Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy

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Biochemical and functional studies have demonstrated major histocompatibility complex (MHC) class II-restricted presentation of peptides derived from cytosolic proteins, but the underlying processing and presentation pathways have remained elusive. Here we show that endogenous presentation of an epitope derived from the cytosolic protein neomycin phosphotransferase II (NeoR) on MHC class II is mediated by autophagy. This presentation pathway involves the sequestration of NeoR into autophagosomes, and subsequent delivery into the lytic compartment. These results identify endosomes/lysosomes as the processing compartment for cytosolic antigens and furthermore link endogenous antigen presentation on MHC class II with the process of cellular protein turnover by autophagy.

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1 Introduction

The two major classes of MHC molecules display peptides derived from different protein pools. MHC class I molecules present antigenic peptides derived from intracellular proteins to $CD8^+$ T cells, whereas MHC class II molecules present antigenic peptides derived from exogenous or cell surface proteins to $CD4^+$ T cells [1, 2]. Although this segregation of the antigen-processing pathways by the immune system has been found to be very efficient, it has proven less stringent than initially assumed. MHC class I loading with exogenous antigens by a subset of antigen-presenting cells (APC) is now considered essential for priming CTL responses to viruses and tumors [3]. Conversely, MHC class II presentation of peptides derived from internally synthesized proteins, referred to as the endogenous pathway of antigen presentation by MHC class II molecules [4, 5], has been demonstrated biochemically and functionally, and evidence for an important role of this alternative pathway in central tolerance induction is beginning to emerge [6].

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Abbreviations: NeoR: Neomycin phosphotransferase II **3-MA:** 3-Methyladenine **RCC:** Renal cell carcinoma **EBV-B cell:** Epstein-Barr virus immortalized lymphoblastoid B cell **GFP:** Green fluorescent protein

Biochemical evidence for this pathway has been obtained by sequence analysis of peptides eluted from MHC class II molecules of transformed human B cell lines [7, 8]. Although the majority of peptides was derived from exogenous and cell membrane-associated molecules, a significant proportion of the peptides was derived from cytoplasmic proteins such as S30 ribosomal protein, c-myc, K-ras and cytochrome B5 reductase.

Functional evidence for the presentation of endogenous antigens by MHC class II molecules has first been provided after measles virus infection by Long and colleagues [9]. Subsequently, presentation of endogenous proteins by class II molecules has been examined with several model antigens, *e.g.* hen egg lysozyme (HEL), immunoglobulin light chain or influenza virus proteins. The common approach in these experiments was to engineer genetic constructs targeting the encoded model antigens to different compartments of the cell and monitoring cell surface presentation by MHC class II-restricted T cells specific for the model antigen. MHC class II-associated presentation of proteins targeted into the cytoplasm or the cell nucleus has been observed in some, but not all studies, suggesting that only selected cytosolic proteins have access to the endogenous pathway of MHC class II presentation [5, 10].

Thus, the identification of the processing and presentation pathways for cytosolic antigens is prerequisite to

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understanding which cytosolic antigens are presented on MHC class II, where processing of these antigens occurs, and what the implications for T helper cell regulation might be. Several potential presentation pathways have been implicated, including bystander presentation following release of antigen by neighboring cells, cytoplasmic processing and transport of peptides into the endoplasmic reticulum (ER), as well as poorly defined mechanisms of lysosomal import [4, 5]. Here we studied MHC class II-restricted presentation of an epitope derived from the cytosolic protein neomycin phosphotransferase II (NeoR). Our experiments show that MHC class II-associated cell surface presentation involves NeoR sequestration in autophagosomes and subsequent processing in the endosomal/lysosomal compartment, in which the pathways of cytoplasmic protein degradation by autophagy and MHC class II presentation converge.

2 Results

2.1 Endogenous presentation of an epitope derived from neomycin phosphotransferase II on HLA-DP3

In a previous effort to generate tumor-specific T cells, PBL of a patient with renal cell carcinoma (RCC) were stimulated *in vitro* with autologous, B7–1 transduced tumor cells (unpublished data). One of the $CD4^+$ T cell clones isolated, termed 20–4/A4, responded against an epitope derived from the gene product of neomycin phosphotransferase II (neomycin resistance gene, *neoR*) used as selection marker for B7–1 transduction of the tumor cells. The HLA-DP3-restricted NeoR protein-derived peptide recognized by 20–4/A4 was identified as amino acids 216–229 DRYQDIALATRDIA. The cytosolic and nuclear localization of this non-self protein made NeoR an attractive model antigen for studying the endogenous pathway of MHC class II antigen presentation. HLA-DP3-restricted presentation of NeoR was analyzed in two types of APC, (i) Epstein-Barr virus immortalized EBV-B cells transiently transfected with a *neoR* expression plasmid (pINCO-NeoR), and (ii) RCC stably transfected with the *neoR* gene (RCC1.24-NeoR). Despite the constitutive expression of *neoR*, the conditional expression of MHC class II in this cell line only after IFN-γ induction facilitates assessment of NeoR antigen presentation. As shown in Fig. 1, both types of APC present internally synthesized NeoR on MHC class II. While RCC1.24-NeoR cells are inefficient in presenting exogenous antigen on MHC class II, incubation of EBV-B1.11 cells with as little as 1 ng/ml of bacterially expressed NeoR protein was sufficient for subsequent detection by T cells (data not shown). Therefore, co-cultivation experiments were performed to make sure that presentation of NeoR was not caused by release from the transfected cells and re-uptake as exogenous antigen. Non-transfected EBV-B cells were incubated with supernatant from transfected cells, and HLA-DP3-negative EBV-B1.25 cells transfected with the *neoR* expression plasmid were co-cultivated with HLA-DP3-positive but non-transfected EBV-B1.11 cells (Fig. 1B). T cell activation was not detected in either of these experiments, excluding presentation of NeoR as exogenous antigen after release in the culture supernatant.

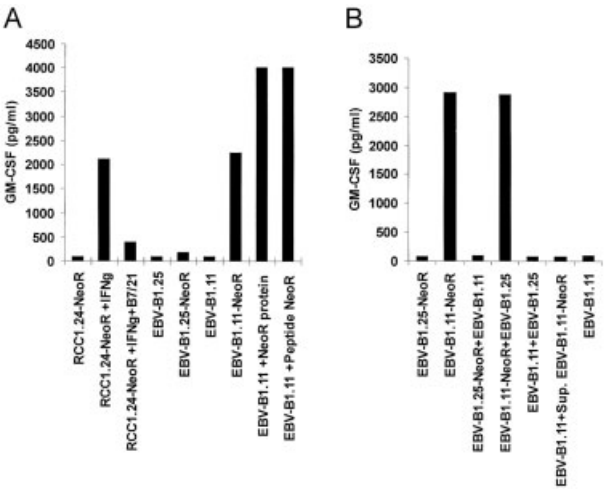

Fig. 1. Endogenous presentation of a peptide derived from cytosolic NeoR on HLA-DP3. (A) The T cell clone 20–4/A4 recognizes an epitope derived from the NeoR protein in the context of HLA-DP3. MHC class II expression on RCC1.24-NeoR cells following IFN-γ treatment confers recognition by 20–4/A4. This recognition is almost completely blocked by the HLA-DP-specific antibody B7/21. EBV-B cells transfected with pINCO-NeoR are able to present endogenous NeoR on MHC class II. As exemplified by the cell lines EBV-B1.11 (HLA-DP3) and EBV-B1.25 (HLA-DP4), recognition by 20–4/A4 is dependent on HLA-DP3 expression. Bacterially expressed NeoR protein and a synthetic peptide encompassing amino acids $NeoR_{216–229}$ served as controls. (B) Cross-presentation experiments to exclude release of NeoR and re-uptake as exogenous protein. EBV-B1.25 cells transfected with the *neoR* expression plasmid pINCO-NeoR, were co-cultured for 24 h with the same number of untransfected EBV-B1.11 cells, or vice versa. Then, T cells were added and co-cultured with the cells for additional 24 h. To test for NeoR release by *neoR*-transfected EBV-B1.11 cells, total supernatant (200 μl) of 1×10^5 EBV-B1.11 cells was harvested 48 h post transfection by centrifugation, and was used as culture media for 1×10^5 untransfected EBV-B1.11 cells. After 48 h, 1×10^5 20–4/A4 T cells were added and GM-CSF concentration determined after additional 24 h.

2.2 NeoR presentation does not depend on key features of the MHC class I pathway

Using minigene constructs encoding only the cognate peptide, Malnati et al. [11] demonstrated that the presentation of an endogenous cytosolic peptide by MHC class II is dependent on a functional transporter associated with antigen processing (TAP). These results implied that protein degradation in the cytoplasm and transport of peptides into the ER by TAP might be a potential mechanism for loading endogenous antigens on MHC class II molecules [5]. Because most cytoplasmic proteins are degraded by the proteasome, a potential participation of this multienzyme complex in NeoR presentation was investigated with the proteasome-specific inhibitor lactacystin [12]. *neoR*-transfected RCC and EBV-B cells were treated with 5 μM lactacystin. Following overnight incubation, cells were fixed with 0.5% paraformaldehyde and NeoR cell surface presentation determined by cocultivation of the cells with the NeoR-specific T cell clone 20–4/A4. As shown in Fig. 2, inhibition of the proteasome did not abolish NeoR cell surface presentation. On the contrary, lactacystin treatment increased antigen presentation, especially in EBV-B cells (Fig. 2B). In control experiments, presentation of a tyrosinase-derived peptide on HLA-A2 was almost completely blocked by lactacystin, indicating that the concentrations of the inhibitor applied were effective (Fig. 2C). Besides the proteasome, a second major proteolytic activity has recently been identified in the cytoplasm, called tripeptidyl peptidase II (TPPII), which could also be involved in NeoR presentation [13]. Similar inhibition experiments were performed using AAF-CMK, a specific inhibitor of TPPII, which again had no effect on NeoR antigen presentation. Although these two proteases are believed to be responsible for the degradation of the vast majority of cytoplasmic proteins, additional and less well characterized cytoplasmic or nuclear proteases might also be involved in NeoR degradation. Therefore, cells were transfected with the Herpes simplex virus gene *ICP47*, which efficiently blocks TAP function and thus prevents transport of peptides from the cytoplasm into the ER [14]. Stable transfection of EBV-B cells with an *ICP47* expression plasmid caused cell surface down-regulation of MHC class I molecules, and HLA-A2-restricted presentation of an epitope derived from the tyrosinase protein was abrogated in this cell line (Fig. 2F). In contrast, MHC class II cell surface expression and NeoR presentation were unaffected, arguing against a role of cytoplasmic processing and TAP in the NeoR presentation pathway.

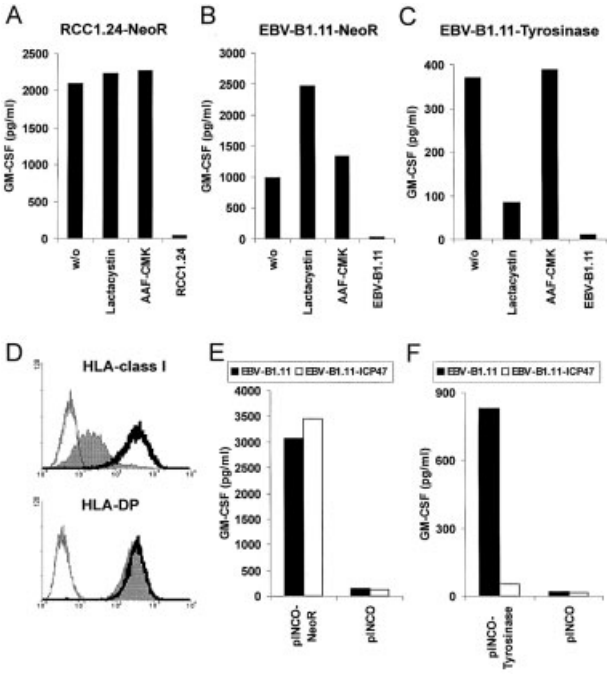

Fig. 2. Endogenous presentation of NeoR on MHC class II does not depend on components of the MHC class I presentation pathway. RCC1.24-NeoR (A), pINCO-NeoR transfected EBV-B1.11 cells (B), and as control pINCO-Tyrosinase-transfected EBV-B1.11 cells (C), were either left untreated or treated with 5 μM lactacystin or 50 μM AAF-CMK for 20 h. Following fixation with 0.5% paraformaldehyde, cells were incubated with T cells, and 24 h later GM-CSF release was determined by ELISA. (D) FACS analysis of EBV-B1.11 cells (open histograms) and EBV-B1.11 cells stably transfected with the *ICP47* gene (shaded histograms). Inhibition of TAP by ICP47 down-regulates HLA class I but not HLA-DP expression. An isotype-matched antibody was used as control (thin-lined histogram). EBV-B1.11-ICP47 cells were transfected with pINCO-NeoR (E), pINCO-Tyrosinase (F), or mock transfected (pINCO) and MHC-restricted presentation monitored with T cells.

2.3 NeoR cell surface presentation requires lysosomal processing

Similar experiments were performed to test whether presentation of NeoR on MHC class II was dependent on lysosomal processing. NeoR-expressing RCC and EBV-B cells were either treated with leupeptin, an inhibitor of lysosomal proteases, or chloroquine, a lysosomotropic agent that prevents acidification of the endosomal/lysosomal compartment. Both substances completely blocked HLA-DP3-restricted presentation of the NeoR epitope in either APC (Fig. 3A, B), indicating that NeoR presentation required processing in the acidic late endosomal/lysosomal compartment. In control experiments, neither drug had any effect on the HLA-A2-restricted presentation of the tyrosinase epitope (Fig. 3C). Because degradation of invariant chain is also sensitive to both

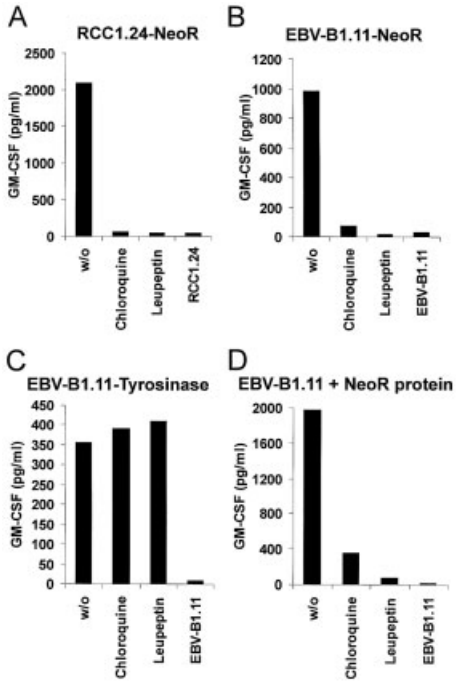

Fig. 3. Lysosomal processing is required for MHC class II-restricted presentation of endogenous and exogenous NeoR. (A) RCC1.24-NeoR incubated with IFN-γ were either left untreated (w/o) or treated with chloroquine or leupeptin for 24 h to block lysosomal processing. Subsequently cells were fixed with 0.5% paraformaldehyde and tested with 20–4/A4 in a GM-CSF release assay. EBV-B1.11 cells transfected with pINCO-NeoR (B) or pINCO-Tyrosinase (C) were tested likewise. Both substances abrogated recognition of neoR-transfected cells, while HLA-A2-restricted presentation of the tyrosinase peptide was not affected. (D) EBV-B1.11 cells were incubated with 200 ng/ml of recombinant NeoR protein and treated with leupeptin or chloroquine as described in (A). Presentation of exogenous NeoR on HLA-DP3 is blocked by both substances as well, indicating that NeoR presentation is dependent on lysosomal processing. The IFN-γ-treated, NeoR-negative parental cell line RCC1.24, and non-transfected EBV-B1.11 cells were used to demonstrate specificity of T cell recognition.

drugs, these results could indicate that maturation of newly synthesized MHC class II molecules and thus loading with NeoR peptides is inhibited. To differentiate between these possibilities, EBV-B cells were incubated with bacterially expressed NeoR in the absence or presence of chloroquine and leupeptin. Uptake of exogenous proteins into early endosomes allows binding to recycling MHC class II molecules, provided that protein processing occurs in this early and only mildly acidic compartment [15]. Both substances completely blocked cell surface presentation of exogenous NeoR (Fig. 3D), which implies that processing of NeoR takes place in the acidic late endosomal/lysosomal compartment. Peptide control studies, furthermore, demonstrated that the inhibitors do not alter class II dissociation from invariant chain and thus class II binding of ligands (data not shown).

2.4 NeoR protein can be detected in endosomes/lysosomes

To test whether NeoR reaches the late endosomal/lysosomal compartment as intact protein, RCC expressing NeoR-GFP fusion protein were examined by UV-fluorescence microscopy. While untreated cells displayed a uniform cytoplasmic and nuclear staining identical to the subcellular distribution observed for NeoR by immunofluorescence (data not shown), inhibition of lysosomal degradation by leupeptin or chloroquine caused rapid formation of brightly fluorescent vesicles, especially at a nuclear proximal region, which implicates a transport mechanism for cytosolic NeoR-GFP into the vacuolar system (data not shown). NeoR-GFP accumulation in these vesicles was also observed in the presence of the fungal metabolite Brefeldin A (BfA), which blocks anterograde transport from the ER to the Golgi complex as well as from endosomes to lysosomes [16]. These results suggested that NeoR accesses the vacuolar compartment through a mechanism different from the secretory pathway.

To identify the port of entry into the vacuolar system, cell fractionation experiments were performed using 293T cells transfected with a *neoR* expression plasmid. Transfected cells were mechanically disrupted and subcellular fractions collected by differential centrifugation [17]. Protein samples from the various fractions normalized to the same number of cell equivalents were subjected to Western blot analysis using a monoclonal antibody directed against the myc-epitope used for tagging NeoR. Fig. 4A shows the distribution of NeoR within the various cell fractions. As expected from the cytosolic and nuclear localization of NeoR, most of the NeoR protein is present in the fractions containing cytoplasm, and nucleus/cell membrane. In addition, a strong signal was detected in the endosomal/lysosomal fraction, suggesting that indeed whole NeoR protein accessed the lytic compartment. To determine which compartment was the entry site for NeoR, the endosomal/lysosomal fraction was further separated by Percoll gradient centrifugation [18], and the resulting fractions analyzed by Western blot. Rab-5 and Lamp-1 were used as markers for early endosomes and late endosomes/lysosomes, respectively. As shown in Fig. 4B, fractions 4–8 stained positive for Lamp-1, while Rab-5 staining was most prominent in earlier fractions and decreased in the late endosomal/lysosomal high density fractions 7 and 8. NeoR was

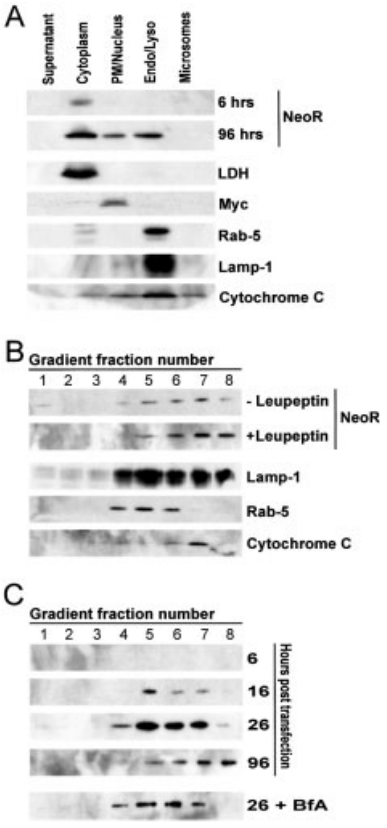

Fig. 4. NeoR accesses an early endosomal compartment. (A) 293T cells transfected with pINCO-NeoR were harvested 6 and 96 h post transfection and NeoR localization within various cell fractions determined by Western blot. In addition, supernatant of pINCO-NeoR transfected 293T cells (96 h post transfection) was included to test for release of NeoR into the culture supernatant. At 6 h post transfection, NeoR protein becomes detectable in the cytoplasm, while at 96 h post transfection a substantial amount of NeoR protein is also localized in the endosomal/lysosomal fraction. For further characterization of the various fractions, blots were stripped and re-probed with antibodies specific for the cytoplasmic protein LDH, the nuclear protein c-myc, early endosomes (Rab-5), late endosomes and lysosomes (Lamp-1) and mitochondria (cytochrome c), which co-purify with endosomes/lysosomes [18]. (B) Endosomal/lysosomal fractions were prepared from pINCO-NeoR-transfected cells 96 h post transfection as in (A). To prevent lysosomal degradation of NeoR, half of the cells were treated with leupeptin for the last 48 h. These fractions were separated on a 27% Percoll gradient and eight different fractions collected. Successful separation into fractions with increasing density was verified by Western blot analysis with antibodies directed against Lamp-1, Rab-5 and cytochrome c. Inhibition of NeoR degradation by leupeptin causes accumulation of NeoR protein in the late endosomal/lysosomal fractions 7 and 8. (C) To determine where NeoR gains access to the vacuolar system, 293T cells were transfected with pINCO-NeoR, treated with leupeptin to prevent NeoR degradation, and the endosomal/lysosomal fractions prepared at various time points post transfection and separated by Percoll gradient centrifugation. Starting at 16 h post transfection, NeoR becomes detectable in the Rab-5-positive fraction 5, and then spreads into late endosomal/lysosomal fractions. At 26 h post transfection an almost identical NeoR distribution pattern is observed in cells treated with or without BfA.

detected again as intact protein without any visible degradation intermediates in Rab-5-positive fractions, suggesting that NeoR accumulated in Rab-5-positive vesicles. Because NeoR is degraded in late endosomes/lysosomes, the weak signal in the lysosomal fractions could be due to protein instability. Therefore, lysosomal degradation of NeoR was blocked with leupeptin, and subcellular compartments prepared in the same way. Under these conditions, the signal for NeoR increased in the high-density, Lamp-1-positive and Rab-5-negative fractions, indicating that NeoR indeed becomes degraded in lysosomes by leupeptin-sensitive proteases. These results indicated that NeoR either accessed lysosomes through endosomes, or that NeoR accessed lysosomes directly and some protein escaped into the endosomal compartment, where it accumulated because of increased stability. To address these possibilities, transfected cells were again treated with leupeptin to block lysosomal degradation and subcellular fractions prepared at various time points post transfection. These time course experiments showed that starting 24 h post transfection, NeoR first becomes detectable in the endosomal compartment and then accumulates in lysosomal fractions (Fig. 4C). In a second set of experiments, transport from endosomes to lysosomes was inhibited by BfA. In these cells, NeoR accumulated almost exclusively in Rab-5-positive fractions (Fig. 4C), demonstrating that NeoR accesses lysosomes through endosomes and not vice versa.

2.5 NeoR accesses the vacuolar system by autophagy

Non-selective bulk degradation of cytoplasmic proteins in the lytic compartment is mediated by a process called autophagy [19, 20]. The initial step in this process involves the nonspecific sequestration of cytoplasm via the formation of a double-membraned autophagosome, which then fuses with vesicles of the endosomal/lysosomal compartment. The sequestration step is subject to feedback inhibition by degradation products like amino acids and purines, and derivatives of these compounds, such as 3-methyladenine (3-MA), are well-established inhibitors of autophagy [21]. To test whether cytosolic NeoR enters the vacuolar compartment by sequestration into autophagosomes, NeoR-GFP-ex-

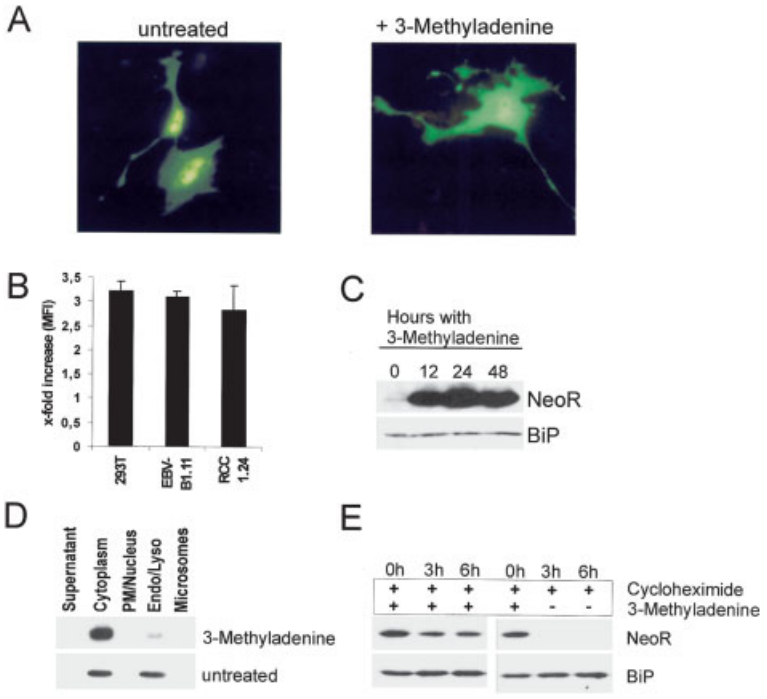

Fig. 5. NeoR accesses the endosomal/lysosomal compartment through autophagy. (A) RCC were transfected with the NeoR-GFP expression construct and subcellular localization of the fusion protein monitored by UV fluorescence. As compared to untreated cells, cells treated with 3-MA show a much brighter UV fluorescence. (B) Semiquantitative analysis of fluorescence intensity of cells transfected with the NeoR-GFP fusion construct. Compared to untreated cells, cells treated for 24 h with 7.5 mM 3-MA show an about threefold increase in mean fluorescence intensity. (C) Western blot analysis of *neoR*-transfected cells. Treatment of cells with 7.5 mM 3-MA causes a dramatic increase in NeoR protein levels in only 12 h. The ER-resident protein BiP was used as gel loading control. (D) This increase in total NeoR protein in 3-MA-treated cells is associated with an increase in cytoplasmic, and a decrease in endosomal/lysosomal NeoR protein levels. (E) Western blot analysis of whole cell lysates prepared from *neoR*-transfected cells. Inhibition of protein synthesis by cycloheximide treatment leads to a rapid decrease in NeoR protein levels. Simultaneous treatment of cells with 3-MA prevents NeoR degradation. BiP was again used as a gel loading control.

pressing RCC were treated with 3-MA, and subcellular distribution of the fusion protein examined by UV fluorescence. Compared to untreated cells, cells treated with 3-MA showed a much brighter staining of the whole cell body (Fig. 5A). This increase in fluorescence intensity under 3-MA treatment was observed in all NeoR-GFP-expressing cell lines tested, including EBV-B cells (Fig. 5B). Moreover, *neoR*-transfected cells treated with 3-MA showed a dramatic increase in cellular NeoR protein levels in only 12 h (Fig. 5C). Besides this increase in total NeoR protein, Western blot analysis of different subfractions also revealed that the ratio of cytoplasmic versus endosomal/lysosomal NeoR protein levels had changed profoundly. In 3-MA-treated cells, most of the NeoR protein is localized in the cytoplasm, while the amount of NeoR protein in the endosomal/lysosomal fraction is strongly reduced (Fig. 5D). To exclude that this increase in NeoR protein levels is due to a stimulatory effect on *neoR* transcription/translation, protein synthesis in *neoR*-transfected cells was blocked with cycloheximide and NeoR protein levels examined by Western blot. As shown in Fig. 5E, NeoR protein levels in cells treated with cycloheximide dropped dramatically, while NeoR protein levels in cells treated with cycloheximide plus 3-MA remained almost constant. These results indicate that 3-MA prevents the degradation of NeoR.

2.6 Inhibition of autophagy blocks NeoR presentation

To determine whether endogenous presentation of the NeoR-derived peptide on MHC class II is dependent on the process of autophagy, RCC1.24 and EBV-B1.11 cells transfected with the *neoR* gene were treated with 3-MA and cell surface presentation monitored with the NeoR-specific T cell clone. As a control, non-transfected RCC1.24 and EBV-B1.11 cells, to which exogenous antigen had been added, were treated likewise. While presentation of exogenously added NeoR protein was not

affected by 3-MA, MHC class II-restricted presentation of endogenous NeoR was inhibited in both cell lines dose dependently. In RCC1.24-NeoR cells, 3-MA almost completely abrogated cell surface presentation (Fig. 6A). Because endogenous antigen presentation in EBV-B1.11-NeoR cells was strongly reduced, but not completely blocked, even by higher concentrations of 3-MA, wortmannin, a structurally unrelated inhibitor of autophagy was used as well. Although higher doses of wortmannin have been shown to affect diverse cellular signal transduction pathways, lower concentrations (<50 nM) of this inhibitor selectively block autophagic sequestration [22]. Endogenous antigen presentation was again inhibited by wortmannin dose dependently and to a similar extent (Fig. 6B). By contrast, neither presentation of endogenously synthesized tyrosinase on MHC class I (Fig. 6C), nor HLA-class I and HLA-DP cell surface expression (Fig. 6D, E) was affected by these inhibitors. These results show that endogenous presentation of the cytosolic antigen NeoR on MHC class II is dependent on the process of autophagy.

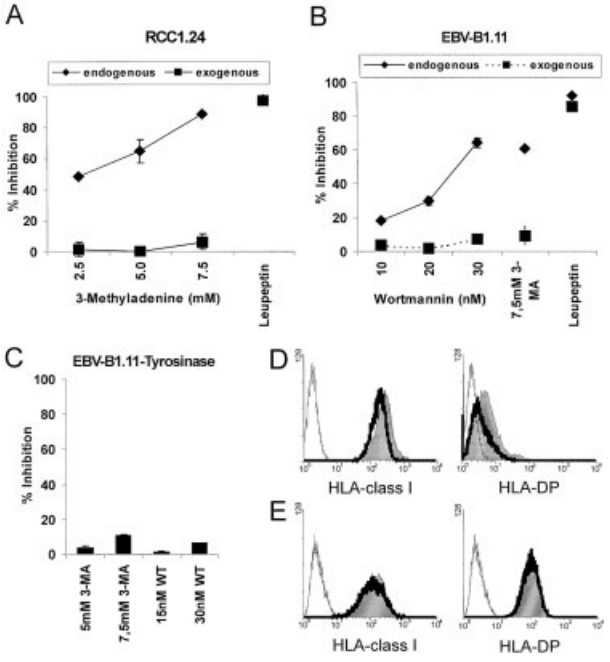

Fig. 6. Inhibition of autophagy blocks endogenous presentation of the NeoR-derived peptide on MHC class II. (A) RCC1.24-NeoR (endogenous), and RCC1.24 cells to which NeoR protein had been added (exogenous), were treated with different concentrations of 3-MA and cell surface presentation of the NeoR-derived peptide on HLA-DP3 monitored with the NeoR-specific T cell clone 20–4/A4. Inhibition of autophagy by 3-MA causes an almost complete inhibition of endogenous antigen presentation, while presentation of exogenous NeoR is not affected. (B) EBV-B1.11 cells transfected with pINCO-NeoR (endogenous), or incubated with recombinant NeoR protein (exogenous), were treated with the inhibitors of autophagy, wortmannin and 3-MA, and HLA-DP3-restricted presentation of the NeoR-derived peptide determined. Inhibition of antigen presentation by leupeptin served as control. (C) Presentation of the HLA-A2-restricted tyrosinase peptide in EBV-B1.11 cells transfected with pINCO-Tyrosinase is not affected by these inhibitors of autophagy. FACS analysis of HLA-class I and HLA-DP expression on RCC1.24 (D) and EBV-B1.11 cells (E). As compared to untreated cells (thick-lined histogram), treatment of cells with 7.5 mM 3-MA (shaded histogram) does not cause down-regulation of HLA-class I or HLA-DP. Isotype control is shown as thin-lined histogram.

3 Discussion

Multiple independent lines of evidence have been reported in the literature demonstrating that MHC class II molecules present peptides derived from cytosolic proteins, but the underlying processing and presentation pathway(s) had remained unknown. Here, we studied MHC class II-restricted presentation of the cytosolic protein NeoR in RCC and EBV-B cells. By using chemical as well as biological inhibitors, we showed that endogenous presentation on MHC class II does not depend upon key features of the classical MHC class I presentation pathway, including proteasome, TPP II and TAP, but is critically dependent on lysosomal processing. Access of the lysosomal compartment via secretion and reuptake as exogenous protein was excluded in cross-presentation experiments. Time course as well as UV-fluorescence experiments indicated that cytosolic NeoR accessed non- or mildly acidic vesicles of the vacuolar system. This vacuolar import was almost completely blocked by inhibitors of autophagy. Concomitant with a dramatic reduction of NeoR protein in the vacuolar compartment, inhibition of autophagy caused a strong increase in cytosolic NeoR protein levels. Most importantly, this dramatic reduction in vacuolar NeoR was paralleled by a similar reduction in MHC class II-restricted cell surface presentation. These results identify autophagy as the main if not exclusive mechanism by which NeoR gains access to the vacuolar compartment, and thus for the endogenous presentation of cytosolic NeoR on MHC class II. Autophagy has been described as a ubiquitous and basic cellular activity in all eukaryotic cell types. It is responsible for the turnover of the majority of intracellular proteins, which may proceed at a maximum rate of about 4%/h [19]. In yeast, multiple means of cytoplasm-to-vacuole transport have been identified, and homologues of the proteins involved also exist in mammalian cells. In macroautophagy, one of the best characterized forms of autophagy, a membranous organelle of unknown origin and composition envelops a

portion of the cytoplasm, eventually enclosing it in a vacuole called an autophagosome. Subsequently, the autophagosome fuses with endosomal/lysosomal vesicles and matures to become an autophagolysosome [19, 23]. Whether NeoR is transported into the vacuolar system by this or a different autophagic mechanism remains to be determined. Autophagy is highly regulated by many factors such as amino acids, second messengers, hormones, and growth factors, and the initial sequestration step is subject to feedback inhibition by degradation products [24]. Because the regulation of autophagy is complex and dependent on cell type and physiological demands, the inhibitory effect of drugs like 3-MA and wortmannin may not always be absolute. This might explain why in the present study antigen presentation was almost completely blocked in RCC, but never exceeded 70% in EBV-B cells even at higher concentrations. Similar variability in antigen presentation depending on the nature of the APC has been observed previously. Brazil et al. [25] described that endogenously synthesized complement C5 was presented on MHC class II molecules in B cells and fibroblasts, but not in macrophages. Interestingly, presentation of this secreted model antigen was also inhibited by 3-MA.

The identification of autophagy as the mechanism responsible for the presentation of a cytosolic antigen on MHC class II implies that potentially all and not just selected cytosolic proteins might have access to the endogenous pathway of MHC class II presentation. Such a model, however, would be in contrast to a number of reports that failed to detect MHC class II-restricted presentation of model antigens when targeted into the cytoplasm. The model antigens used, however, were often signal sequence-deleted versions of secreted or cell surface proteins. As reported for HEL, such ectopic expression may cause misfolding and rapid degradation of the protein in the cytoplasm [26]. These model antigens, therefore, may never reach protein levels sufficient for MHC class II loading and subsequent detection by T cells. The importance of protein stability and consequently protein level for the endogenous presentation of cytosolic proteins on MHC class II has been demonstrated before [27], and is further supported by our observation that presentation of NeoR on MHC class II is enhanced when proteasomal degradation is inhibited. Studies with additional cytosolic antigens will be required to determine to which extent the process of autophagy contributes to the endogenous presentation of cytosolic antigens on MHC class II.

Peptide elution studies had suggested that endogenous presentation of cytosolic antigens is a minor event and thus of limited impact on $CD4^+$ T helper cell regulation. Amino acid sequences, however, had only been obtained for the most abundant peptides. Recent analysis of a larger number of MHC class II-bound peptides revealed a surprisingly high representation of peptides derived from cytosolic proteins [28]. Furthermore, accumulating evidence suggests an important role of this alternative pathway in central tolerance induction [6]. Studies in transgenic mice demonstrated that tolerance of $CD4^+$ T cells to a nuclear model antigen is induced by cells of the medullary thymic epithelium (mTE), but not by bone marrow-derived APC [29]. Importantly, tolerance resulted from endogenous and not from cross-presentation of the model antigen, indicating that mTE utilize the endogenous pathway of MHC class II presentation to induce tolerance to internal proteins. Moreover, cells of the cortical thymic epithelium involved in positive selection of thymocytes efficiently present cytosolic, but not exogenous, antigens on MHC class II, restricting the peptide repertoire to endogenous self antigens [6]. Circumstantial evidence also suggests an important role of this pathway outside of the thymus. The correlation of MHC class II expression on *e.g.* laryngeal or breast cancer cells with disease prognosis infers that direct recognition of tumors by T helper cells is of critical importance to antitumor immunity [30]. Interestingly, two antigens recognized by melanoma-specific $CD4^+$ T cells are derived from cytoplasmic proteins [31]. Furthermore, three out of four $CD4^+$ T cell clones generated from a patient with RCC by repeated *in vitro* stimulation of PBL with autologous tumor cells recognize cytoplasmic antigens (unpublished data), suggesting that the endogenous pathway of MHC class II presentation might be of greater importance than previously recognized, and further understanding of this pathway will allow manipulation of antigen presentation in the future.

4 Materials and methods

4.1 Cell culture

The EBV-transformed B cell lines EBV-B1.11 and EBV-B1.25, and the renal carcinoma cell lines were grown in media consisting of RPMI 1640, 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin. The RCC line RCC1.24 had been established from a primary tumor biopsy [32]. Retroviral transduction of this cell line with *neoR* yielded RCC1.24-NeoR. 293T cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The stable transfectant EBV-B1.11-ICP47 was generated by electroporation of EBV-B1.11 cells with the plasmid pINCO-ICP47 and puromycin selection. The 293T-CytoNeoR cell line was generated by stable transfection of 293T cells with the pINCO-NeoR plasmid. The $CD8^+$ T cell clone specific for the tyrosi-

nase epitope YMNGTMSQV presented on HLA-A2 was isolated from a healthy individual by peptide stimulation as described [33]. T cells were cultured in T cell media consisting of RPMI 1640, 10% human serum, 2 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 0.1 mg/ml streptomycin.

4.2 Construction of plasmids

The coding sequence of *NeoR* was cloned into the pCMV/cyto plasmid (Invitrogen, Groningen), fusing the myc epitope to the C terminus of *neoR*. For subcellular localization studies, the coding sequence of the green fluorescent protein (GFP) was fused to the *neoR* gene, resulting in the plasmid pCMV/cytoNeoR-GFP. The open reading frame of the *tyrosinase* gene was cloned into the pCMV/cyto plasmid. For the generation of stable cell lines the pINCO plasmid [34] (kindly provided by Dr. Pelicci, Perugia, Italy) was used. The above-described ORF were cloned into the pINCO plasmid, giving rise to the plasmids pINCO-NeoR, pINCO-NeoR-GFP and pINCO-Tyrosinase. The pINCO-ICP47 plasmid was generated by inserting the ICP47 ORF from plasmid pKEX2XR/ICP47 [35] (kindly provided by Dr. Rammensee, Tübingen, Germany) into pINCO.

4.3 Antigen processing and presentation assays

EBV-B cells (1×10^7) were transfected with the indicated plasmids (20 μg) by electroporation (Gene Pulser, Bio-Rad), and protein expression allowed for 48 h. Transfected EBV-B cells were either left untreated or treated with different inhibitors for the indicated period of time and subsequently fixed with 0.5% paraformaldehyde (Sigma-Aldrich, Deisenhofen, Germany). RCC1.24-NeoR cells were induced to express MHC class II by addition of 50 U/ml IFN-γ for 48 h, before incubation with the inhibitors. When presentation of exogenous NeoR protein was examined, EBV-B cells were incubated with 200 ng/ml recombinant protein with or without inhibitors for 24 h and subsequently fixed. RCC1.24 cell were first induced with IFN-γ (50 U/ml) for 24 h; 1 μg/ml recombinant NeoR protein was added (+/– inhibitors) for an additional 24 h, and the cells fixed. Unless otherwise indicated, the following concentrations of inhibitors were used: 1 μg/ml BfA (Sigma-Aldrich), 50 μM AAF-CMK (Bachem, Heidelberg, Germany), 100 μM chloroquine (ICN Biomedicals, Eschwege, Germany), 5 μM lactacystin (ICN Biomedicals), 200 μg/ml leupeptin (Biomol, Hamburg, Germany), 1–10 mM 3-MA (Sigma-Aldrich), 10–30 nM wortmannin (Sigma-Aldrich), and 50 μg/ml cycloheximide (Sigma-Aldrich). T cell recognition of the cells was assessed by culturing 1×10^5 T cells with 1×10^5 EBV-B cells or 2×10^4 RCC1.24-NeoR cells in a final volume of 200 μl. Following 20 h of co-culture, the amount of GM-CSF in the cell culture supernatant was determined by ELISA (R&D Systems, Wiesbaden, Germany).

4.4 Subcellular fractionation and Western blot

All subcellular fractionations were done essentially as described [17, 18]. For the separation of endosomes and lysosomes, the 100,000×g pellet was resuspended in 1 ml of fractionation buffer, loaded on a 27% Percoll gradient and centrifuged for 1 h at 22,000 rpm in a Ti-70 rotor. Eight different 1-ml fractions were collected starting from the top of the tube, separated on a 10% SDS-polyacrylamide gel and analyzed by Western blot, using the following antibodies: anti-Myc antibody (Invitrogen), anti-Lamp-1, anti-Rab-5, anti-cytochrome c, and anti-BiP antibodies (all from Becton Dickinson, Heidelberg, Germany), anti-lactate dehydrogenase (LDH; DPC Biermann, Bad Nauheim, Germany). Proteins were visualized by using a horseradish peroxidase coupled anti-mouse Ig antibody (Promega, Madison, WI) and the ECLplus detection system (Amersham, Freiburg, Germany).

4.5 FACS and UV-fluorescence analysis

For cell surface staining, 5×10^5 cells were labeled with saturating amounts of antibodies directed against MHC class I (W6/32, Sigma-Aldrich), and HLA-DP (B7/21, Becton Dickinson). Following incubation with FITC-conjugated goat anti-mouse (IgM and IgG, Dianova, Hamburg, Germany) secondary antibody, cells were analyzed in a FACScan flow cytometer (Becton Dickinson) using the CellQuest software.

For GFP staining, RCC1.24 cells were transfected with pINCO-NeoR-GFP by lipofection. At 48 h post transfection, cells were incubated with inhibitors, and intracellular protein distribution evaluated using a Zeiss Axiovert microscope.

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