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Chronic Viral Infections Interfere with the Activation of CD8+T Cells

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ABSTRACT

To optimally prime naïve CD8+ T cells pAPCs, such as dendritic cells and macrophages, must present appropriate viral peptides to CD8+ T cells. Priming of CD8+T cells can be either via direct presentation by using endogenous antigens or cross-presentation by utilizing exogenous antigens. Licensing of pAPCs to activate CD+T cells are greatly affected by infection with some viruses that interfere with pathways of antigen presentation. In our results, LCMV-infected macrophages have upregulated their phagocytic activity up to 2 folds than normal, *in vitro*. Besides, BM-MØ prepared from LCMV-WE and Cl13-infected mice have shown an interesting variation in up-regulating some surface markers. Importantly, while LCMV-Cl13 has increased CD86 expression rates, LCMV-WE had no effect. Additionally, BM- MØ derived from LCMV-WE and Cl13-infectd mice have activated LCMV-specific CD8+T cells equally. In contrast, Sp-MØ prepared from LCMV-WE, but not from LCMV-Cl13-infected mice, have lost their ability to activate the same CD8+T cells profile. In other hand, cross-priming of OVA-specific CD8+T cells was greatly affected by subjecting mice to LCMV-WE and Cl13 infection. While LCMV-WE improved the cross-priming efficiency of OVA-specific CD8+T cells, LCMV-Cl13 downregulated the cross-priming efficiency of OVA-specific CD8+T cells. Collectively, induction of CD8+T cells to exert their cytotoxic activity against infection, via either direct or cross- presentation pathways, is greatly influenced by acute vs. chronic virus infection.

Keywords - *Cross-presentation; Phagocytosis; Surface markers; CD8+T cells.*

INTRODUCTION

Acute viral infections are often characterized by substantial activation and expansion of CD8+ T cells. In contrast, chronic virus infection results in the functional impairment (exhaustion) and/or physical deletion of CD8+T cells. As a model, different LCMV strains can result in either an acute or chronic infection, for example, LCMV-Arm causes an acute infection, while LCMV-Cl13 causes chronic infection. In the acute LCMV model, about 10% of effector cells differentiate into long-lived memory cells. The protective CD8+T cell response is directed against several epitopes from the glycoprotein (GP) and nucleoprotein (NP) of LCMV. In chronic LCMV infections, there is an exhaustion of NP396-specific T cells and altered immunodominance in T cell specificities.[1-5] Interestingly, these two strains differ by only two amino acids, but they preserve all known T-cell epitopes.[6,7] Therefore, the LCMV infection models are excellent systems to investigate cell mediated immunity during virus infections because they induce different CD8+ responses. To optimally prime naïve CD8+ T cells, pAPCs such as dendritic cells and macrophages must present appropriate viral peptides to CD8+ T cells. Because pAPCs are essential in adaptive immunity, they represent an ideal target for immunosuppressive viruses. For example, LCMV clone-13 (LCMV-Cl13), which establishes persistent infections, negatively affects CD11c^{+high} DCs.[8]

Infection with high doses of the LCMV-Cl13 impairs, but does not totally abolish, the expression of MHC and co-stimulatory molecules on CD11c^{+} DCs.[8] As a result, these DCs do not efficiently stimulate T cell proliferation *ex vivo*.[8] However, other pAPCs populations such as macrophages are not significantly affected by LCMV-Cl13 infection.[8] The activation status of pAPCs can affect immunity during viral infections. Recent data indicate that rapid activation of DCs occurs in the spleen after acute LCMV infection and is associated with an increase in splenic pDCs and a decrease in classical DCs as early as day three post infection.[9]

We previously provided evidence that cross-priming is an important mechanism in CTL immune responses.[10-13] In that, pAPCs acquire exogenous antigens and utilize it to generate 8-11 amino acids epitopes that are loaded on MHC-I molecules and expressed on the surface to activate CD8+T cells. Nevertheless, currently there is little understanding of the functionality of cross-priming during the onset of persistent virus infections in the host. With regard to cross presentation, we now recognize that pAPC subsets can exhibit differences in their abilities to cross-process diverse antigens.[12] For example, CD11c^{+} CD8^{+} DCs are the most efficient population in inducing CD8+T

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cell activation to exogenous cell-associated antigens.[14-17] Such efficiency could not merely be attributed to their uptake capabilities but are related to unknown mechanisms, when compared to less efficient pAPCs.[18] If a specialized cross-processing mechanism, which allows certain pAPC populations to efficiently cross-prime, does exist, it is plausible that persistent viruses will interfere with it during infections.

In this work, we utilize the LCMV infection model[19] to study an important immune concept in T cell priming which has not previously been investigated: the cross-priming mechanism during the establishment of persistent viral infections.

MATERIALS AND METHODS

Mice, cells and virus

C57BL/6 (H-2^b) mice were purchased from Charles River (St. Constant, QC, Canada), and were used between 6 to 8 weeks of age. Animal experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care. DC2.4 a dendritic cells line[20] were cultured in Roswell Park Memorial Institute Medium (RPMI) [Invitrogen, Burlington] containing 10% FBS. Lymphocytic Choriomeningitis virus (LCMV) – WE and Cl13 strains [obtained from F. Lehmann-Grube, Hamburg, Germany] were propagated in a L929 fibroblast cell line [ATCC] and used in subsequent experiments at the indicated pfu. Mice were infected i.v with 10^6 pfu of LCMV-WE and Cl13.

Intracellular staining of LCMV-NP

DC2.4 were infected at a multiplicity of infection (m.o.i) of 1 pfu/cell in serum free medium for 1 hr at 37ºC. DC2.4 (control or LCMV-WE-infected) and LCMV-infected DC2.4 were harvested for fixation with 4% formalin for 30 min at room temperature and washed with PBS. The cells were then permeabilized with 1% Triton X-100 for 20 min at room temperature and incubated for 1 hr with VL4 antibody.[21] After washing with PBS twice, FITC-conjugated AffiniPure F(ab')$_2$ fragment goat anti-rat IgG (H+L) Ab (1 µg/ml) was left with the cells for 1 hr or overnight at 4°C. Cells were washed and resuspended in ~ 400µL FACS buffer (0.8 g NaCl, 0.02 g KCl, 0.115 g Na_2HPO_4, 0.013 g NaN_3 in 100 ml). Cells were acquired with an Epics XL-MCL flow cytometer [Beckman Coulter, Miami, FL].

Phagocytosis assay

LCMV-infected, at m.o.i. of 1 for 24 hr at 37ºC, and non-infected DC2.4 were prepared in round-bottom 96-well plates at 1 x 10^5 cells/well. The DC2.4 were then co-cultured with antigen donor cells (ADCs) at a ratio of 3:1 (ADCs: DC2.4) for 1 hr at 37°C. ADCs were HEK cells labeled with the fluorescent marker carboxyfluorescein diacetate succinimidyl ester (CFSE). The cells were labeled according to the manufacturer's instruction (Invitrogen). Briefly, HEK cells were harvested and resuspended in PBS followed by incubation with CFSE dye 0.2 µg/ml at 37 ºC for 15 min. The cells were washed twice following incubation and resuspended in fresh DMEM medium. The CFSE-labeled HEK cells (ADCs) were treated to undergo death by a treatment referred as LyUV. [22]After then the un-up-taken ADCs were washed out and DC2.4 were then stained with anti-mouse H-2K^b R-PE antibody for 15 min at 4 ºC to distinguish them from the antigen donor cells. Cells were analyzed by FCM and the percentage of phagocytosis was calculated based on the number of double positive DC2.4 that indicated uptake of the CFSE-labeled LyUV-treated HEK cells

Preparation of bone marrow-derived and spleen macrophages

BM-MØ were prepared as previously described.[23] Femurs and tibias from 6-8 weeks old C57BL/6 mice were collected and the marrow was flushed with warm PBS using a 26g$^{3/8}$ sterile needle (Becton-Dickinson, Rutherford, N.J, U.S.A). Bone marrow cells were washed twice with warm PBS and re-suspended in lysis buffer (1.66% w/v ammonium chloride) for 5 min with gentile shaking to lyse red blood cells. The cells were then washed twice with warm PBS. Debris was removed by passing the cells suspension through a metal sieve. Cells were cultured in 6-well tissue culture plates (3-5 x 10^6 cells/ well in conditioned medium (CM), consisting of RPMI containing 10% FBS and 20% of L929 supernatant as a source of M-CSF or 5 ng/ml of recombinant mouse M-CSF (Shenandoah Biotechnology, PA). After 3 days, the non-adherent cells were washed out and fresh medium was added. The medium was changed every 2 days and the cells were harvested with 1x Trypsin-EDTA and tested at the indicated times.

Spleen macrophages (Sp-MØ) were prepared as previously described.[24] Macrophages were either isolated from single individual spleens or from 3 pooled spleens if higher cell numbers were required. To prepare Sp-MØ, homogenized splenocytes were passed through a metal sieve to remove debris, and the cells were suspended for 3-5 min at 37ºC in lysis buffer (1.66 % ammonium chloride) to lyse the red blood cells. After two washing steps with warm RPMI 10% FBS medium, the cells were cultured in recombinant M-CSF (5 ng / ml) or CM as described above. After 3 days of culture, non-adherent cells were removed as the entire medium was replaced on day 3 to enrich it for adherent cells. This was repeated again on day 6 and adherent cells were harvested on day 7. The cells were collected with 1x Trypsin-EDTA and tested at the indicated times of culture

Flow cytometry analysis

The macrophages were stained directly with fluorchrome-labeled Abs against surface markers; PE-conjugated anti-mouse MHC-I, H-2K^b (clone CTKb), FITC-conjugated anti-mouse MHC-II, I-Ab, (clone 25-9-17s) PE-Cy5-conjugated anti-mouse CD11b (clone M1/70.15), PE-conjugated Hamster anti-mouse CD11c (clone N418), PE-conjugated anti-mouse CD80 (clone RMMP-1), PE-conjugated anti-mouse CD86 (clone RMMP-2), and FITC-conjugated anti-mouse F4/80 (clone C1:A3-1) were purchased from Cedarlane, (Ontario, Canada). For cell surface staining, the cells were stained for 15-20 min at

4ºC. After two washing cycles with cold PBS buffer, the cells were prepared for measurement with flow cytometry (FCM). Data were acquired with the Epics XL-MCL flow cytometer and analyzed with the Expo 32 Advanced Digital Compensation Software package (Beckman Coulter, Miami, FL).

Antigen presentation assays employing peptide-specific CD8+ T cells

For direct presentation assays, peptide-specific CD8+ T cells were generated as described previously.[22] Briefly, B6 mice were injected i.v. with 200 pfu LCMV and left for 4-6 weeks before spleens were removed and lymphocytes were purified by ficoll-gradient centrifugation with lymphocytes separation medium (Fisher, Whitby, On). Purified splenocytes were then re-stimulated with peptide-pulsed (10^{-7} M) γ-irradiated BMA cells in the presence of IL-2 (20 U/ml). On day 5 or 6 post stimulation, an additional ficoll-gradient centrifugation step was conducted 2 days before testing in the antigen presentation assays. At this stage, the cells were found to be specific for the peptide used for stimulation with purity reaching > 80% as determined with ICS assays. Four CTLs specific for LCMV-NP396, NP205, GP33, and GP276 were involved. BM- and Sp-MØ prepared from LCMV-WE and CL13-infected mice were co-incubated with peptide-specific CD8+ T cells for 3 hr in presence of BFA 10 µg/ml at a ratio of 1:1. The intracellular cytokine staining was carried out as described.[22] Briefly, the cells were stained for $CD8^+$ with Tri-color conjugate (The Texas Red, Alexa Fluor and Pacific Blue), Rat anti-mouse CD8a (Cedarlane, Hornby, On) for 20 min on ice, washed twice with PBS, and fixed with 1% paraformaldehyde for 20 min at room temp. After two washing steps, the cells were stained with FITC-conjugated Rat anti-mouse IFN-γ (CEdarlane, Hornby, On) in PBS/0.1% saponin for overnight at 4ºC. FACS was performed and the percentage of the activated CTLs was calculated based on the number of double positive cells.

In vivo cross-priming of OVA

To test for cross-priming, two groups of B6 mice were injected first with 10^6 pfu of LCMV-WE and Cl13 i.v. followed by subcutaneous (s.c.) immunization with 25 mg/ml OVA and intravenous (i.v.) injection with Sp-MØ incubated *in vitro* with OVA at 10^6 cells/mouse. After 7 days, splenocytes were obtained and expanded *in vitro* with peptide-pulsed (10^{-7} M) γ-irradiated BMA cells (specific for NP396, NP205, GP33, and GP 276) in the presence of IL-2 (20 U/ml). On day 5 or 6 post stimulation, an additional ficoll-gradient centrifugation step was conducted 2 days. The CTL lines were then tested against peptide-pulsed DC2.4 in presence of BFA 10µg/ml and the intracellular cytokine staining was performed as described above.

RESULTS

The phagocytic capacity of uninfected vs. LCMV-infected DC2.4

We first sought to determine whether the internalization process of DC2.4 (a dendritic cell line) cells is increased or decreased during infection with LCMV-WE. The comparison of the phagocytic capacity of infected and non-infected pAPCs will help in providing insight into the mechanisms whereby these cells regulate this activity. As we described in materials and methods LyUV-treated HEK cells were employed as antigen donor cells or target cells, as described elsewhere.[22]

DC2.4 cells, first, were tested for their susceptibility to LCMV-WE infection by subjecting them to direct infection with LCMV-WE at m.o.i of 1 for 24 hr at 37°C. The cells were then examined for *de novo* synthesis of the major LCMV nucleoproteins (LCMV-NP) which was used as an indicator of the infectivity. Importantly, DC2.4 cells were shown to be highly susceptible to LCMV-WE infections, evident by the abundant expression of LCMV-NP (Figure 1a).

For the phagocytic experiment, HEK cells were labeled with CFSE at a concentration of 0.2 µg/ml, LyUV treated, and then co-incubated with LCMV-infected DC2.4, as previously described, and non-infected DC2.4 at a ratio of 1:3 for 1 hr at 37Cº. It is well known that pAPCs, including DCs and MØ, have high a capacity to capture and internalize antigens from the surroundings using different mechanisms including phagocytosis, pinocytosis, and receptor-mediated endocytosis.[25,26] Interestingly, LCMV-infected DC2.4 cells showed more than a 100 fold increase of taking up LyUV-treated HEK cells than non-infected DC2.4 (Figure 1b). The condition of the ADCs was not critical, since we obtained similar results with LCMV-infected HEK cells.

Changes in the surface marker profiles of BM-MØ prepared from mice infected with LCMV-WE and LCMV-Cl13

To further study and compare the effect of LCMV-WE versus Cl13 on the differentiation and expression of some surface markers of pAPCs, two groups of B6 mice were injected i.v.: one with 10^6 LCMV-WE, and the other with the same dose of LCMV-Cl13 for 7 days. This investigation was initially directed to test the effect of LCMV infection on bone marrow-derived macrophages (BM-MØ). These MØ were used 7 days after isolation from bone marrow and cultivation in 20% M-CSF containing RPMI medium. The results we show here indicate that the prepared MØ were highly pure, since about 98% of them were expressing F4/80, a specific macrophage marker (Figure 2).

BM-MØ prepared from LCMV-WE (solid line) and Cl13(dashed line)-infected mice have shown marked differences in the expression rates of some surface markers. The results have shown that the expression rates of MHC-I, II, CD11c, and F4/80 in both conditions was clearly high, except the CD80 (costimulatory molecule) surface marker which was totally absent in both conditions

(Figure 2). As compared to the uninfected control, the expression rate of CD11b surface marker was markedly higher in LCMV-Cl13 than in LCMA-WE. Although BM-MØ prepared from LCMV-Cl13-infected mice have shown a high expression rate of CD86 (costimulatory molecule), the expression level of this marker on the LCMV-WE-infected BM-MØ was nearly undetectable.

BM- and Sp-MØ prepared from LCMV-WE and Cl13-infected mice have activated CTLs differently

To expand upon the previous observations and investigate the capacity of BM- and Sp-MØ prepared from LCMV-WE and Cl13-infected mice to activate CTL responses, we evaluated the activation profile of LCMV-specific CTLs. Experimentally, two groups of B6 mice were infected with LCMV-WE and Cl13, and BM-MØ and spleen-derived macrophages (Sp-MØ) were tested for their capacity to activate LCMV-specific CTLs prepared from memory mice against four epitopes (NP396, NP205, GP33, and GP276). MØ and CTLs were co-incubated in presence of BFA 10μg/ml and the production of IFN-γ by the activated CTL was assessed by applying the ICS technique.

Based on the results depicted in (Figure 3a and 3b), both LCMV-WE and Cl13 targeted cells in the secondary lymphoid organs such as bone marrow cells and splenocytes *in vivo*. BM- and Sp-MØ were susceptible to LCMV-WE and Cl13 infection as evidenced by their capacity to activate all four LCMV-specific CTLs. Remarkably; activation of LCMV-specific CTLs by Sp-MØ from LCMV-Cl13-infected mice was much higher than those from LCMV-WE-infected mice. In both cases, the GP33 and NP396 epitopes were dominant, while the response to GP276 and NP205 was subdominant (Figure 3b). Interestingly, the scenario is completely different in the bone marrow, in that BM-MØ prepared from LCMV-WE and Cl13-infected mice have shown a high and comparable capacity to activate LCMV-specific CTLs (Figure 3a). Notably, and in contrast to observations by Sp-MØ, the activation profiles of LCMV-specific CTLs were very close, with a slight domination of GP33 and NP396-specific CTLs. Thus, LCMV-WE and Cl13 strains can induce a potent state of infection in bone marrow progenitor cells that persist for long time as compared to splenocytes.

The effects of LCMV-WE v.s. Cl13 on cross-priming of OVA

In an attempt to understand the effect of persistent virus infection on *in vivo* cross-priming, we compared the influence of LCMV-WE v.s. LCMV-Cl13 on the ability of pAPCs to cross-prime OVA-specific CTLs. To optimally prime naïve CD8+T cells pAPCs, such as DCs and MØ, must present appropriate viral peptides to CD8+T cells.

Our data obtained during the early stages of LCMV infections and depicted in Figure 4 has demonstrated a significant difference in the outcome of cross-priming during the initiation of acute or chronic infections. In this experiment (n=2), 4 mice were injected intraperitoneally (i.p.) with 1x10^6 pfu of either LCMV-WE or LCMV-Cl13, or left uninfected. After 24 hr post infection, all mice (except negative controls) were injected subcutaneously (s.c.) with OVA protein (Sigma, 25 mg/mouse). After 8 days, the splenocytes were isolated and OVA-specific CTLs were prepared and expanded *in vitro* using the MHC class I SIINFEKL epitope (OVA MHC class I peptide) as well as medium supplemented with 25 U/ml IL-2 as previously described.[22] Figure 4 shows that cross-priming of OVA in untreated mice resulted in 50% of activated CTLs. Prior infection with LCMV-WE increased the percentage of activated OVA-specific CTLs to 70%, whereas infection with the persistent strain LCMV-Cl13 resulted in a profound reduction of CTLs (35%) specific for the exogenous antigen OVA. It is also interesting that the LCMV-Cl13 infections actually inhibited cross-priming when compared to uninfected mice.

In a parallel experiment, the same infection protocol was followed, except instead of OVA, we inject mice i.v with Sp-MØ incubated *in vitro* with 20 mg/ml OVA at 10^6 cells/mouse. Interestingly, Sp-MØ internalized OVA could efficiently induce OVA-specific CTL responses in untreated mice which resulted in 80% of activated OVA-specific CTLs. In contrast, Sp-MØ internalized OVA failed to induce any significant OVA-specific CTL responses in mice infected early with either LCMV-WE or LCMV-Cl13. These results indicate that the virus infection induced a state where the CD8+T cells could not be reached by Sp-MØ-OVA, or there was competition between CD8+T cells specific for LCMV and OVA.

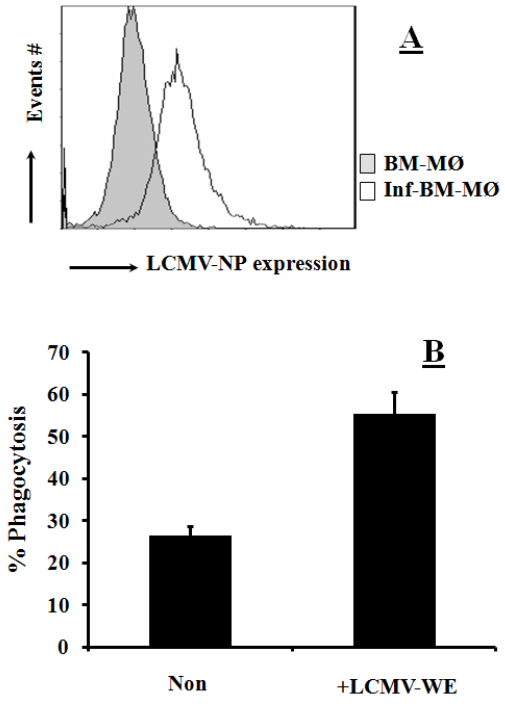

Figure 1: The phagocytic capacity of uninfected vs. LCMV-infected BM-MØ:

A) Susceptibility of BM-MØ cells to LCMV infection. BM-MØ were infected for 24 hr with LCMV-WE. The LCMV-NP intensity was detected by intracellular staining using VL4 supernatant as a source of the first antibody (anti-NP mAb). LCMV-infected BM-MØ are represented

by the unfilled histogram, while non-infected BM-MØ is represented by the gray-filled histogram. The result represent one 3 independent experiments with similar results.

B) The phagocytic capacity of the LCMV infected vs. non-infected BM-MØ. LCMV-infected and non-infected BM-MØ were co-cultured with CFSE (2ug/ml)-labeled LyUV-treated HEK cells at a ratio of 1:3 for 1 hr at 37°C. FACS was performed and the percentage of phagocytosis was calculated based on the number of double positive cells. The up-taken capacity is expressed as % up-taken. Results are expressed as mean ± s.d. for three repeats of one experiment out of three experiments.

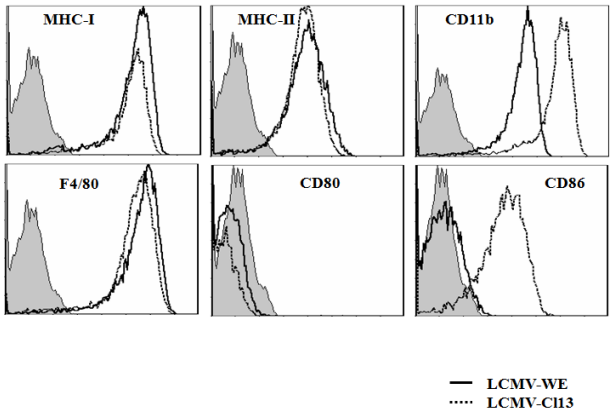

Figure 2: Analysis of surface marker expression on macrophages.

Histograms show staining with monoclonal antibodies specific for MHC-I, MHC-II, CD11b, F4/80, CD80, and CD86 after incubation with the specific antibodies on ice for 15 min. Negative controls are depicted by filled histograms. Macrophages (bone marrow; BM) from LCMV-WE and Cl13-infected mice were collected after 7 days of infection and tested for the above surface markers after 7 days of *in vitro* culturing. Data was acquired by FCM and is presented as overlay plots to reflect the different time points. This is one representative experiment from 2 independent trials.

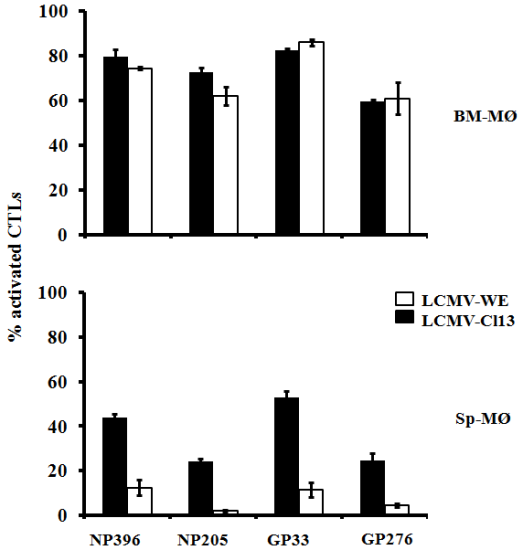

Figure 3: Antigen presentation by BM- and Sp-MØ.

Two groups of B6 mice were challenged i.v with 10^6 pfu LCMV-WE and Cl13. After 7 days, bone marrow cells and splenocytes were collected and cultured for 7 days in RPMI 10% FCS medium containing 20% M-CSF to generate BM- MØ and Sp-MØ, respectively. Macrophages were co-incubated with CTLs specific for four LCMV epitopes (NP396, NP205, GP33, and GP 276) at a ratio of 1:1 in presence of BFA 10µg/ml for IFN-γ production by ICS assay. The cells were stained for CD8+ and intracellular IFN-γ produced. FACS was performed and the percentage of the activated CTLs was calculated based on the number of double positive cells. Results are expressed as mean ± s.d. for three repeats of one experiment

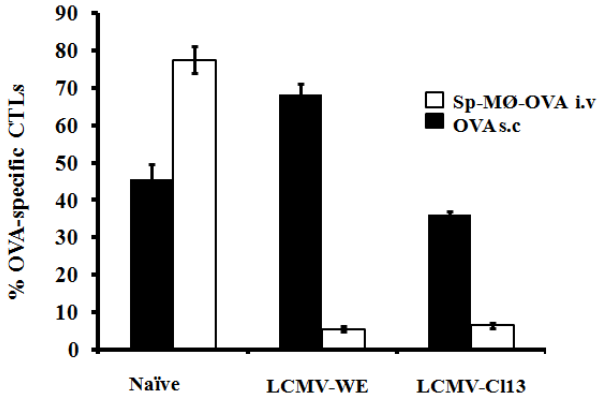

Figure 4: Influence of LCMV infection on in vivo cross-priming of OVA.

Two groups of B6 mice were challenged i.v with 10^6 pfu LCMV-WE and Cl13. After 2 days, these mice were challenged s.c with 20 mg/ml soluble OVA and i.v with 10^6 Sp-MØ incubated with 20 mg/ml soluble OVA *in vitro*. After 7 days, splenocytes were obtained and expanded *in vitro* for 8 days with peptide-pulsed (10^{-7} M) γ-irradiated BMA cells (specific for NP396, NP205, GP33, and GP 276). The CTL lines were then tested against peptide-pulsed DC2.4 in presence of BFA 10µg/ml for IFN-γ production by ICS assay. The activation of OVA-specific CTL was determined by FACS, which is based on the number of double positive cells. Results are expressed as mean ± s.d. for three repeats of one experiment out of 3 independent experiments.

DISCUSSION

We have chosen to focus our study on three major changes that could happen during LCMV infection: the phagocytic capacity of MØ, expression of MØ surface markers, and the changes in the cross-priming activity of pAPCs. The effect of viral infection on the capacity of MØ to internalize antigens has not been fully studied, partly because of difficulties in purifying and isolating these cells with high efficiency. In our system we used LYUV-treated HEK cells which serve a good source to supply different antigenic forms ranging from small soluble debris to big large fragments.[24-27] Interestingly, Krysko et al., has reported that apoptotic cells fragments were taken up via phagocytosis while necrotic cell debris were entering via macropinocytosis in a mouse MØ cell line.[28,29] As shown in our results, LCMV-infected MØ upregulated their phagocytic activity as evident by their ability to internalize double quantity of LYUV-treated ADCs as compared to non-infected MØ. The generated new state after infection most likely due to several factors: the increase in the size of MØ which may result in a marked

increase in the external surface area of macrophages. Therefore, increase the capacity to attach and engulf much more quantity of antigens than normal non-infected MØ. The other possible factor is the increase in the expression of some surface markers that could work as receptors to facilitate and increase the phagocytic processes of MØ. Interestingly, Alatery et al., has published that Sp-MØ and BM-MØ have upregulated their phagocytic capacity during their maturation stages.[24] However, our results may indicate to some interesting issues such as; during virus challenge, MØ will upregulate their phagocytic capacity as their primarily roles in cleaning up and removing dying cells from the surroundings.

With regard to the effect of LCMV-WE versus Cl13 on the differentiation and expression of some surface markers on bone marrow-derived macrophages (BM-MØ), we have noticed slight difference in one or two markers, CD86 and CD11b. While BM-MØ prepared from LCMV-Cl13-infected mice have shown a high expression rate of CD86, the expression level of this marker on the LCMV-WE-infected BM-MØ was nearly undetectable. In general, the expression rate of co-stimulatory molecules determines the activation status of effector immune cells that participate in eradicating and terminating the viruses' challenges. But on the other hand, the high expression rates of co-stimulatory molecules will activate high percentages of virus-specific CD8+T cells and for long time which will absolutely induces a state of exhaustion for these cells. In acute infections the antigenic stimulation occurs at an adequate intensity resulting in optimal T cell activation needed for antigen clearance and the establishment of functional memory.[3] While in chronic infections, there is continuous antigenic stimulation due to antigen persistence. This occurs due to high levels of viral replication resulting in functional exhaustion of CD8+ T cells.[1,4] Therefore, this would explain one the possible ways by which why LCMV-Cl13 induces persistent infection. In a separate *in vivo* work done to test the impact of LCMV-Arm and Cl13 on spleen DCs, Sevilla et al., has shown that LCMV-Cl13, but not LCMV-WE, reduced the surface expression of MHC-I, II, CD80, and CD86.[8] These results contradict our observation and suggest that different pAPCs respond differently to virus infections. In support, Sevilla et al., has found that MØ were not significantly affected by LCMV-Cl13 infection.[8]

Further *in vivo* investigation on the effects of LCMV-WE and Cl13 on MØ in the secondary lymphoid organs such as bone marrow cells and splenocytes was carried out by employing four LCMV-specific CD+T cell lines. The obtained results indicate that LCMV-WE was cleared rapidly from the spleen, while LCMV-Cl13 remained in this organ longer. It is well known that LCMV-WE causes acute infection and is cleared rapidly from the spleen by day 8 p.i., whereas LCMV-Cl13 cause chronic infection and persisted longer than one month.[30,31] Interestingly, the scenario is completely different in the bone marrow, in that BM-MØ prepared from LCMV-WE and Cl13-infected mice have shown a high and comparable capacity to activate LCMV-specific CTLs. This means clearly that both virus strains stay longer in the bone marrow. Thus, LCMV-WE and Cl13 strains can induce a potent state of infection in bone marrow progenitor cells that persist for long time as compared to splenocytes. In a related work by Wherry et al., has shown that infection with LCMV-arm and CL13 resulted in LCMV-specific CTL activation in different tissues including the bone marrow.[30] However, our results might imply to some extent that clearance the virus from tissues require some kinds of immune cells that exist in the spleen but are absent in the bone marrow. With this regard, several studies have shown that marginal zone macrophages are critical in early to control an infection with lymphocytic choriomeningitis virus (LCMV).[32,33]

The remarkable and interesting observation we have shown is about the impact of LCMV-WE and Cl13 on *in vivo* cross-priming. While much is known about the effect of chronic virus infection on modulating some of the immune mechanisms, including suppression of CTL activation, the exact mechanism by which these viruses interfere with the cross-priming pathway is not addressed yet. We show here for the first time, clear evidence that, in contrast to LCMV-WE, LCMV-CL13 that establishes chronic infection downregulated the *in vivo* cross-priming of OVA-specific CTLs. It well known that to optimally cross-prime naïve CD8+ T cells pAPCs, such as DCs and MØ must uptake antigens from the surroundings, process them and present appropriate viral peptides to CD8+ T cells.[12] Because pAPCs are essential in adaptive immunity, they represent an ideal target for immunosuppressive viruses. LCMV clone-13 (LCMV-Cl13), which establishes persistent infections negatively affects $CD11c^{+high}$ DCs.[8] Infection with high doses of the LCMV-Cl13 impairs, but does not totally abolish the expression of MHC and co-stimulatory molecules on $CD11c^{+}$ DCs.[8] As a result, these DCs do not efficiently stimulate T cell proliferation *ex vivo*.[8] This might be suggested as one of the possible reasons to explain the downregulation effect of LCMV-Cl13 on *in vivo* cross-priming. However, other pAPC populations such as MØ are not significantly affected by LCMV-Cl13 infection.[8] It has been shown that during acute LCMV infections, cross-priming of the exogenous antigen ovalbumin (OVA) is increased due to IFNα production as a result of virus acute virus infection.[34] These findings indicate that DCs require a special "license", provided by type I interferon after acute virus infection, to present exogenous antigens to CTLs. The above studies raise many questions in other virus infection models such as in persistent infections or chronic virus infection. Finally and based on our results, persistent virus infections interfere, for some unknown reasons, with the ability of certain pAPCs subpopulations to cross-prime CD8+ T cells.

REFERENCES

[1] Wherry EJ, Ahmed R (2004) Memory CD8+T-cell differentiation during viral infection, *J Virol* **78**(11), 5535-5545.

[2] Van der Most RG, Murali-Krishna K, Lanier JG, Wherry EJ, Puglielli MT, Blattman JN, Sette A, Ahmed R (2003) Changing immunodominance patterns in antiviral CD8+T-cell responses after loss of epitope presentation or chronic antigenic stimulation, *Virology* **315**(1), 93-102.

[3] Williams MA, Holmes BJ, Sun JC, Bevan MJ (2006) Developing and maintaining protective CD8+ memory T cells, *Immunol Rev* **211**,146-153.

[4] Oldstone MB (2006) Viral persistence: parameters, mechanisms and future predictions, *Virology* **344**(1),111-118.

[5] Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, Ahmed R (1998) Viral immune evasion due to persistence of activated T cells without effector function, *J Exp Med* **188**(12), 2205-2213.

[6] Matloubian M, Kolhekar SR, Somasundaram T, Ahmed R (1993) Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus, *J Virol* **67**(12),7340-7349.

[7] Matloubian M, Somasundaram T, Kolhekar SR, Selvakumar R, Ahmed R (1990) Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice, *J Exp Med* **172**(4),1043-1048.

[8] Sevilla N, McGavern DB, Teng C, Kunz S, Oldstone MB (2004) Viral targeting of hematopoietic progenitors and inhibition of DC maturation as a dual strategy for immune subversion, *J Clin Invest* **113**(5),737-745.

[9] Montoya M, Edwards MJ, Reid DM, Borrow P (2005) Rapid activation of spleen dendritic cell subsets following lymphocytic choriomeningitis virus infection of mice: analysis of the involvement of type 1 IFN, *J Immunol* **174**(4), 1851-1861.

[10] Basta S, Chen W, Bennink JR, Yewdell JW (2002) Inhibitory effects of cytomegalovirus proteins US2 and US11 point to contributions from direct priming and cross-priming in induction of vaccinia virus-specific CD8(+) T cells, *J Immunol* **168**(11), 5403-5408.

[11] Basta S, Bennink JR (2003) A survival game of hide and seek: cytomegaloviruses and MHC class I antigen presentation pathways, *Viral Immunol* **16**(3), 231-242.

[12] Basta S, Alatery A (2007) The Cross-priming Pathway: A Portrait of an Intricate Immune System, *Scand J Immunol* **65**(4), 311-319.

[13] Dunbar E, Alatery A, Basta S (2007) Cross-Priming of a Single Viral Protein from Lymphocytic Choriomeningitis Virus Alters Immunodominance Hierarchies of CD8(+) T Cells during Subsequent Viral Infections, *Viral Immunol* **20**(4), 585-598.

[14] Schulz O, Reis e Sousa C (2002) Cross-presentation of cell-associated antigens by CD8alpha+ dendritic cells is attributable to their ability to internalize dead cells, *Immunology* **107**(2), 183-189.

[15] Iyoda T, Shimoyama S, Liu K, et al. (2002) The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and *in vivo*. *J Exp Med* **195**(10), 1289-1302.

[16] den Haan JM, Bevan MJ (2002) Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(-) dendritic cells *in vivo*, *J Exp Med*. **196**, 817-827.

[17] den Haan JM, Lehar SM, Bevan MJ (2000) CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells *in vivo*, *J Exp Med*. **192**, 1685-1696.

[18] Schnorrer P, Behrens GM, Wilson NS (2006) The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture, *Proc Natl Acad Sci* USA **103**, 10729-10734.

[19] Zinkernagel RM (2002) Lymphocytic choriomeningitis virus and immunology, *Curr Top Microbiol Immunol*. **263**, 1-5.

[20] Shen Z, Reznikoff G, Dranoff G, Rock KL (1997) Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules, *J Immunol* **158**, 2723-2730.

[21] Battegay M, Cooper S, Althage A, Banziger J, Hengartner H, Zinkernagel RM (1991) Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates, *J Virol Methods* **33**, 191-198.

[22] Basta S, Stoessel R, Basler M, van den Broek M, Groettrup M (2005) Cross-presentation of the long-lived lymphocytic choriomeningitis virus nucleoprotein does not require neosynthesis and is enhanced via heat shock proteins, *J Immunol* **175**, 796-805.

[23] Johnson CR, Kitz D, Little JR (1983) A method for the derivation and continuous propagation of cloned murine bone marrow macrophages, *J Immunol Methods* **65**, 319-332.

[24] Alatery A, Basta S (2008) An efficient culture method for generating large quantities of mature mouse splenic macrophages, *J Immunol Methods* **338**, 47-57.

[25] Brode S, Macary PA (2004) Cross-presentation: dendritic cells and macrophages bite off more than they can chew, *Immunology* **112**, 345-351.

[26] Lin ML, Zhan Y, Villadangos JA, Lew AM (2008) The cell biology of cross-presentation and the role of dendritic cell subsets, *Immunol Cell Biol* **86**, 353-362.

[27] Alatery A, Siddiqui S, Chan M, Kus A, Petrof EO, Basta S (2010) Cross, but not direct, presentation of cell-associated virus antigens by spleen macrophages is influenced by their differentiation state, *Immunol Cell Biol* **88**, 3-12.

[28] Krysko DV, Denecker G, Festjens N, Gabriels S, Parthoens E, D'Herde K, Vandenabeele P (2006) Macrophages use different internalization mechanisms to clear apoptotic and necrotic cells, *Cell Death Differ*. **13**, 2011-2022.

[29] Krysko DV, Brouckaert G, Kalai M, Vandenabeele P, D'Herde K (2003) Mechanisms of internalization of apoptotic and necrotic L929 cells by a macrophage cell line studied by electron microscopy, *J Morphol*. **258**, 336-345.

[30] Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R (2003) Viral persistence alters CD8+T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment, *J Virol*. **77**, 4911-4927.

[31] Ahmed R, Salmi A, Butler LD, Chiller JM, Oldstone MB (1984) Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence, *J Exp Med*. **160**, 521-540.

[32] Seiler P, Aichele P, Odermatt B, Hengartner H, Zinkernagel RM, Schwendener RA (1997) Crucial role of marginal zone macrophages and marginal zone metallophils in the clearance of lymphocytic choriomeningitis virus infection, *Eur J Immunol*. **27**, 2626-2633.

[33] Oehen S, Odermatt B, Karrer U, Hengartner H, Zinkernagel R, Lopez-Macias C (2002) Marginal zone macrophages and immune responses against viruses, *J Immunol*. **169**, 1453-1458.

[34] Le Bon A, Etchart N, Rossmann C, Ashton M, Hou S, Gewert D, Borrow P, Tough DF (2003) Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon, *Nat Immunol*. **4**, 1009-1015.