulate MHCII expression, while knockdown of HDAC2 had only a minor effect (Fig. 1b and 1c). HDAC1 and HDAC2 have partly overlapping functions and can compensate for each other (48), therefore we also silenced both HDACs together. This strongly increased IFN γ -induced MHCII expression, and decreased the sensitivity of cells to Keap1 depletion (Fig. 2b and 2c). Thus, HDAC1 and HDAC2 together inhibit IFN γ -induced MHCII expression in a pathway that is antagonized by Keap1.

Keap1 regulates histone acetylation levels

The rescue of Keap1-mediated regulation of MHCII expression by HDAC inhibitors implies that Keap1 controls MHCII expression by controlling histone acetylation. Indeed, cells silenced for Keap1 displayed reduced acetylation of H3 and H4, which was independent of addition of IFN γ (Fig. 3a), indicating Keap1 regulates global histone acetylation. This effect was observed in multiple cell types (Fig. 3b), suggesting broad regulation of steady state histone acetylation by Keap1. Inhibition of HDAC1/2 mostly compensated for reduced histone acetylation (Fig. 3c), suggesting that Keap1 inhibits HDAC activity rather than promoting histone acetyl transferase (HAT) activity. HDAC1/2 levels were not increased by loss of Keap1 (Fig. 3a), and total HDAC activity in the cells was unaltered by Keap1 silencing (Fig. 3d). Thus, Keap1 does not affect total HDAC activity but does affect histone acetyl modification and hereby MHCII expression.

P62/SQSTM1 interacts with Keap1 and controls histone acetylation and MHCII expression

How do Keap1 and HDACs intermingle to modify MHCII expression in non-APC? Keap1 mainly serves as a substrate adaptor for Cul3 and binds many interactors, including Nrf2 and other substrates containing an ETGE motif, via its Kelch-domain, to properly position it for Cul3-mediated ubiquitination (49). Two point mutations of Keap1 were generated, one that renders Keap1 unable to bind ETGE motif containing proteins (Y572A)(50) and one that eliminates ubiquitin transfer to substrate proteins (G186R)(51). Stable expression of wild-type RNAi resistant Keap1 allowed rescuing IFN γ -induced MHCII expression upon Keap1 silencing, confirming that the effect of the siRNAs indeed relied on Keap1 depletion. In contrast, the effects on MHCII expression were not rescued by either point mutant of Keap1 (Fig. 4a and 4b), indicating a role for both substrate binding and ubiquitination in IFN γ -induced MHCII expression by Keap1. This effect was independent of the canonical substrate Nrf2, as co-depletion of Nrf2 did not restore IFN γ -induced MHCII expression to normal levels (Fig. 4c).

To identify proteins that cooperate with Keap1 to control IFN γ -induced MHCII transcription, we performed an RNAi screen targeting all described Keap1 interactors. Using this screen, BPTF/FALZ and p62/SQSTM1 were identified as additional partners in the regulations of IFN γ -induced MHCII expression (Fig. 4d). BPTF is a chromatin remodeler that binds histone modifications H3K4me3 and H4K16ac and

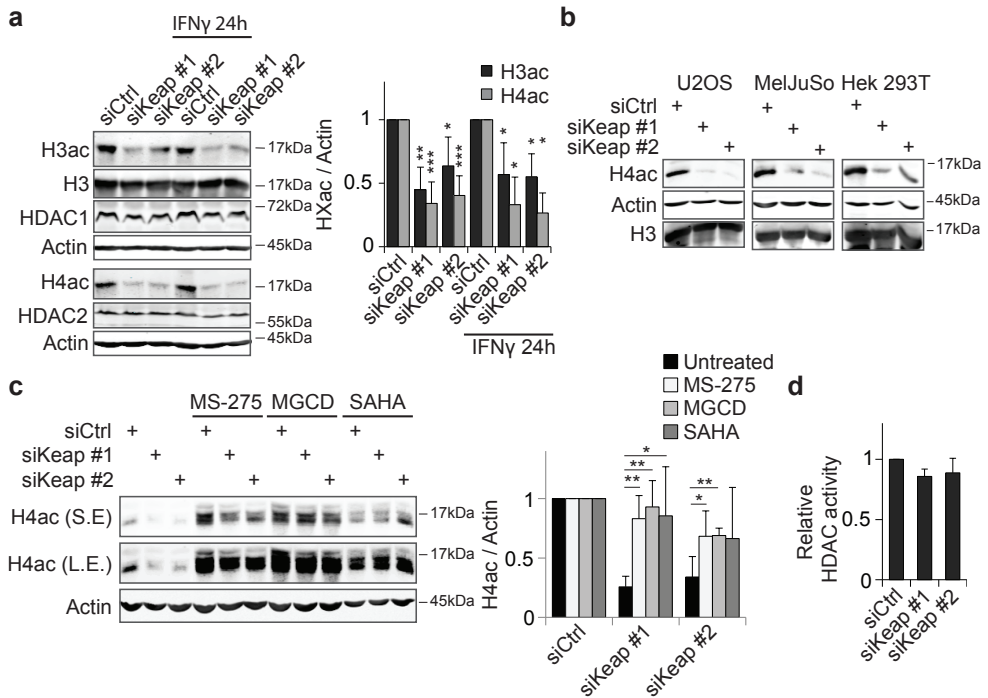


Figure 3: Keap1 regulates histone acetylation. (A) Western blot analysis for the indicated proteins from HeLa cells transfected with the indicated siRNAs and treated for 24h with IFN γ when indicated. Right: quantification of H3ac and H4ac signals relative to corresponding siCtrl and normalized for actin. (B) HeK293T, MeJuSo and U2OS cells transfected with the indicated siRNAs were lysed and analysed by Western Blot for the indicated proteins. (C) HeLa cells transfected with siRNAs were treated with HDAC inhibitors when indicated and subjected to Western Blot analysis. Bottom: quantification of H4ac signals relative to corresponding siCtrl and normalized for actin. All experiments shown represent mean $\pm$ SD of at least three independent experiments. Statistical significance was calculated compared to control cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

unwinds the local chromatin to allow transcription (52, 53), while p62 is an adaptor protein involved in autophagy, endosome positioning and cell signalling (54-56). p62 interacts with Keap1 in a ETGE-motif dependent manner and can promote its degradation via autophagy (57), while BPTF does not contain an ETGE(-like) motif but does bind the Kelch domain of Keap1 (58). Whereas depletion of BPTF did not decrease histone acetylation as detected by H4ac, p62 depletion reduced histone acetylation (Fig. 4e), suggesting Keap1 cooperates with p62 to drive MHCII expression. In agreement, p62 knockdown decreased the transcription and expression of MHCII and Ii, but not CIITA (Fig. 4f). In addition, the reduced histone acetylation levels following p62 depletion could be restored by inhibition of HDACs (Fig. 4g), analogous to earlier observations with Keap1 depletion. Thus, p62 is somewhere in the Keap1 controlled pathway of histone acetylation that modifies IFN γ -induced MHCII expression in non-immune cells.

Arsenite regulates IFN γ -induced MHCII expression

Keap1 contains several exposed cysteine residues that are modified during oxida-

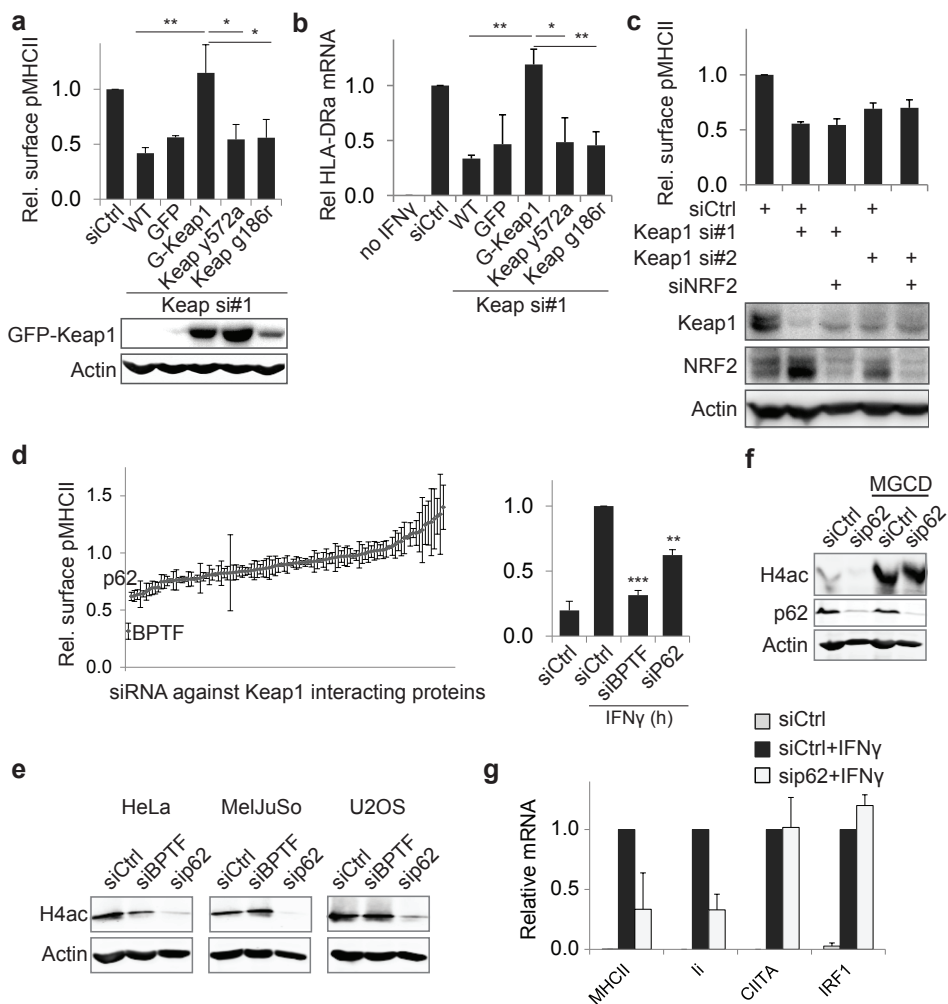


Figure 4: Keap1 cooperates with p62 to regulate histone acetylation. (A) HeLa cells stably expressing GFP or RNAi resistant GFP-Keap1 with the indicated mutations were transfected with the indicated siRNAs and stimulated with IFN γ for 48h and analysed by flow cytometry. Below: Western Blot for expression of the indicated GFP-Keap1 constructs. (B) HeLa cells from (A) were stimulated for 24 hours with IFN γ and mRNA levels of HLA-DR α were measured using qRT-PCR. (C) pMHCII levels on HeLa cells transfected with the indicated siRNAs and stimulated with IFN γ for 48h were measured using flow cytometry. Bottom: Western blot for expression of the indicated proteins (D) Screen for effect of silencing Keap1 interacting proteins on MHCII surface levels. HeLa cells transfected with 106 different siRNAs targeting Keap1-interacting proteins were stimulated with IFN γ for 48h and analysed by flow cytometry. Right: summary of the independent triplicate experiment data for siRNAs targeting BPTF and p62. (E) p62 or BPTF was silenced in the indicated cell line and expression of the indicated proteins was assessed using Western blot. (F) HeLa cells transfected with the indicated siRNAs were treated with 1 μ M MGCD0103 for 24 hours and analysed for the indicated proteins by Western Blot. (G) HeLa cells transfected with the indicated siRNAs were stimulated for 24 hours with IFN γ and mRNA levels were measured using qRT-PCR. All experiments shown represent mean $\pm$ SD of at least three independent experiments. Statistical significance was calculated compared to control cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

tive stress, rendering it inactive and hereby facilitating the NRF2-dependent expression of antioxidant genes. This raises the possibility that by inactivating Keap1, oxidative stress impairs IFN γ -induced MHCII expression. Interestingly, *in vivo* exposure to AS(III), an oxidative stressor that activates NRF2, has already been reported to decrease the expression of different MHCII-alleles and is linked to an impaired immune response (59, 60). To assess a direct role for AS(III) in IFN γ -induced MHCII expression, HeLa cells were exposed to different concentrations of sodium arsenite (AS(III)) during stimulation with IFN γ . A dose-dependent decrease in MHCII expression was observed, indicating a direct role for AS(III) in the regulation of IFN γ -induced MHCII expression (Fig. 5a). Similar to Keap1 depletion, this decrease was transcription-dependent and confined to MHCII and Ii, but not CIITA (Fig. 5b). As for Keap1 depletion, treatment with HDAC-inhibitor MGCD0103 fully restored IFN γ -induced MHCII expression (Fig. 5a). However, as reported earlier (61), no decrease was observed in global histone acetylation levels upon treatment with AS(III) (Fig. 5c), arguing against AS(III)-mediated inhibition of Keap1. AS(III) is also known to directly target the H4K16-specific histone acetyltransferase MYST1 (61), suggesting it could exert its effect via MYST1. In support of this, MYST1 knockdown reduced

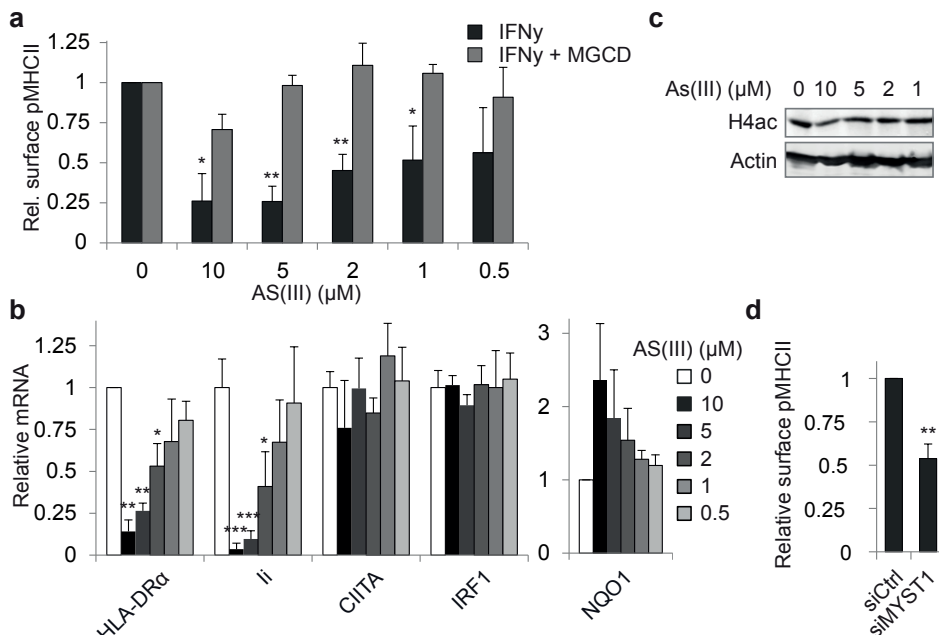


Figure 5: Arsenite controls histone acetylation dependent MHCII expression. (A) HeLa cells were stimulated with IFN γ for 48 hours in combination with the indicated concentration of NaAs₂O₃ and or 1 μM MGCD0103 were analysed for pMHCII expression using flow cytometry. MGCD0103-treated samples were normalized to its corresponding no NaAs₂O₃ treatment sample. (B) HeLa cells treated or not with NaAs₂O₃ were stimulated for 24 hours with IFN γ and mRNA levels were measured using qRT-PCR. (C) HeLa cells treated or not with NaAs₂O₃ for 24h were subjected to analysis of the indicated proteins by Western Blot. (D) pMHCII levels on HeLa cells transfected with the indicated siRNAs and stimulated with IFN γ for 48h were measured using flow cytometry. All experiments shown represent mean +SD of at least three independent experiments. Statistical significance was calculated compared to control cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

IFN γ -induced MHCII expression (Fig. 5d). Thus, sodium arsenite impairs IFN γ -mediated MHCII expression, probably by targeting specific histone acetylation at H4K16 by MYST1, and this effect could be negated by HDAC-inhibitors.

Antioxidants control IFN γ -induced MHCII expression

Keap1 senses oxidative stress and is a primary target for antioxidants such as tert-butylhydroquinone (tBHQ) and dimethyl fumarate (DMF) (36, 62). Both of these drugs display immunomodulatory activity, with their mechanism of action not fully defined (63, 64). DMF has been approved by the FDA for the treatment of psoriasis and multiple sclerosis (MS) (65-67). Interestingly, both of these autoimmune diseases have been linked to IFN γ and activation of CD4 $^{+}$ T-cells (25, 68, 69), implying a possible role for IFN γ -induced MHCII expression in disease pathology. To address whether DMF, like Keap1 inhibition, reduces IFN γ -induced MHC class II expression, we treated various cells with IFN γ in the absence or presence of DMF. In all these cells, DMF reduced IFN γ -induced MHCII expression in a dose dependent manner, whereas constitutive MHCII expression on monocyte-like THP-1 cells was unaffected (Fig. 6a). Similarly, tBHQ specifically reduced IFN γ -induced MHCII expression (Fig. 6a). In multiple sclerosis (MS), as well as experimental autoimmune encephalomyelitis (EAE, a mouse model that mimics MS), macrophages play an important role in the initiation of the inflammatory response (70). To assess whether DMF also affects MHCII expression on these cells, monocyte derived macrophages (MDMs), as well as MDMs that were cultured in the presence of myelin to generate foamy macrophages, which are present in brain lesions of MS patients (71), were treated with different doses of DMF. In both types of macrophages, DMF caused a dose dependent decrease in IFN γ -induced MHCII expression but not constitutive MHCII expression (Fig. 6b and c).

mRNA analysis of these macrophages revealed that the effect was transcriptional, since levels of HLA-DR α and Ii transcripts were reduced (Fig. 6d). No significant decrease was observed for IRF1, whereas the response of CIITA was highly variable. As reported before for keratinocytes and peripheral blood mononuclear cells (PB-MCs) (72), DMF also reduced the expression of the pro-inflammatory chemokines CXCL9 and CXCL10 in macrophages, suggesting a broad reduction of the IFN γ -induced pro-inflammatory signature following DMF treatment (Fig. 6d). To our surprise, the inhibitory effect of DMF (and tBHQ) was independent of histone acetylation, as this inhibition could not be relieved by HDAC-inhibitors (Fig. 6e), histone acetylation levels were not affected by DMF (Fig. 6f), and the activity of the DR300-luciferase construct was affected by DMF (Fig. 6g). Thus, DMF inhibits IFN γ -induced MHCII and chemokine expression via a mechanism independent of Keap1, possibly aiding its anti-inflammatory properties.

Discussion

Cytokine stimulated non-hematopoietic cells have often been discarded as functional contributors to MHCII-dependent immune activation, due to their lack of co-stimulatory receptors. However, several reports have recently challenged this dogma by demonstrating that tumour recognition, transplantation rejection, graft-versus-host disease and specific autoimmune diseases rely on MHCII expression by non-profes-

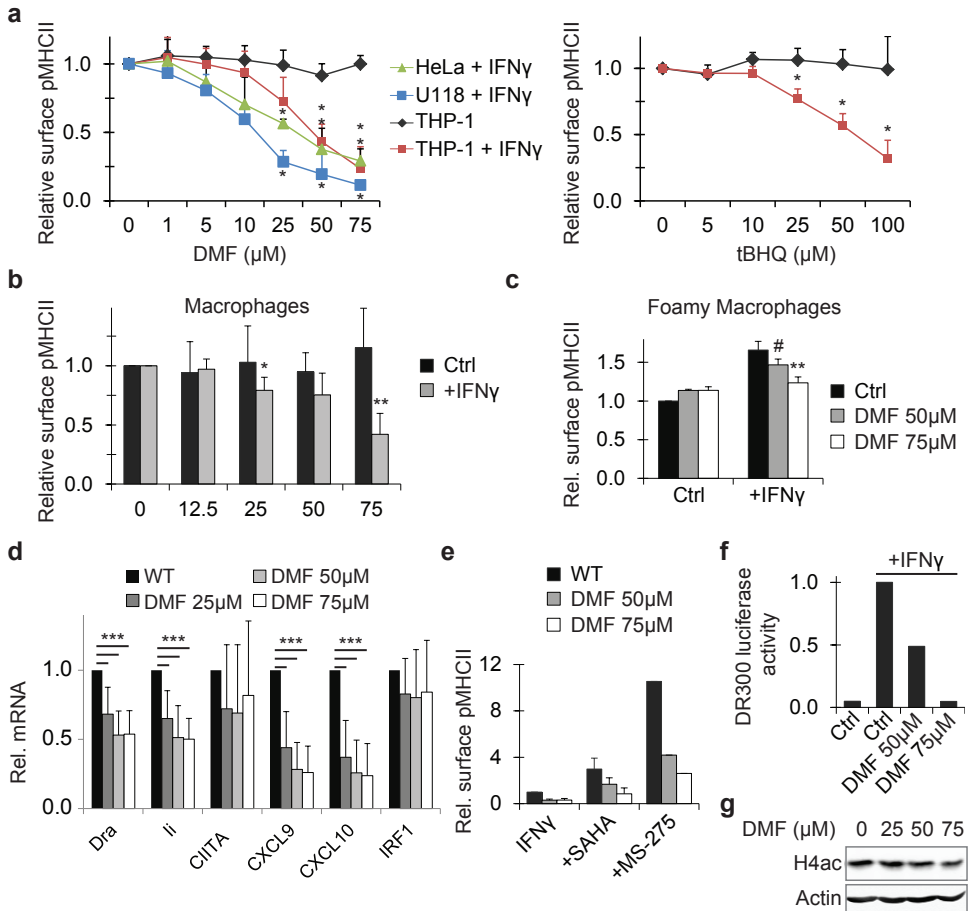


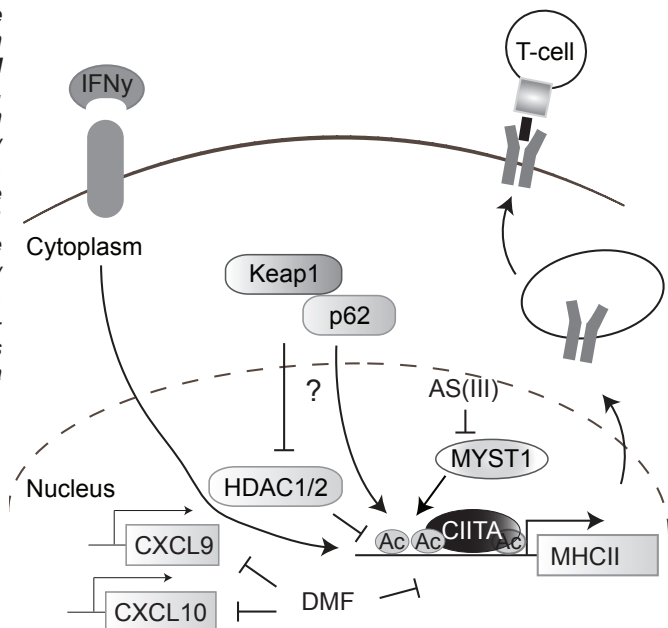
Figure 6: Dimethylfumarate inhibits IFN γ -induced MHCII and chemokine expression. (A) THP-1, HeLa and U118 cells were treated with the indicated concentration of DMF or tBHQ as well as IFN γ for 48h when indicated and pMHCII expression was analysed using flow cytometry. For THP-1+IFN γ samples, signal for THP-1 without IFN γ was subtracted to correct for upregulation by IFN γ . $n=3$. (B) Macrophages were treated with the indicated concentration of DMF as well as IFN γ for 48h when indicated and pMHCII expression was analysed using flow cytometry. For IFN γ treated samples, the signal of wt macrophages was subtracted. $n=4$. (C) Foamy macrophages were treated with the indicated concentration of DMF as well as IFN γ for 24h when indicated and pMHCII expression was analysed using flow cytometry. $n=3$. (D) Macrophages were treated with the indicated concentration of DMF as well as IFN γ for 24h and mRNA expression of the indicated proteins was analysed using qRT-PCR, using GAPDH as loading control. $n=8$. (E) HeLa cells were stimulated for 48h with IFN γ and inhibitors and expression of pMHCII was analysed by flow cytometry. Concentrations: SAHA (5 μM), MS-275 (0.1 μM). (F) MHCII promoter activity (DR300) was analysed in HeLa cells treated or not with IFN γ and the indicated concentration of DMF. (G) HeLa cells were treated with DMF as indicated and analysed by Western Blot for the indicated proteins. Statistical significance was calculated compared to control cells (# $p = 0.069$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

sional APCs, sparking interest in modulating this process. Here, we identify factors and compounds that interfere with IFN γ -induced MHCII expression in non-immune cells. We show that oxidative stress sensor Keap1 and autophagy adaptor p62 up-hold global histone acetylation levels to facilitate IFN γ -induced MHCII expression

(Fig. 7). Their loss can be compensated for by inhibition of HDACs, arguing for a role in the epigenetic regulation of MHCII expression. Furthermore, we identify DMF, an antioxidant used in the treatment of psoriasis and MS, as an inhibitor of the IFN γ response, suggesting an additional mechanism of action for this immunomodulatory drug.

Dimethylfumarate (DMF) has recently been FDA approved for the treatment of relapsing-remitting MS, but its exact mechanism of action is still unresolved (73, 74). Several immunomodulatory actions have been ascribed to this drug, including skewing dendritic cell differentiation to a protective type II subset (75, 76), inhibition of dendritic cell and monocyte maturation (64, 77), inhibition of T-cell activation (78), and skewing T-cells to a Th2 subtype (79, 80). In addition, other modes of proposed action include inhibition of leukocyte infiltration and migration (81). Many of these cellular outcomes are key to the pro-inflammatory action of IFN γ , as this cytokine induces CXCL9 and CXCL10 production to induce T-cell migration and attraction, as well as MHCII expression to facilitate activation of CD4 $^{+}$ T-cells. We show that DMF inhibits IFN γ -induced chemokine expression in macrophages, as also shown for keratinocytes and PBMCs (72). The inhibitory effect of DMF extends to IFN γ -induced expression of MHCII, indicating that DMF acts to dampen antigen presentation and immune cell recruitment in both hematopoietic and non-hematopoietic origin. Since DMF did not affect constitutive expression of MHCII on macrophages and B-cells, it probably acts as a context-specific inhibitor of MHCII expression. The sensitivity of different cell types to DMF varied, with non-hematopoietic cell types significantly reducing MHCII expression at concentrations as low as 10 μ M, while macrophages required at least 50 μ M DMF for a similar response. This could be the result of the higher expression of antioxidant genes in macrophages, which help quench DMF (82). While the exact mechanism of action for DMF remains unclear, recent data in

Figure 7: Model for the regulation of transcription of IFN γ -induced MHCII expression by Keap1, p62, arsenite and DMF. Transcription of MHCII genes is regulated by CIITA and histone acetylation. HDAC1/2 represses histone acetylation, while p62, Keap1 and MYST1 promote it. Arsenite inhibits MYST1 and hereby impairs MHCII expression. Dimethylfumarate inhibits IFN γ -induced expression of MHCII, as well as CXCL9 and CXCL10, via a yet undefined mechanism.



a mouse model for MS suggest that the immunomodulatory and protective activity of DMF does not involve NRF2 (64). This observation is in line with our data that suggest that the effect of DMF on the IFN γ -axis is independent of the Keap1-NRF2 antioxidant response pathway. Since many proteins are targeted by DMF (78), its immunomodulatory action could result from interfering with multiple pathways.

Besides antioxidants, we also tested a role for oxidative stressor arsenite in IFN γ -induced MHCII expression. Arsenic contamination of drinking water is a persistent problem in many countries and can lead to carcinogenesis and immunotoxicity (83). Our data suggest that the latter could in part be mediated via impaired IFN γ -induced expression of MHCII. This is corroborated by *in vivo* data where a decrease in MHCII expression on leukocytes was observed in mice exposed to arsenite, as well as people living in areas with high arsenic levels in drinking water (59, 60). Interestingly, arsenic alleviates graft-versus-host-disease (GVHD) and transplantation rejection of MHCII-mismatched donors (84, 85), which could arise from decreased IFN γ -induced expression of MHCII on respectively host and donor cells. Quenched IFN γ -induced MHCII expression was restored by inhibition of HDAC1/2, arguing that arsenite alters epigenetic regulation of MHCII expression. In agreement with this, arsenite reduces global histone acetylation levels in *Drosophila* (86), as well as H3K9 and H4K16, but not global, histone acetylation in human cells (87, 88), the latter by targeting the histone acetyltransferase MYST1 (61). Depletion of MYST1 also impaired IFN γ -induced MHCII expression, suggesting that arsenite inhibits MYST1 to control MHCII expression. Thus, arsenic controls IFN γ -mediated MHCII expression via epigenetic regulation, possibly contributing to its immunosuppressive activity.

Apart from chemical modulators of MHCII expression, our data indicate a function for Keap1 and p62/SQSTM1 in control of IFN γ -induced MHCII expression. Keap1 and p62 instil their effects by modulating the epigenetic environment of the MHCII locus, as no significant effect of their loss was observed on an ectopically introduced MHCII promoter or expression of CIITA. Depletion of Keap1 and p62 was accompanied by a reduction in global histone acetylation levels, which could be restored by inhibition of HDAC1 and HDAC2. Thus, Keap1 and p62 either inhibit HDAC1/2 or activate HATs. p62 is known to inhibit HDAC6 (89) via a direct interaction. However, we failed to detect an interaction of either Keap1 or p62 with HDAC1. Loss of Keap1 did not change total HDAC activity in the cell, nor did it alter the localization of HDAC1 (data not shown), raising the possibility that HDAC targeting to the DNA via recruitment factors is affected. The predominantly cytosolic nature of Keap1 and p62 versus the nuclear localization of HDAC1 presuppose that this regulation is indirect. In assembly, Keap1 and p62 alter the epigenetic histone acetylation landscape to facilitate expression of MHCII after IFN γ stimulation.

Physiologically, many stimuli regulate global cellular histone acetylation levels, either via HATs (as in the case of NF κ B signalling (90)), or via HDACs (in the case of cell cycle related kinase CK2, nuclear actin and sumoylation (89, 91, 92)). Keap1 and p62 could be involved in histone acetylation regulation due to their role as stress sensors, respectively being degraded by oxidative stress and autophagy. This would translate extracellular conditions to epigenetic effects. The notion that overall histone acetylation levels are decreased upon removal of Keap1 and p62 infers that the transcriptional changes are not limited to MHCII. For Keap1, this could function to

minimize expression of novel proteins that are subjected to oxidation, whereas for p62 this could attenuate transcription of MHCII and other genes to favour the expression of autophagy-related genes, as observed for mTOR inhibition, which inhibits H4K16ac to drive autophagy gene expression (93). This integrates regulation of histone acetylation into the stress response that is activated following downregulation of Keap1 and p62.

Mutations in or depletion of Keap1 and p62 potentially impairs MHCII expression by tumour cells, subsequently hampering immune recognition. In agreement with this, epigenetic silencing of IFN γ target genes in tumours has been widely observed, and its reversal by HDAC-inhibitors aides immunotherapy treatment in several mouse models (33, 34). Keap1 is frequently mutated in lung cancer (94), providing a rationale for treatment selection with HDAC-inhibitors in lung patients. However, we observed no clear correlation between expression of Keap1-target NRF2 and MHCII in lung tumour tissues from the Human Protein Atlas database (95). Furthermore, both Keap1 mutant and wild-type lung cancer cell lines are sensitive to HDAC-inhibitors (34), indicating that multiple factors are involved in the epigenetic silencing of IFN γ target genes. Nevertheless, our data support the notion that HDAC-inhibitors, specifically HDAC1 and HDAC2 inhibitors, are interesting tools to stimulate immune recognition of tumour cells.

Collectively, our data identify several novel regulators of IFN γ -induced MHCII expression, as occurs in inflamed tissue. Two stress-related proteins, Keap1 and p62, induce histone acetylation and IFN γ -mediated MHCII expression in non-immune cells. Mutation of the p62-binding site in Keap1 failed to restore expression of MHCII, implying that Keap1 and p62 functionally cooperate in this process. Arsenite, an immunosuppressive compound that induces oxidative stress, also controls IFN γ -mediated MHCII expression. The effects of Keap1 or p62 depletion and arsenite on MHCII expression could be overcome by HDAC-inhibitors, implying epigenetic regulation by these factors. Finally we demonstrate that the drug dimethylfumarate (DMF) specifically impairs IFN γ -induced MHCII expression, providing an additional mechanism of action for this drug that is used for the treatment of autoimmune diseases.

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Materials and Methods

Cell culture, treatments and constructs

HeLa, U118, U2OS and Hek293T cells were cultured in DMEM supplemented with 10% FCS, MelJuSO cells in IMDM with 10% FCS. For IFN γ stimulation, cells were stimulated with 100 ng/ml IFN γ (Ebioscience) for the indicated time. For genera-

tion of stable cell lines, Keap1 was cloned into a GFP-C1 vector and mutagenized to avoid targeting by Keap1 siRNA#1 using the primers: fw 5'-ggggcttgacggga-caaatcgtctaaactcagctgagtggtac-3', rv 5'-gggtagtaacactcagctgagtttagacgattgtcccg-ccaaagc-3'. Subsequent mutagenesis was performed using the following primers: Y572A fw: 5'-ctacgtccttgaggcgctgatggtcacacgttc-3', Y572A rv: 5'-actgtccaggaacgt-gtgaccatcagcgctccaaggacg-3' G186R fw: 5'-cccagcaatgccatccgcatcgccaactcg-3', G186R rv: 5'-gctcagcgaagttggcgatgcggatggcattg-3'. GFP and GFP-Keap1 mutants were recloned into a retroviral pMX vector and upon retroviral transduction cells selected using Puromycin (4 μ g/ml, Gibco).

Transfections

For expression studies, HeLa cells were transfected using Effectene (Qiagen) according to the manufacturer's instructions. For siRNA mediated depletion, cells were reverse transfected according to the manufacturer's protocol with DharmaFECT transfection reagent #1 and 50 nM siRNA (except for double and triple transfections). Briefly, siRNAs and DharmaFECT were mixed and incubated for 20 minutes in a culture well, after which cells were added and left to adhere. Three days later, cells were used for analysis. Catalog numbers: siCtrl: D00120613-20, siSTAT1 D-003543, siHDAC1 D-003493, siHDAC2 D-003495, siBPTF M-004025-01-0005, sip62 D-010230 of the Human siGenome SMARTpool, Dharmacon. siRNA sequences targeting Keap1: #1 GGACAAACCGCCUUAUUC and #2 GGGCGUGGCUGUCCUCAU, siRNA sequences targeting p62: #1 and #2, all from Dharmacon.

Reagents and antibodies

Rabbit α HDAC1 NB100-56340 (Novus Biologicals), Rabbit α HDAC2 SC-7899, mouse α p62 SC-28359 (both from Santa Cruz), Rabbit α H3ac 06599 (Millipore), rabbit α H4ac ab177790, rabbit α H3 ab1791 (both from Abcam), mouse α Keap1, mouse α Actin (both from Sigma), rabbit α GFP (as described before (96)). Sodium (meta)Arsenite and Dimethylfumarate were acquired from Sigma.

Flow Cytometry

Three days after siRNA transfection, HeLa cells were trypsinized and stained with Cy5-labeled L243 antibody(97), before analysis using flow cytometry (BD FACSArray, or BD FACS Calibur for GFP co-detection).

Co-immunoprecipitation and Western blotting

For protein expression analysis, cells were directly lysed in sample buffer and proteins were separated by SDS-PAGE and transferred to Western Blot filters. Antibody incubations and blocking were done in PBS supplemented with 0.1 (v/v)% Tween and 5% (w/v) milk powder. Blots were imaged using the Odyssey Imaging System (LI-COR) or Chemidoc (Biorad).

cDNA synthesis and qPCR

RNA isolation, cDNA synthesis and quantitative RT-PCR were performed according to the manufacturer's (Roche) instructions. Signal was normalized to GAPDH and calculated using the Pfaffl formula. Primers used for detection were: GAPDH fw: 5'-TGTTGCCATCAATGACCCCTT-3', GAPDH rv: 5'-CTCCACGACGTACT-CAGCG-3', HLA-DR α fw: 5'-CATGGGCTATCAAAGAAGAAC-3', HLA-DR α rv: 5'-CTTGAGCCTCAAAGCTGGC-3', li fw: 5'-CACCTGCTCCAGAATGCTG-3', li rv: 5'-CAGTTCCAGTGACTCTTTCG-3', IRF1 fw: 5'-GCACCAAGTGATCTGTACAAC-3', IRF1 rv: 5'-GCTCCTCCTTACAGCTAAAG-3', CIITA fw: 5'-CCTGCTGTTCGG-

GACCTAAA-3, CIITA rv: 5'-GGATCCGCACCAGTTTGG-3', Keap1 fw: 5'-CTG-GAGGATCATACCAAGCAGG-3', Keap1 rv: 5'-GAACATGGCCTTGAAGACAGG-3', NQO1 fw: 5'-GGGCAAGTCCATCCCAACTG-3', NQO1 rv: 5'-GCAAGTCAGG-GAAGCCTGGA-3', CXCL9 fw: 5'-GTGGTGTTCCTTTCTCTTG-3', CXCL9 rv: 5'-GTAGGTGGATAGTCCCTTGG-3', CXCL10 fw: 5'-TGATTTGCTGCCTTATCT-TTCTGA-3', CXCL10 rv: 5'-CAGCCTCTGTGTGGTCCATCCTTG-3'.

Luciferase assays

HeLa cells reverse transfected with the indicated siRNAs were transfected the next day with a luciferase construct under the control of the MHCII locus (DR300), as well as a SV40-Renilla pGL3 reporter construct. A day later, cells were stimulated with IFN γ when indicated and lysed and analysed the day after using the Dual Luciferase Reporter Assay (Promega). Data were normalized to Renilla luciferase signal.

HDAC activity assay

Three days after transfection, HDAC activity in HeLa cells were analysed using the Fluor de Lys assay (Enzo Life Sciences) according to the manufacturer's instructions. Read-out was performed on the BMG Labtech Clariostar.

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