Existence of a weak cytotoxic CD4⁺ T cell population in mice infected with ectromelia virus^{*)}

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Summary

This study set to delineate MHC class II immunogenic peptides encoded in proteins expressed by A33R, 14 kDa fusion protein and p42 genes of ectromelia virus (ECTV) Moscow strain (ECTV-Mos), a virus related to variola virus (*Variola vera virus*) responsible for smallpox in humans. A search for a safe and efficacious vaccine against poxviruses is still required mostly because of the emerging nature of certain viruses among poxviruses. In silico prediction of peptides from the 3 protein sequences revealed 6 potential candidates. Investigations included assessment of the peptide's ability to bind to MHC class II molecules on antigen-presenting cells and to induce the proliferation, cytokine synthesis and cytotoxicity of CD4⁺ T cells originating from mice previously infected with ECTV-Mos. The results show that peptide ENHAETLRAAMISLA (Pep3) predicted from the protein sequence 14 kDa fusion protein induced significant proliferation, cytokine synthesis and cytotoxicity. Also Pep3 was able to bind strongly to MHC class II molecules on A20 cells. These results suggest that a small population of CD4⁺ T cells play a protective role dependent on cytotoxicity and possibly complement the CD8⁺ T cells population in this regard.

Keywords: Ectromelia virus, CD4⁺ lymphocytes, epitopes

Conventionally, CD4⁺ T cells are important for their role as helper T cells to both T and B cell antiviral immune functions. It is mostly believed that CD4⁺ T cells are a source of both Th1 and Th2 cytokines, but little attention has been paid to the possible role of CD4⁺ T cells in the cytotoxic function. Many reports in this regard deal with the possible role of CD4⁺ T cells in anti-tumor immune responses. Quezada et al. (7) showed CD4⁺ T cell cytotoxic function in a mouse melanoma model under lymphopenic conditions. Antigen recognition was entirely dependent on MHC class II expression on tumor cells. An intriguing finding was the observation of a cytotoxic function of CD4⁺ T cells in a bystander manner against MHC class II-negative tumor cells (2). Yet other studies have shown that it is possible to engineer CD4⁺ T cells that serve as helper T cells and cytotoxic MHC class II-restricted CD4⁺ T cells (15). The referenced studies show that CD4⁺ T cells contribute to the overall cytotoxicity against tumors and therefore have a potential application in antitumor therapy.

Because of the intracellular nature of virus infections, their control is effectively achieved by CD8⁺ T cells, whereas CD4⁺ T cells are rarely described as cytotoxic T cells in viral infections. In a retroviral infection model, it was possible to demonstrate significant CD4⁺ T cell cytotoxicity only after depletion of both regulatory T cells and CD8⁺ T cells (4). Also polyfunctional CD4⁺ T cells have been reported in viral infections. In terminal liver disease resulting from viral hepatitis, a population of highly cytotoxic CD4⁺ T cells have been reported (1). A recent report ascribes to the existence of a CD4⁺ T cell cytotoxic response in the acute phase of vaccinia virus infection (6). Although a large body of literature exists, many aspects regarding development of CD4⁺ cytotoxic T cells remain unexplained (5, 10). With the knowledge of antigenic peptides that are immunodominant in viral infections it would be possible to design antiviral T cell vaccines that incorporate both CD8⁺ and CD4⁺ T cell antigenic peptides. Such a rational approach to vaccine design could optimize vaccine design capable of inducing cytotoxic memory responses in both CD8⁺ and CD4⁺ T cell compartments.

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The Poxviridae family of viruses includes the variola virus (VARV, smallpox). Fortunately, the relentless vaccination program instituted by the World Health Organization has led to the elimination of this virus (17). However, other poxviruses, such as the zoonotic monkeypox virus (MPXV), still pose a threat to human health (16). Therefore, investigations into ways to improve the current vaccines against poxviruses are warranted. An earlier publication (12) indicated lytic activity of a small population of CD4⁺ T cells against A20 cells previously infected with the Moscow strain of mouse pox virus. We show that at least one peptide epitope was able to stimulate CD4⁺ T cells from infected mice and show a low-level cytotoxic activity against A20 cells infected with mouse pox virus. Therefore, it is highly possible that CD4⁺ T cells in pox virus infection may play a polyfunctional role in immune response. The aim of this study was to delineate the epitopes that may be responsible for activation of this CD4⁺ T cell population to elicit a cytotoxic function.

Material and methods

Cells and virus. Ectromelia virus Moscow strain (ECTV-Mos) was purchased from ATCC (ATCC® VR-1374™) and propagated and titrated in Vero cells (ATCC® CCL-81™) and kept at -80°C until use. Vero cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic.

Mice and infection. BALB/c mice were purchased from the Maria Skłodowska-Curie Oncology Center in Warsaw and acclimatized before use in any experiments. All experiments were performed with permission of the ethics committee (Permit no. 41/2010). The mice were infected in the footpad with the Moscow strain of ectromelia virus at a dose of 1×10^4 pfu/ml in 20 µl. They were observed for 5 days. At least 5 mice were used in the virus infected or control group.

Peptides. Peptides were predicted by *in silico* analysis of amino acid sequences translated from 3 genes encoded by the mouse pox virus: A33R, fusion protein 14 kDa and p42. Only peptides with the highest probability score were selected for *in vitro* investigations. Table 1 shows the amino acid sequences of MHC class II epitopes for H-2A mice predicted by SYFPEITHI database (<http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm>). Synthesis of peptides was outsourced to Polipharm (Poland). Before use, all peptides were first reconstituted in DMSO and then diluted in RPMI-1640. Additionally, fluorescently labelled standard MHC class II peptides (8) for binding assays were purchased from Sigma (USA).

Cell harvest and stimulation. To assess the capability of the predicted peptides to stimulate CD4⁺ T cells, a proliferation was performed. Briefly, splenocytes from mice were harvested at 5 days post infection (dpi) and suspended in RPMI-1640 medium supplemented with 10% FBS and glutamine. CD4⁺ and CD8⁺ T cells were sorted out with the MACS cell separation system and stained with CFSE (Carboxyfluorescein succinimidyl ester, Molecular Probes, USA) as described by the manufacturer. The remaining cells from the flow-through of the sorting procedure were pulsed with

Tab. 1. Amino acid sequences of predicted CD4⁺ T cell epitopes

Gene	Peptide	Amino acid sequence
A33R	Pep1	T D S A V D V A A T S S T H R
	Pep2	L L S M I T M S A F L I V R L
14 kDa	Pep3	E N H A E T L R A A M I S L A
	Pep4	D K H E A T V K A D G D D N E
p42	Pep5	I D W I T D V I V Q S I L R G
	Pep6	Q F R A W K K R I A G R D Y M
F2L	Pep7	S N H A A G Y D L*

Explanations: *A CD8⁺ specific peptide, used here as a control peptide for MHC class I, reported by Tschärke (14).

individual peptides (Tab. 1) at 5 µg/ml for 2 h, inactivated with mitomycin D and later incubated with sorted CFSE-stained CD4⁺ or CD8⁺ T cells at a concentration of 2×10^4 for 72 h. Proliferation was measured by flow cytometry, and proliferation indices were calculated as described by FlowJo (LLC, USA). To assess cytokine synthesis, CD4⁺ or CD8⁺ T cells were similarly prepared but not stained with CFSE and incubated with peptide pulsed, mitomycin D inactivated splenocytes. Brefeldin A was added at least 5 h before the end of the 72 h incubation period. Cytokine synthesis was assessed by Cell Fixation/Permeabilization Kit for Intracellular Cytokine Analysis and flow cytometry (FACs Calibur, BD Biosciences, USA).

MHC binding assay. A competitive binding assay was used to determine the binding efficiency of peptides to MHC class II molecules on A20 cells. Standard peptides labelled with FITC at a concentration of 5 µg/ml were mixed with an equal amount of the assessed peptides and added to A20 cells or EMT6 cells. The mixture was incubated at 37°C for 4 h, and binding efficiency was measured by flow cytometry.

Cytotoxicity assay. To assess cytotoxic capability of T cells, sorted CD4⁺ or CD8⁺ T cells were incubated with splenocytes pulsed with peptides and inactivated with mitomycin D for 5 days. Later, A20 (MHC II) and EMT6 cells (MHC I) encoding GFP were infected with ECTV-Mos for 2 h, washed and incubated with peptide-activated CD4⁺ T cells for 5 hours. A flow cytometry-based cytotoxicity assays were performed as described in detailed in (13) with a modification regarding target cells. EMT6 and A20 encoding GFP were used instead.

Statistical analysis. The non-parametric Wilcoxon test for paired samples was used to compare the means, where $p \leq 0.05$ was considered significant.

Results and discussion

A flow cytometry-based assay involving measurement of CFSE dilution was employed to ascertain if the predicted peptides were capable of stimulating a proliferative response of CD4⁺ T cells originating from mice previously infected with ECTV-Mos. Figure 1 shows proliferation indices of all investigated peptides. Out of 6 predicted peptides, only Pep3 was capable of inducing statistically significant ($p \leq 0.05$) proliferation of CD4⁺ T cells in comparison to other cells, not stimulated, or other tested peptides. Pep7, a control peptide specific for cytotoxic CD8⁺ T cells, was able to induce proliferation of CD8⁺ T cells only, indicating technical soundness of

the technique used. These data suggest that 14 kDa of ECTV-Mos encodes a peptide specific for CD4⁺ T cells. Furthermore, these proliferation data were supported by MHC binding data, where Pep3 for MHC class II competitively displaced the standard labelled peptides as shown in Figure 2. This indicates that the fusion protein of ECTV-Mos contains a CD4⁺ T cell epitope that binds MHC class II molecules with considerable affinity

Fig. 1. Proliferation indices showing reactivity of CD4⁺ T cells isolated from mice previously infected with ECTV-Mos, upon stimulation with individual peptides predicted from A33R, 14 kDa and p42 amino acid sequences. Pep7 was used as a control to stimulate CD8⁺ T cells. It is a peptide predicted from F2L and reported earlier (14), which is specific for CD8⁺ T cells. Explanations: * – $p \leq 0.05$ when compared to NS (non-peptide stimulated) cells.

Fig. 2. Binding of peptides to MHC class II molecules on A20 cells. A competitive assay with fluorescently labelled peptides as described in “Material and methods”

Fig. 3. Cytokine synthesis. CD4⁺ T cells isolated from mice infected with ECTV-Mos were stimulated with Pep3 followed by intracellular cytokine detection

Explanations: NP – no peptide stimulation; * – $p > 0.05$ in comparison to NP; ** – $p \leq 0.05$ in comparison to NP. Statistics were based on data from at least 3 experiments.

ity and can induce proliferation. The drawback to the technique used here was the inability to calculate IC_{50} , which would have made it possible to precisely define the degree of affinity. Activated T cells also engage in cytokine synthesis and release. To confirm this, both Th1 and Th2 cytokines were measured for Pep3 only. Figure 3 shows that non-stimulated CD4⁺ T cells synthesized a background level of all investigated cytokines. However, when the CD4⁺ T cells were stimulated with Pep3 IL-4, IL-6, IL-10 and IL-13 were synthesized more than 3-fold higher compared to non-stimulated control counterparts. The only exception was IFN-γ, which was synthesized at an equal level with non-stimulated control cells. This is interpreted as lack of CD4⁺ T cell activation in this regard. All increases in cytokine synthesis were statistically significantly different from controls. Th1 cytokines, i.e., IL-2 and IFN-γ, were synthesized but not significantly different from non-stimulated cells. IFN-γ was synthesized at a higher level in CD8⁺ (data not shown) T cells stimulated with already described Pep7 (14) specific for MHC class I. The cytokine profile derived from stimulation with Pep3 suggests that this population of CD4⁺ T cells may be inclined to perform functions suited to a Th2 immune response.

In a previous report, CD4⁺ T cells with a cytotoxic profile were reported following infection of BALB/c mice with ECTV-Mos. In this study, further investigations were carried out in respect to Pep3, which showed activation of CD4⁺ T cells. A20 cells which express MHC class II molecules were used as target cells in flow cytometry-based cytotoxicity assays. Double positive (GFP/7AAD) cells indicated cytotoxicity of CD4⁺ T cells (Fig. 4). Pep7 served as a positive control for

Fig. 4. Cytotoxicity of CD4⁺ T cells against targets cells. (A) A20 cells were infected with ECTV-Mos and later co-cultured with CD4⁺ T cells stimulated with Pep3. (B) EMT6 cells against CD8⁺ T cells

Explanations: E : T – Effector to Target ratio; NS – cells from infected mice not re-stimulated with peptide; * – $p \leq 0.05$ compared to NS and control cells from non-infected mice.

the technique used. Clearly, CD4⁺ T cells re-stimulated with Pep3 were activated to elicit cytotoxicity against A20 cells. However, the killing level was rather low compared to that of CD8⁺ T cells against EMT6 cells, but consistent with that achieved with CD4⁺ T cells not re-stimulated but originating from infected mice and with that in (12) and similar to that achieved in (1). The results reported here show that although cytotoxicity is always associated with CD8⁺ T cells, there are viral infections in which this particular role is also shared with CD4⁺ T cells. It suggests that besides CD8⁺ T cells, a complementation of the cytotoxic function is available from a population in the CD4⁺ T cell compartment. However, a different issue which requires further investigation is the mechanism of epitope acquisition by antigen presenting cells. In influenza virus infection, cytotoxic CD4⁺ T cells are readily induced in the primary immune response through dependence on type I IFNs and IL-2/IL-2 receptor signalling pathway (3). In the case of Dengue virus infection, the development of cytotoxic CD4⁺ T cells is reported to occur during the secondary stimulation, when the primed CD4⁺ T cells increase the expression of CD45RA and downregulated CCR7 (11). Although, many studies now point to the presence of cytolytic CD4⁺ T cells in virus infections, the significance of the role they play is still not well defined. It is rather difficult to measure this significance because the phenotype of these cytotoxic CD4⁺ T cells appears to differ depending on the type of the infecting virus. Only models that exclude the entire CD4⁺ T cells long term or transiently show that there is little if any influence on the development of CD8⁺ T cell memory (9). However, definition of epitopes can aid construction of subunit vaccines. Such vaccines do not require the entire pathogen. The essential antigens lower the chances of adverse effects of a vaccines and increase the potential of eliciting efficient immune response. In conclusion, the results reported in this study show that a 14 kDa fusion protein of ECTV-Mos encodes an epitope to which CD4⁺ T cells are primed to elicit a low-level cytotoxic immune response, and therefore possibly complementing the CD8⁺ T cells compartment.

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