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Running Title: CTL priming by non-infectious virus

ABSTRACT

Respiratory syncytial virus causes lower respiratory tract infections in infancy and old age, affecting also immunocompromised patients. The viral fusion protein is an important vaccine candidate eliciting antibody and cell mediated immune responses. Cytotoxic $CD8^+$ T lymphocytes (CTL) are known to play a role in both lung pathology and viral clearance. In BALB/c mice the fusion protein epitope F249-258 is presented to CTL by the murine MHC class I molecule K^d. In cells infected with recombinant vaccinia viruses encoding the fusion protein, F249-258 is presented by MHC class I molecules through pathways that are independent of the transporters associated with antigen processing, TAP. We have now found that F249-258 can be generated from non-infectious virus from an exogenous source. Antigen processing follows a lysosomal pathway that appears to require autophagy. As a practical consequence, inactivated virus suffices for *in vivo* priming of virus-specific $CD8^+$ T lymphocytes.

Keywords: Antigen processing, Autophagy, CTL, MHC class I, TAP, Virus.

INTRODUCTION

Cytotoxic $CD8^+$ T lymphocytes (CTL) specifically recognise MHC class I molecules presenting viral peptides on the surface of infected cells. In general, presented peptides are produced after cytosolic proteolytic processing by the proteasome of newly synthesised viral proteins. Cytosolic viral peptides are then efficiently translocated by the transporter associated with antigen processing (TAP) to the lumen of the endoplasmic reticulum (ER), from where the MHC/peptide complexes reach the cell surface following the constitutive secretory route. Alternative pathways can present antigens independently of TAP by diverse mechanisms (reviewed in[1,2]). Peptides presented on MHC class I molecules are usually processed from antigens synthesised within the infected cell. However, professional antigen presenting cells can process exogenous antigens and cross-present peptides to CTL.

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection in infants and young children[3], affecting also immunocompromised patients and the elderly. The fusion (F) glycoprotein of RSV is a major vaccine candidate as it is an important target for neutralizing antibodies[4] and virus-specific CTL[5]. Studies in the mouse model of human RSV have shown that CTL play a role both in lung pathology and viral clearance[6,7]. In previous studies, CTL epitope F249-258 presented by the murine MHC class I molecule K^d was identified in the RSV F protein[8], and found to be presented only through TAP-independent pathways[9] in cells infected with RSV or recombinant vaccinia virus (rVACV). We now report on the further characterisation of TAP-independent presentation of epitope F249-258 from

rVACV and reveal that epitope F249-258 is processed from exogenous precursors present in the virus inoculum. Exogenous antigen follows a TAP-independent lysosomal MHC class I antigen presentation pathway that appears to require autophagy for CTL activation. Interestingly, $CD8^+$ T lymphocytes are also activated by UV-inactivated (UVi) non-infectious rVACV *in vivo*.

RESULTS AND DISCUSSION

TAP-independent presentation of F249-258 follows an exogenous lysosomal pathway

In a previous study[9] we found that epitope F249-258 is presented by K^d independently of TAP in cells infected with RSV or with rVACV expressing either native F (vvF) or even cytosolic $Fsig^-$ protein ($vvFsig^-$) lacking the signal peptide (Figure 1a). In cells infected with rVACV, TAP-independent presentation of epitope F249-258 was partially resistant to brefeldin A (BFA), a metabolite that specifically blocks anterograde transport from the Golgi complex in the endogenous secretory pathway[9]. Presentation was also partially resistant to lactacystin (LC), a proteasome inhibitor[9]. Such an atypical behaviour of epitope F249-258 suggested an exogenous processing pathway. To assess the requirement for infectious virus, rVACV vvF and $vvFsig^-$ infectious particles were inactivated by UV light. Absence of infectivity was controlled for the UVi rVACV by titration on CV-1 monolayers that revealed no p.f.u., and absence of protein expression from UVi rVACV was confirmed by flow cytometry in infected CV-1 and $T2/K^d$ cells (Figure 1b). Virus titration was always used as it is more sensitive to control inactivation of UVi rVACV, since CV-1 cells infected with vvF at m.o.i. 0.01 were not stained with F-specific antibody but however p.f.u. were observed. Of note, the full-length proteins were not detected by cytometry when UVi viruses adsorbed to cells (Figure 1b).

To detect viral antigen presentation, intracellular cytokine staining (ICS) assays were performed in which TAP-deficient $T2/K^d$ cells served as targets to

activate CTL F/F249-258. This mono-specific CTL line was generated *in vitro* by stimulating splenocytes obtained from vvF-infected BALB/c mice with peptide F249-258. Figure 1c shows CTL were activated by T2/K^d cells and by dendritic cells either infected with rVACV (light grey bars) or with the equivalent volume of the UVi counterparts (dark grey bars). These results suggest that TAP-independent presentation may be due to an exogenous source of F249-258 that was not synthesised endogenously in infected cells, but present in the purified virus inoculum. The equal behaviour of infectious and non-infectious rVACV suggests the existence of a sole exogenous processing pathway. In contrast, in cells expressing the native F protein from its natural context, RSV, we described TAP-independent endogenous presentation of F249-258[9]. Detection of CTL activation occurs above a threshold established by a certain amount of MHC/peptide complexes exposed on the surface of the infected cell. Thus we cannot rule out the possibility that both endogenous and exogenous TAP-independent F249-258 presentation pathways coexist in cells infected with rVACV, but we are currently unable to discriminate among them in our experimental setting. However, we are certain of addressing exclusively the exogenous pathway when UVi rVACV is used.

Dependence on protein synthesis was studied by infecting T2/K^d cells with vvF and vvFsig$^-$ or with their UVi counterparts in the presence of protein synthesis inhibitor cycloheximide (CH). Conventional presentation of viral proteins requires their endogenous expression in infected cells, and thus activation of CTL is completely blocked by CH. In contrast, Figure 1d does not show a strict protein synthesis requirement for F249-258 presentation to CTL. This result is compatible with the presence of epitope precursors in the virus

inoculum that, in order to complete antigen processing, may require some protein synthesis of processing proteases or other cellular factors.

To evaluate if the peptide that represents the final epitope was already present in the virus inoculum, infection of T2/K^d cells with vvF or vvF UVi was not allowed to proceed beyond viral adsorption (Figure 1e). Limiting concentrations of the synthetic peptide representing the final epitope were added in parallel to target cells immediately before fixing, and strong CTL activation was observed (x in Figure 1e). Absence of CTL activation at time 0 min after virus addition thus allows concluding the final peptide itself was not present in the purified rVACV inoculum. Indeed, time was required to induce activation of CTL, probably for the processing of precursors present in the virus inoculum.

TAP-independent processing followed by loading of exogenous antigen-derived peptides onto recycling MHC class I molecules has been shown to occur in endolysosomal acidic compartments[10-12]. MHC class II extracellular antigen processing by dendritic cells is also affected by macroautophagy[13,14]. The requirement found for further processing of precursors of epitope F249-258 may in fact point to a lysosomal or autophagy pathway. In general, autophagy supplies cytoplasmic components to lysosomes for their degradation. However, the F glycoprotein might reach lysosomes either by autophagy or as a final step after signal-peptide-mediated protein insertion into the ER. Therefore, to address different lysosomal processing pathways, including autophagy, cells were infected with virus expressing cytosolic $Fsig^-$, thus theoretically allowing antigen access to lysosomal compartments endogenously from the cytosol only through autophagy ($vvFsig^-$) or exogenously from the inoculum ($vvFsig^-$ and

vvFsig$^-$ UVi). ICS assays were performed in the presence of NH_4Cl, a lysosomotropic agent that raises intralysosomal pH; leupeptin (Leup), which inhibits trypsin-like and cysteine lysosomal proteases[15]; NH_4Cl combined with Leup, which blocks different forms of autophagy, including chaperone-mediated autophagy, as well as lysosomal degradation; and 3-methyladenine (3MA)[16], which blocks both endocytic and endogenous protein degradation through macroautophagy[17]. Figure 1f shows strong inhibitions of CTL activation by all the inhibitors tested except by Leup. In addition, similar results were obtained with chloroquine, another lysosomotropic agent (data not shown). In all cases inhibitions seemed higher for UVi rVACV than for infectious rVACV. Therefore, the data suggest that precursors of epitope F249-258 followed a lysosomal pathway to be processed to the final epitope presented to CTL. MHC class I presentation of an exogenous antigen has been shown to be carried out in these acidic compartments by the Leup-sensitive lysosomal protease cathepsin S[18]. Our results suggest that epitope F249-258 precursors from exogenous sources follow a proteasome-independent and TAP-independent lysosomal pathway that does not require Leup-sensitive endolysosomal serine or cysteine proteases and appears to involve autophagy for epitope presentation to CTL. At this stage it is unclear how exogenous precursors may reach lysosomes by a form of autophagy, but a role for the fusion activity of the F protein was unlikely, as the cytosolic Fsig$^-$ protein was equally presented to CTL by this exogenous, TAP-independent lysosomal pathway. Alternatively, rather than for processing, intact lysosomes may be required to allow entry into the cytosol of infectious or UVi virion cores[19] if they carried epitope F249-258 precursors, or to allow entry of such precursors if they used the same route as the virions. Finally,

exogenous precursors may employ some of the autophagy machinery recruited to the plasma membrane as described for bacterial infection[20].

Lysosomes[12] and, more recently, autophagy have been reported to participate also in endogenous MHC class I presentation of viral epitopes through a proteasome- and TAP-dependent pathway[21] and a TAP- and proteasome-independent pathway[22]. Autophagy also supplies MHC class II molecules with peptides for presentation of cytosolic antigens[23]. In particular, presentation of epitope F249-258 from cells expressing the cytosolic $Fsig^-$ protein is very suggestive of an additional TAP-independent endogenous processing of this F protein by autophagy. No evidence was found for an involvement of chaperone-mediated autophagy, as the inhibitory effect of NH_4Cl combined with Leup was not higher than that of 3MA (Figure 1f). However, the presence of the exogenous pathway, revealed by testing UVi rVACV, precludes the detailed study of additional endogenous pathways and their contribution to overall TAP-independent presentation of these proteins.

Cross-priming of $CD8^+$ T lymphocytes specific for F249-258 in vivo

To assess *in vivo* the relevance of the exogenous lysosomal presentation pathway described in cell culture for epitope F249-258, mice were vaccinated with infectious vvF and non-infectious UVi vvF. The acute $CD8^+$ T lymphocyte response was studied 5 or 6 days after BALB/c mice immunization by performing *ex vivo* ICS assays with cells obtained from the spleen or peritoneal exudate (PEC). vvF (light grey bars) primed $CD8^+$ T lymphocytes specific for F249-258 at levels previously described[8]. Non-infectious UVi vvF (light grey

bars) activated $CD8^+$ T lymphocytes at lower levels, decreasing with respect to vvF eight to three times in spleen and PEC, respectively (Figure 2a), supporting the hypothesis of coexisting endogenous and exogenous TAP-independent F249-258 presentation pathways in cells infected with vvF. Interestingly, when splenocytes from UVi vvF-immunized mice were cultured, stable CTL lines were generated, and they not only recognized peptide-pulsed cells (data not shown) but also cells infected with $vvFsig^-$ (Figure 2b). Therefore, F249-258 specific CTL are cross-primed *in vivo* by non-infectious virus, presumably by the exogenous proteasome-independent TAP-independent lysosomal pathway. Although we do not know the exact nature of the antigenic precursor source of epitope F249-258, it must include a portion of the F protein harbouring the epitope, which is interestingly located near important antigenic sites for neutralising antibodies[24]. Our findings have important implications for RSV vaccine studies as they open the possibility of priming RSV-specific CTL and antibodies with a non-infectious agent.

METHODS

Mice

BALB/c mice (H-2^d haplotype) (The Jackson Laboratory) were bred in our colony in accordance to national regulations. Animal studies have been reviewed and approved by the appropriate institutional review committee. Mice were infected i.p. with 10^7 p.f.u. of vvF or with an equal volume of UVi vvF containing less than 10 p.f.u. after inactivation.

Recombinant vaccinia viruses (rVACV)

Construction of rVACV vvF containing the F gene of the Long strain of RSV has been described[25]. Recombinant vvFsig$^-$ encodes a cytosolic F protein in which the signal peptide has been deleted[26]. As a result, the protein is rapidly degraded and is barely detectable in infected cells. All viruses were based on the Western Reserve (WR) wild type vaccinia strain. Virus stocks were purified by ultracentrifugation of crude infected cell lysates through a 36% sucrose cushion. Inactivation of rVACV was performed by exposing rVACV to 254 nm UV light for 30-60 min at 4ºC. Inactivation of the UVi rVACV was controlled by viral titration on CV-1 cell monolayers to confirm absence of p.f.u. after 24 h infection. Lack of protein expression was monitored by flow cytometry staining with anti-RSV F protein monoclonal antibody (clone 18B2, Argene). Infectious titer was reduced below detection limit by at least 10^6-fold.

Cell lines and virus infection

T2/K^d is a human lymphoblastoid T2 cell line deficient in TAP and transfected with K^d, and was provided by Dr. G. Hämmerling (German Cancer Research Centre, Heidelberg, Germany). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 5×10^{-5} M 2-ME in a 5% CO_2 atmosphere at 37ºC. CV-1 cells are green monkey kidney fibroblasts and were grown in DMEM. Bone marrow derived BALB/c dendritic cells were obtained as described[27].

Infection of cells with rVACV was performed at a m.o.i. of 3-15 as described[9,28]. Protein synthesis inhibitor CH (Sigma) was used at 50 μg ml^{-1} during infection. NH_4Cl was prepared fresh and used at 20 mM. Leup (Sigma) was used at 100 μM. 3MA (Sigma) was prepared fresh in medium and used at 10 mM.

CTL lines and detection of $CD8^+$ T-lymphocyte activation by intracellular cytokine staining (ICS)

Generation of polyclonal CTL line CTL F/F249-258 has been described[8]. Briefly, splenocytes were obtained from vvF-infected or UVi vvF-inoculated BALB/c mice and CTL lines generated by *in vitro* stimulation with 10^{-9} M peptide F249-258 (TYMLTNSELL).

ICS assays to measure $CD8^+$ T lymphocyte activation were performed as described[9]. Target cells were fixed in 1% paraformaldehyde (Electron Microscopy Sciences) at 4ºC for 10 min and washed twice before being used to activate CTL by coculture. In *ex vivo* ICS, splenocytes or PEC were obtained from BALB/c mice 5 or 6 days post-infection with 10^7 p.f.u. vvF or UVi vvF,

stimulated with an excess 10^{-7} M peptide for up to 2 h, and further stimulated for 3 h in the presence of BFA. Following stimulation during coculture, cells were stained and events acquired and analysed as described[9].

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AUTHOR CONTRIBUTIONS

CJ performed the experiments, CJ, MR, DL, and MDV conceived and designed the experiments, CJ and MDV analyzed the data, CJ, JAM and MDV wrote the manuscript, BGB and JAM provided reagents.

The authors declare that they have no financial conflict of interest.

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FIGURE LEGENDS

FIGURE 1. Proteasome-independent and TAP-independent presentation of epitope F249-258 follows a lysosomal exogenous pathway. (**a**) rVACV encoding different forms of RSV F glycoprotein used in this study were based in the Western Reserve (WR) wild-type vaccinia strain. Virus vvF encodes the wild type F protein of RSV. Hydrophobic regions are signal peptide (sp), fusion peptide (fp), and transmembrane region (tm). Processing by furin-like proteases in the ectodomain (arrows) yields the F1 and F2 chains. Virus vvFsig$^-$ encodes a cytosolic form of the F protein lacking the signal peptide. The CTL epitope (▲) presented by K^d assessed in this study is F249-258 (TYMLTNSELL). (**b**) CV-1 cells were infected for 24 h with the indicated virus at the indicated m.o.i., p.f.u. counted, and cells stained with anti-RSV-F antibody. For UVi an estimated m.o.i. ≤ 10^{-7} was calculated. T2/K^d cells were infected for 5 h with indicated rVACV (m.o.i. 5) or with their UVi counterparts (m.o.i. ≤ 10^{-5}). Background staining by cells infected with WR is shown in grey. (**c-f**) T2/K^d cells were infected with rVACV at a m.o.i. of 3-15 (light grey bars) or with the same volume of UVi non-infectious virus, equivalent to a m.o.i. ≤ 10^{-5} (dark grey bars). ICS assays were performed by stimulating CTL F/F249-258 monospecific for epitope F249-258 with the indicated fixed targets in the presence of BFA to intracellularly accumulate IFNγ produced upon CTL activation. Represented data are the percentages of total $CD8^+$ lymphocytes that expressed IFNγ or the percentage of inhibition of CTL activation. When the mean is represented the standard deviation is shown by error bars. Lack of side effects by inhibitors was controlled by measuring CTL

activation by limiting concentrations of exogenous peptide in the presence of inhibitors, as well as their effect on rVACV protein expression. (**c**) DC, dendritic cells, Data are the mean of at least five independent experiments. (**d**) Cells were infected with the indicated virus in presence of protein synthesis inhibitor CH. Data in the left panel are the mean of one to five independent experiments. A representative experiment is shown in the right panel. (**e**) Cells were incubated with vvF, UVi vvF or parental strain WR at 37ºC for the indicated times. As control, CTL activation induced by cells incubated with synthetic peptide just before fixation and washing is shown (x). Results from a representative experiment are shown. (**f**) Cells were infected with vvFsig$^-$ in the presence of inhibitors NH_4Cl (lysosomotropic agent), Leup (inhibitor of lysosomal serine and cysteine proteases) or 3MA (autophagy inhibitor). Percentage activated CTL in the absence of inhibitors ranged from 11 to 38. Data are from one to two independent experiments.

FIGURE 2. CD8$^+$ T lymphocytes specific for epitope F249-258 are cross-primed *in vivo* by non-infectious UVi vvF. (**a**) *Ex vivo* ICS assays were performed with splenocytes or peritoneal exudate cells (PEC) obtained from BALB/c mice 5 or 6 days after immunization with 10^7 p.f.u. vvF (light grey bars) or UVi vvF (dark grey bars). Percentage of total CD8$^+$ lymphocytes that were stained with anti-IFNγ after activation with peptide F249-258 is represented. The mean of 2 independent experiments with a total of 2 and 5 mice infected with vvF or UVi vvF, respectively, is represented and the standard error of the mean indicated by error bars. (**b**) Splenocytes obtained from one of the five BALB/c mice inoculated with UVi vvF were stimulated *in vitro* to generate a CTL

F/F249-258 line. An ICS assay was performed with the obtained CTL line and T2/K^d cells infected with the indicated virus. Percentage of total $CD8^+$ lymphocytes that expressed IFNγ is represented. F249-258 peptide-coated cells activated 60% of the CTL line $CD8^+$ T lymphocytes.

FIGURE 1(a-f). Johnstone *et al*.

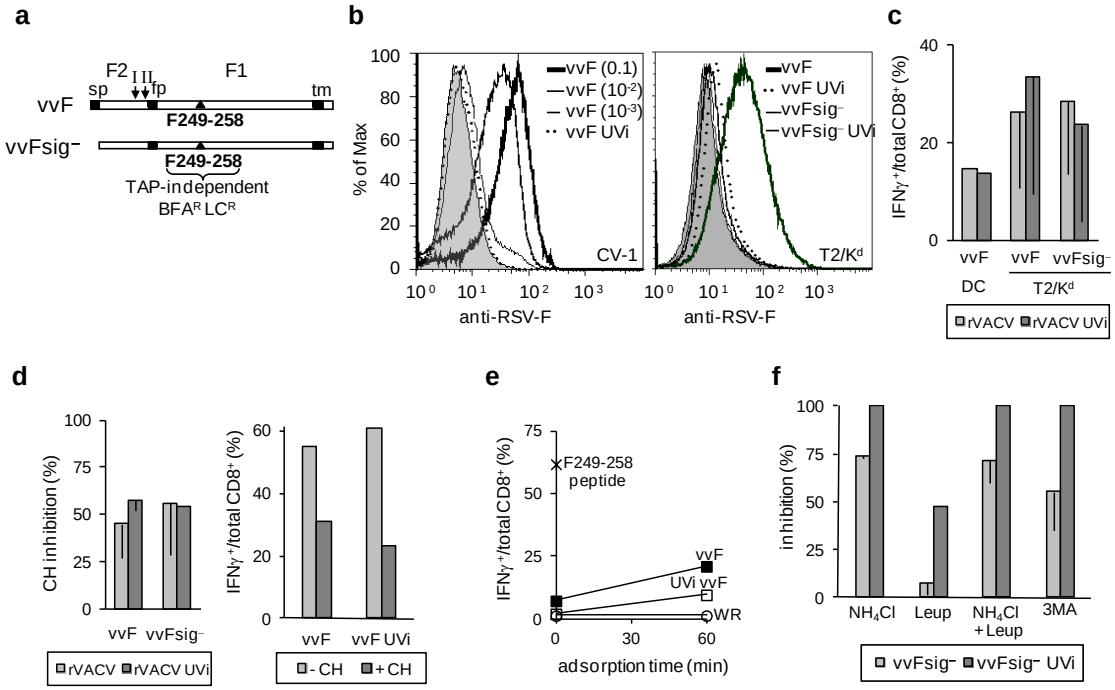

FIGURE 2(a-b). Johnstone *et al*.