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Strain-specific helper T cell profile in the gut-associated lymphoid tissue

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ABSTRACT

C57BL/6, BALB/c and NOD mice are among the most frequently used strains in autoimmunity research. NOD mice spontaneously develop type 1 diabetes (T1D) and they are prone to induction of experimental autoimmune encephalomyelitis (EAE). Both diseases can be routinely induced in C57BL/6 mice, but not in BALB/c mice. Also, C57BL/6 mice are generally considered T helper (Th)1-biased and BALB/c Th2-biased mice. Having in mind increasingly appreciated role of gut associated lymphoid tissue (GALT) cells in autoimmunity, especially in relation to gut Th17 and regulatory T (Treg) cells, our aim was to determine if there are differences in proportion of $CD4^+$ T cell populations in mesenteric lymph nodes and Peyer's patches of these mouse strains. Lower proportion of Treg was observed in NOD PP, Th2 cells dominated in BALB/c mice in mesenteric lymph nodes (MLN) and Peyer's patches (PP), while Th1 cells prevailed in C57BL/6 MLN. Intradermal immunization of mice with complete Freund's adjuvant resulted in significant difference in Th cell distribution in GALT of NOD mice. Differences were less pronounced in C57BL/6 mice, while GALT of BALB/c mice was almost unresponsive to the immunization. The observed strain- and tissue-dependent changes in Treg proportion after the immunization was probably a consequence of different CCR2 or CCR6-related migration patterns and/or *in situ* Treg proliferation. In conclusion, NOD, a highly autoimmunity-prone mouse strain, exhibits more profound GALT-related immune response upon immunization compared to the strains that are less prone to autoimmunity.

1. Introduction

Autoimmune reactivity is initiated in the lymphoid organs where self-antigens are presented to T cells. However it is still not clear which lymphoid organs are the site of primary activation of encephalitogenic and diabetogenic T cells that initiate multiple sclerosis and type 1 diabetes (T1D), respectively. These could be lymphoid tissues that drain organs affected by the particular autoimmunity, e.g. deep cervical lymph nodes draining brain and spinal cord in multiple sclerosis and pancreatic lymph nodes draining pancreas in T1D [1,2]. However, these could also be distant lymphoid organs in which self-reactive T cells are activated by molecular mimicry, bystander activation or superantigens [3,4]. Also, increasing evidence suggest that certain processes in the initiation and development of these diseases take place in the gut-associated lymphoid tissue (GALT) which is largely influenced by gut microbiota [5,6]. Indeed, alterations in gut microbiota composition, including antibiotic treatment, have been shown to change susceptibility/resistance of different mouse strains to experimental autoimmune encephalomyelitis (EAE) and T1D [7,8]. This effect is mediated through modulation of the balance between T helper 17 (Th17) cells and regulatory T (Treg) cells in GALT [7,8].

Different mouse strains exhibit diverse susceptibility for autoimmune response development. NOD mice develop T1D spontaneously and are prone to EAE induction, C57BL/6 are prone to induction of both T1D and EAE, while BALB/c mice are more resistant to induction of these diseases [9–12]. It is already known that these strains vary in their gut microbiota content [13,14]. Here, we were interested in detailed phenotypic analysis of mesenteric lymph nodes (MLN) and Peyer's patches (PP) of these strains, particularly in determining proportions of Th1, Th17 and Treg cells. Analyses were performed on intact mice, as well as on mice immunized with complete Freund's adjuvant (CFA). Obtained results showed that GALT-related immunological profile of C57BL/6 and BALB/c mice were Th1-biased and Th2-biased, respectively. Peyer's patches of normoglycemic NOD mice had lower Treg cell proportion (compared to other two strains) that was significantly increased upon immunization. Also, the

Abbreviations: CFA, complete Freund's adjuvant; DLN, draining lymph nodes; GALT, gut-associated lymphoid tissue; IFN-γ, interferon-gamma; IL, interleukin; PBS, phosphate buffer saline; PP, Peyer's patches; MLN, mesenteric lymph nodes; SD, standard deviation; T1D, type 1 diabetes; Th, T helper cells; Treg, regulatory T cells

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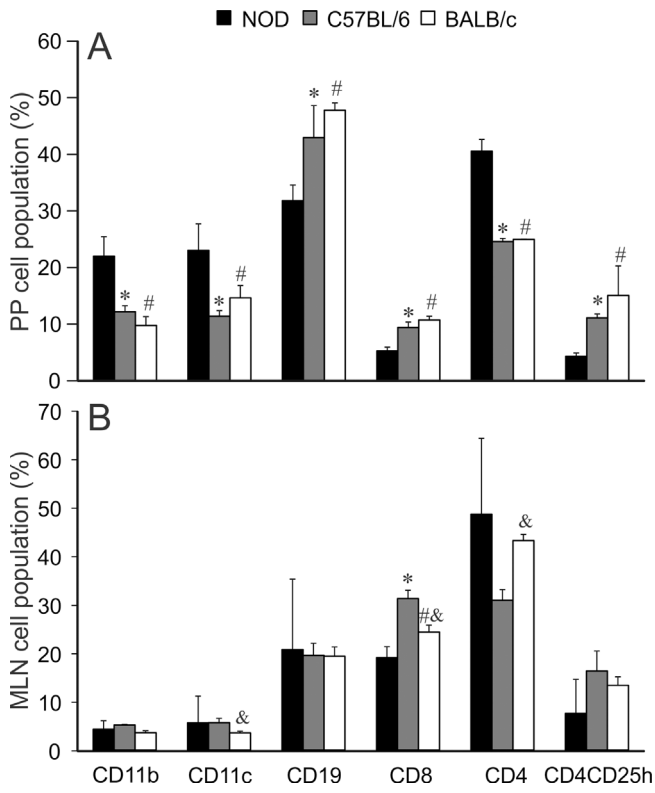

Fig. 1. Phenotype of MLN and PP. Proportion of cell populations in PP (A) and MLN (B) was determined by flow cytofluorimetry. CD4CD25 h represents $CD4^+CD25^{high}$ (Treg) population. Data are presented as mean + SD of data obtained from 3 mice per group. *$p < 0.05$ represents statistically significant difference between C57BL/6 and NOD mice, #$p < 0.05$ between BALB/c and NOD mice and &$p < 0.05$ between BALB/c and C57BL/6 mice.

immunization led to major changes in Th profile in GALT of NOD mice, moderate in C57BL/6 mice and limited in BALB/c mice.

2. Materials and methods

2.1. Animals and immunization

NOD, C57BL/6 and BALB/c strains were bred and housed at the Animal Facility of the Institute for Biological Research "Sinisa Stankovic", University of Belgrade. Female mice 2 months of age were used in the experiments that were approved by the local ethics committee (Institute for Biological Research "Sinisa Stankovic", N° 01-2262). Immunization was performed by injecting 200 µl of complete Freund's adjuvant (CFA, Difco, Detroit, MI, USA, supplemented with *Mycobacterium tuberculosis* to 5 mg/ml) intradermally into both hind limbs and the base of the tail.

2.2. Cell isolation, preparation and culture

Cells were isolated from mesenteric lymph nodes, lymph nodes draining the site of injection (popliteal and inguinal), and Peyer's patches 10 days after the immunization with CFA.

Mesenteric lymph nodes (MLN) and draining lymph nodes (DLN) were isolated from mice and then mechanically disrupted by gentle pressing through 70 µm cell strainer. Cells were collected by centrifugation at 550*g* for 5 min. Peyer's patches (PP) were excised from the surface of the small intestine, which was previously mechanically cleaned out of content. Collected PP were washed thoroughly in PBS supplemented with antibiotics (penicillin/streptomycin, Sigma Aldrich, St. Luis, MO, USA) to remove excess mucus. Mechanical disruption of PP was performed by applying mild pressure through the cell strainer (BD Bioscience, Bedford, USA) in PBS with antibiotics. Cells were collected by centrifugation at 550*g* for 5 min and resuspended in RPMI-1640 medium (with 25 mmol/l HEPES, 2 mmol/l L-glutamine) supplemented with 10% FCS (PAA Chemicals, Pasching, Austria), penicillin/streptomycin and 5 µM β-mercaptoethanol (both from Sigma Aldrich, St. Luis, MO, USA). Cells were plated at 2.5×10^6/ml in 24-well plates (Sarstedt, Numbrecht, Germany) and cultivated in RPMI 1640 medium supplemented with 5% fetal calf serum (PAA Laboratories) in humidified atmosphere with 5% CO^2.

2.3. Immunofluorescence analysis

The phenotype of MLNC and PP cells was assessed by flow cytometry using the anti-mouse antibodies specific for CD4, CD8, CD11b, CD11c, CD19, CD25, CCR2 and CCR6 (eBioscience, San Diego, CA). Prior to intracellular cytokine staining, cells were stimulated with phorbol myristate acetate (PMA, 100 ng/ml) and ionomycin (400 ng/ml) (both from Sigma-Aldrich) in the presence of Brefeldin A (eBioscience) (5 µM) for 4 h, stained with anti-CD4 FITC antibody, fixed in 2% paraformaldehyde, permeabilized with Permeabilization buffer (eBioscience) and then stained for the intracellular cytokines with the following antibodies: anti-mouse antibodies against IL-17, IFN-γ, IL-4, IL-10 coupled with phycoerythrin. Isotype-matched controls were included in all experiments (eBioscience). Stained cells were detected on CyFlow Space (Partec, Görlitz, Germany). Cells were initially gated on live cells using FSC vs. SSC dot plots. For Th/Treg profile analysis cells were additionally gated on $CD4^+$ cells. For determination of Treg cells, $CD25^{high}$ cells were identified. Proportion and mean fluorescence intensity (MFI) as a measure of cytokine expression per cell were analyzed by FlowMax Software (Partec).

2.4. Proliferation assay

One day prior to cell isolation and analysis animals were treated intraperitoneally with bromodeoxyuridine (BrdU, Sigma Aldrich) at 100 mg/kg body weight. After isolation cells were stained with CD25-FITC (eBioscience) for 1 h at 4 °C and then permeabilized overnight. The next day, cells were treated with 300 µg/ml DNAse II (Sigma Aldrich) for 1 h at 37 °C, followed by washing in Permeabilization buffer (eBioscience). Cells were stained with anti-BrdU antibody (eBioscience) for 1 h at 4 °C. After two washing steps, cells were analyzed by the flow cytometer and the obtained% of $BrdU^+$ cells represented proportion of cells proliferating *in vivo*.

2.5. Statistical analysis

The results are presented as mean ± SD of values obtained in repeated experiments. Two-way ANOVA followed by Tukey's multiple comparisons test (Fig. 1) and Student's *t*-test (Figs. 2 and 3) were performed for statistical analysis. A *p* value less than 0.05 was considered statistically significant. To analyze correlation between the strains according to the analyzed immune parameters (cells: $CD4^+$, $CD4^+$IFN-γ^+, $CD4^+$IL-4^+, $CD4^+$IL-17^+, $CD4^+CD25^{high}$, expression: IFN-γ, IL-4, IL-17, IL-10), Pearson's coefficient of correlation was calculated. GraphPad 6 (GraphPad Software, San Diego, CA) and Statistica 8.0 (Quest Software Inc., Aliso Viejo, CA) were used for analyses.

3. Results and discussion

3.1. Cellularity and cellular composition of PP and MLN

BALB/c mice PP and MLN had higher cellularity in comparison to C57BL/6 and NOD mice (Table 1). Proportion of $CD11c^+$ dendritic cells, $CD4^+$ T lymphocytes and $CD11b^+$ cells was higher, while proportion of $CD19^+$ B cells, $CD8^+$ T cells and $CD4^+CD25^{high}$ regulatory T cells (Treg) was lower among PP cells in NOD mice in comparison to the