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LETTER TO THE EDITOR

Flow cytometric analysis of intracellular IL-17A in T-cells during ectromelia virus infection

L. SZULC*, M. GIERYŃSKA, A. BORATYŃSKA, L. MARTYNISZYN, M. NIEMIAŁTOWSKI*

Division of Immunology, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences-SGGW, Ciszewskiego Str. 8, 02-786 Warsaw, Poland

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Ectromelia virus (ECTV), an orthopoxvirus, is a causative agent of mousepox, a disease which is very similar to smallpox in human, caused by variola virus. Various inbred strains of mice have been classified as genetically susceptible, e.g. BALB/c and DBA (both H-2^d haplotype) and C3H (H-2^k), or resistant, e.g. C57BL/6, C57BL/10 and 129/Sv (all H-2^b), to lethal infection with ECTV (*1*). Recovery of resistant mice from a primary ECTV infection depends on multiple mechanisms of host immune response, including natural killer (NK) cells and tissue macrophage activity, production of interferons, function of cytotoxic $CD8^+$ T-cells, production of antibodies and activation of complement system (*2-4*). Moreover, resistant mice generate during ECTV infection a type 1 cytokine immune response, which is associated with strong cell-mediated immune response and recovery, whereas susceptible strains of mice predominantly elicit a type 2 cytokine response, which leads to weak and delayed cellular response resulting in high mortality rates (*5*). In viral infections caused by many viruses, including human herpes virus type 1, respiratory syncytial virus, rotaviruses and human T-cell leukemia virus type 1, T_h17 immune response is induced, where interleukin (IL)-17 is suggested to enhance the inflammatory responses to those viruses, possibly resulting in higher mortality (*6*). Meanwhile, strong infiltration of $CD11b^+$ (neutrophils and macrophages) to the sites of virus replication observed during acute stages of ECTV infection in spleen of BALB/c mice (*7*) may promote tissue damage through the release of lysosomal enzymes and reactive oxygen species. For that reason, evaluation of the IL-17A production may be an important determinant in mousepox pathogenesis.

Production of IL-17A by $CD4^+$ and $CD8^+$ T-cells isolated from draining lymph nodes (DLNs: popliteal and superficial inguinal) and spleen of BALB/c and C57BL/6 mice was assessed by intracellular staining with Cytofix/Cytoperm and Perm/Wash (both from BD Biosciences) according to the manufacturer's protocol and flow cytometry analysis. Mice were injected into the hind foot pad (f.p.) with 50 PFU of highly virulent Moscow strain of ECTV (ECTV-MOS)/ mouse in 20 µl/f.p. Negative control BALB/c mice were injected with sterile PBS. Organs were aseptically removed at 7, 14 and 21 days post infection (p.i.) and single cell suspensions were prepared. Cells were then placed in 96-well plates and stimulated with 50 ng/ ml phorbol 12-myristate 13-acetate (PMA, Sigma) and 500 ng/ ml ionomycin (Sigma) in the presence of 10 µg/ ml brefeldin A (BD Biosciences) for 4 hrs at 37°C in 5% CO_2 (*8–9*). After surface staining with monoclonal antibodies anti-CD4-PerCP (H129.9) and anti-CD8-FITC (53-6.7), cells were permeabilized with Cytofix/ Cytoperm and stained intracellularly with monoclonal antibody anti-IL-17A-PE (TC11-18H10). All antibodies and appropriate isotype controls were purchased from BD Biosciences. Percentage of IL-17A producing cells was measured by FACSCalibur flow cytometer and analyzed by

*Corresponding authors. E-mail: marek_niemialtowski@sggw.pl, lidiaszulc@gmail.com; fax: +4822-5936066.

Abbreviations: DLN = draining lymph nodes; ECTV = ectromelia virus; ECTV-MOS = Moscow strain of ECTV; IL = interleukin; p.i. = post infection; VACV = vaccinia virus

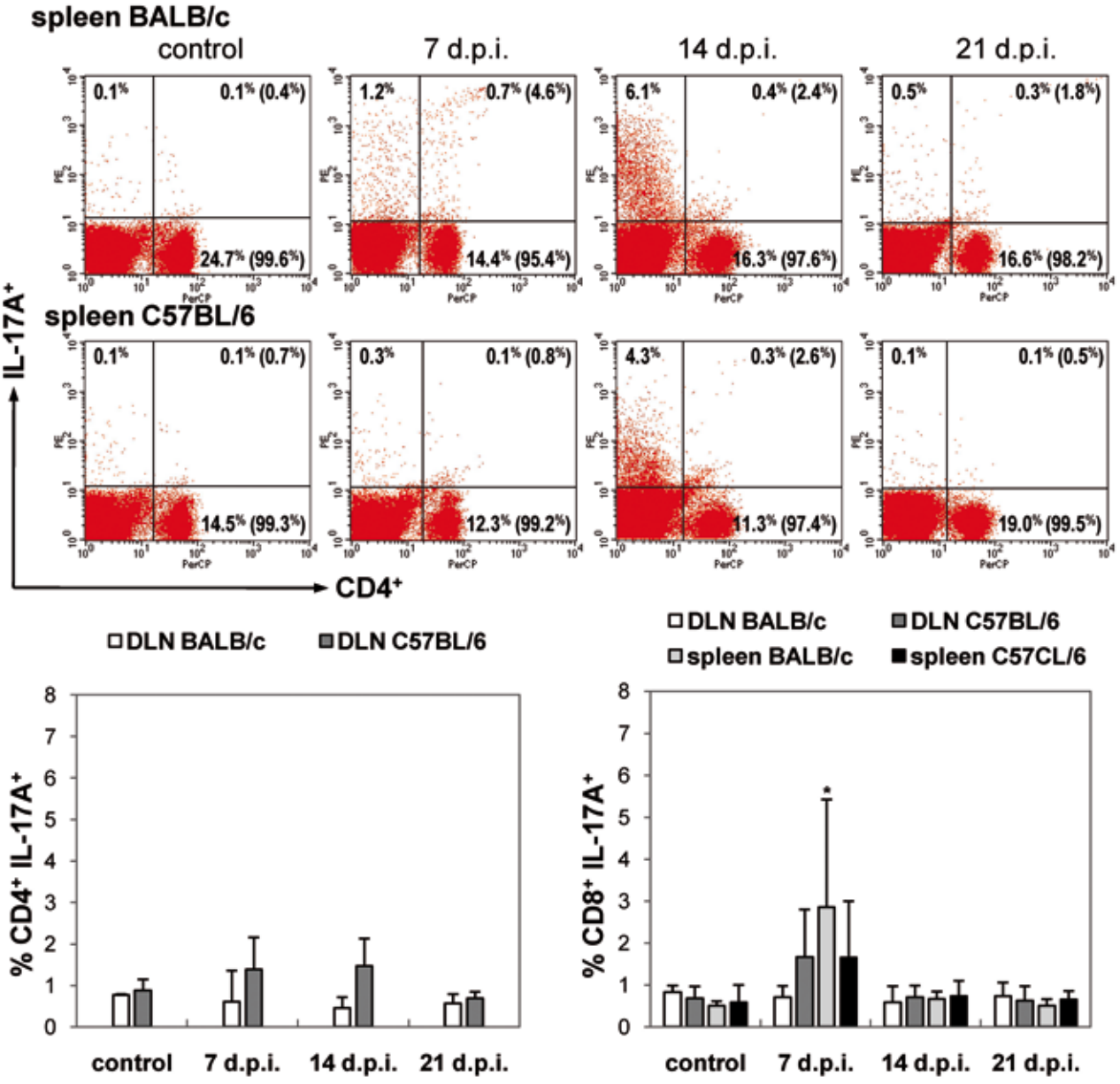

Fig. 1

Kinetics of IL-17A production by CD4+ and CD8+ T-cells during ECTV-MOS infection

CellQuest software (BD Biosciences). Using forward (FSC) and side (SSC) scatter profiles a gate was set on lymphocyte population, where single-cell cytokine production was evaluated. The percentage of cytokine producing $CD4^+$ and $CD8^+$ T-cells was calculated as mean ± SD (standard deviation). Statistical differences between control mice and infected animals at indicated time points were calculated using Mann-Whitney U test (STATISTICA 6.0 software, StatSoft). The value of $P < 0.05$ was considered to be significant.

No changes in the percentage of IL-17A-producing $CD4^+$ and $CD8^+$ T-cells were detected in DLNs of both strains of mice during the course of infection. However, the kinetics of IL-17A production in spleen varied between BALB/c and C57BL/6 mice. In spleens of BALB/c mice we observed an increase of IL-17A-producing $CD4^+$ (4%) and $CD8^+$ (2.8%) T-cells at day 7 p.i., when strong replication of the virus occurred in the organ. IL-17A, which is secreted by T_h17-cells, promotes neutrophil maturation through the induction

of cytokines important in granulopoiesis and neutrophil chemotaxis by promoting the production and release of C-X-C chemokines (CXCL1/KC and CXCL8/IL-8) (*10*). On the other hand, T_h17-cells are thought to be involved in tissue destruction during inflammation (*11*). We cannot exclude that IL-17A promotes tissue destruction in spleens of BALB/c mice by increasing the afflux of $CD11b^+$ cells, the influx of which activates the release of multiple potent factors leading to tissue damage, e.g. reactive forms of oxygen and proteolytic enzymes. In contrary, it has been shown that IL-17-knockout BALB/c mice were more sensitive to vaccinia virus (VACV) infection than wild type BALB/c mice (*12*). It has been found that IL-17 is an important cytokine in host defense against VACV and participates in the IL-23-regulated resistance to VACV infection. BALB/c mice infected with a mutant VACV expressing IL-17, despite the fact, that they looked very sick on the first few days after infection, eventually recovered from the illness. Meanwhile, BALB/c mice infected with the wild type VACV succumbed to the disease (*12*). IL-17A signaling in T-cells can also modulate T_h1 differentiation, probably by suppressing the transcription of T-bet and other T_h1-associated genes (*13*), however, it has also been reported that IL-17 does not significantly alter T_h1 or T_h2 differentiation and IFN-γ or IL-4 expression (*14*).

The percentage of $IL\text{-}17A^+CD4^+$ T-cells started to decline at day 14 p.i. in spleens of BALB/c mice, whereas in C57BL/6 spleens achieved a maximum value. A statistically significant ($P < 0.05$) increase in percentage of IL-17A-producing $CD4^+$ T-cells was still detected on day 21 p.i. in BALB/c mice spleens. Moreover, IL-17A was mainly produced by T-cells in spleens of BALB/c and C57BL/6 mice at day 7 p.i, whereas at day 14 p.i. a reduced percentage of T-cells producing IL-17A was detected within all splenic IL-17A-producing cells. At this time point, other cell types of the immune system were responsible for IL-17A production. It has been reported that in mice infected with *Mycobacterium tuberculosis* the majority of IL-17A-producing T-cells were TCR γδ T-cells (*15*). Moreover, TCR γδ T-cells expressing TCR Vγ4 or Vγ6 developed into IL-17A producing T-cells, which were essential for early protection against *Listeria monocytogenes* infection (*16*). TCR γδ T-cells and $CD4^-CD8^-$ TCR αβ T-cells were identified as FasL-induced IL-17A-producing cells and were found in adhesion molecule-deficient $CD18^{-/-}$ and $selectin^{-/-}$ mice and were called neutrophil regulatory T-cells (Tn) (*16*). IL-17A can also be secreted by neutrophils and from this cellular source regulates NKT-cell activation, IFN-γ production, neutrophil infiltration and tissue destruction in mouse kidney ischemia-reperfusion injury (*17*). Thus, the importance of IL-17A in the ECTV-MOS infection should be considered to play a role in both elimination of viral particles and promoting the tissue injury during the acute phase of infection.

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