

Kinetics of immune cell responses in the multiple low-dose streptozotocin mouse model of type 1 diabetes

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Abstract

In type 1 diabetes (T1D), the insulin-producing β cells are destructed by immune mechanisms. It has been hypothesized that the very first immune response in T1D onset comes from innate immune cells, which further activates the adaptive immune cells to attack the islets. Despite intensive research on characterization of islet-infiltrating immune cells, the kinetics of different immune cells in multiple low-dose streptozotocin (MLDSTZ)-induced T1D mouse model is still much unclear. Therefore, we investigated the proportions of innate immune cells such as neutrophils, dendritic cells (DCs), plasmacytoid dendritic cells (pDCs), macrophages, natural killer (NK) cells, and adaptive immune cells (T and B lymphocytes) in thymic, pancreatic-draining lymph nodes, and spleens of MLDSTZ mice on days 3, 7, 10, and 21 after the first injection of STZ by flow cytometry. The proportions of DCs and B cells were increased from day 3, while the proportions of B-1a lymphocytes and interferon- γ^+ cells among NK cells were increased, but NK cells were decreased on day 10 in MLDSTZ-treated mice, illustrating that the initial immune response is induced by DCs and B cells. Later, the proportions of T helper 1 and cytotoxic T cells were increased from day 7, suggesting that the innate immune cells precede adaptive immune cell response in MLDSTZ mice. Altogether, our data demonstrate a possible sequence of events regarding the involvement of DCs, pDCs, NK cells, B-1a lymphocytes, B, and T cells at the early stage of T1D development.

KEYWORDS

adaptive immune cells, B-1a lymphocytes, dendritic cells, innate immune cells, NK cells, type 1 diabetes

Abbreviations: APC, antigen-presenting cell; CD, cluster of differentiation; CRAMP, cathelicidin-related antimicrobial peptide; DC, dendritic cell; IFN, interferon; IL, interleukin; LADA, latent autoimmune diabetes mellitus in the adult; MLDSTZ, multiple low-dose streptozotocin; NK, natural killer; NOD, non-obese diabetic; pDC, plasmacytoid dendritic cell; PDLN, pancreatic-draining lymph node; STZ, streptozotocin; T1D, type 1 diabetes; T2D, type 2 diabetes; Tc cell, cytotoxic T cell; TGF, tumour growth factor; Th cell, T helper cell.

Zhengkang Luo and Charlotte Soläng have equal contribution for first authorship.

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1 | INTRODUCTION

In type 1 diabetes (T1D), the insulin-producing β cells of pancreas are destructed by immunological mechanisms.^{1,2} This leads to insulin deficiency which causes hyperglycemia and further severe complications such as kidney failure, heart disease, and retinopathy.³ The prevalence of T1D is increasing worldwide and in 2014 about 40 million individuals were estimated to have T1D.⁴ Hitherto, the contributing factors behind T1D onset are considered to be both genetic and environmental.² Among the main mediators are autoreactive T cells, which infiltrate the islets and induce insulinitis.⁵⁻⁸ However, innate immune cells such as antigen-presenting cells or dendritic cells (DCs), neutrophils, macrophages, and B cells are also believed to contribute to insulinitis.^{6,7,9-14} Diana et al have shown that neutrophils, DCs, and B-1a lymphocytes initiate an autoreactive response in pancreas of non-obese diabetic (NOD) mice through pro-inflammatory mediators such as interferon (IFN)- α and cathelicidin-related antimicrobial peptide (CRAMP) during the early development of T1D in young NOD mice.¹⁵ Several studies have further confirmed the involvement of innate immune cells in autoimmune diseases.¹⁶⁻¹⁸

It has also been speculated that the β cells in pancreas are damaged by unknown reasons such as viral infections, bacterial infections, genetic factors, and/or environmental factors. This putative damage on β cells causes genetic material as dsDNA to leak out from damaged β cells, which will be processed by DCs as antigens that will further recruit more immune cells to the pancreatic islets in an attempt to prevent the damage of β cells.^{15,19}

Neutrophils comprise the main fraction of the human peripheral blood immune cells. Murine neutrophils can be characterized by their expression of Ly6G.²⁰ In T1D patients the cell count of circulating neutrophils have been reported to be decreased in peripheral blood, indicating that neutrophils have migrated to localized organs to induce inflammation.¹⁶ It has also been observed that neutrophils are promoting certain autoimmune reactions in the pancreas, by secretion of CRAMP to further activate the IFN- α -secreting plasmacytoid dendritic cells (pDCs).¹⁵ pDCs have been reported to be involved in T1D and other autoimmune diseases, such as psoriasis, rheumatoid arthritis, and systemic lupus erythematosus.^{15,17,18,21} It has also been suggested that pDCs induce the development of T1D in the NOD mouse model^{15,22} through IFN- α production, and that depletion of the pDCs reduces the frequency of T1D among the NOD mice.¹⁵ Murine pDCs can be detected by their expression of CD11c, B220, and PDCA-1 surface proteins,²³ which distinguishes them from conventional dendritic cells that express CD11b instead of CD11c and lack expression of B220.¹⁵

B1-a lymphocytes belong to a subset of B lymphocytes that are important mediators in antibody production. However, they also tend to be autoreactive due to high production of autoantibodies²⁴ and it has also been suggested that B-1a lymphocytes activates autoreactive T cells.²⁵ The increased number of B-1a lymphocytes have been observed in early age of NOD mice, and their depletion resulted in a reduction of dsDNA-specific IgGs in pancreatic islets.¹⁵ While all B lymphocytes express CD19 at the cell surface, B-1a lymphocytes can be further characterized by their expression of CD5,²⁴ and the lack of CD21 expression.¹⁵

Natural killer (NK) cells are a group of cytotoxic innate immune cells that are crucial for immune defence and homeostasis. In mice, NK cells can be identified by the surface marker NK1.1 and the activating receptor Nkp46. It has been shown that the depletion of NK cells prevent development of T1D.^{26,27} The blockade of Nkp46 has been reported to prevent the development of diabetes in NOD mouse model.²⁸ In addition, we have reported a higher of proportion of NK cells in latent autoimmune diabetes mellitus in the adult (LADA) patients compare to healthy controls and T2D patients, but similar to T1D patients, suggesting a role of NK cells in T1D.²⁹ However, the kinetic response of NK cells has not been studied in animal models of T1D.

The previous studies have mainly focused on pancreas, pancreatic islets, and spleens of NOD mice. NOD mice develop T1D spontaneously, which hampers the examination of the kinetics of immune cells response during the course of disease progression. Therefore, in the present study, we used multiple low-dose streptozotocin (MLDSTZ) mouse model. In this animal model, mice develop hyperglycemia gradually after the five consecutive daily low doses of STZ injection, which allow us to follow the kinetics of disease progression in relation to immune cell involvement.³⁰ It has been hypothesized that β cells are damaged by known and/or unknown factors, releasing islet antigens from β cells.³¹ The islet antigens are then taken up by antigen-presenting cells in the pancreatic-draining lymph nodes (PDLNs) and presented to T cells, leading to insulinitis. If insulinitis is not controlled with intervention, mass immune attacks occur, thus the onset of diabetes.³¹ We investigated the proportion of DCs, macrophages, neutrophils, CD8⁺ DCs, pDCs, B-1a lymphocytes, B cells, NK cells, and CD4⁺ and CD8⁺ T cells in thymic glands, PDLNs, and spleens of MLDSTZ mice to specify which of them are the first altered cell populations in the early development of T1D.

2 | MATERIALS AND METHODS

2.1 | Animals

All the animal experiments were approved by the local ethical committee in the Uppsala County. Male CD-1 mice

obtained from Charles River (Hannover, Germany) were kept at pathogen free conditions at the Animal Department, Biomedical Center, Uppsala University, Uppsala, Sweden. As previously described, the mice were injected intraperitoneally (i.p) with either 40 mg/kg of streptozotocin (STZ; Sigma-Aldrich) dissolved in saline, or 200 μ L of only saline (vehicle) for five consecutive days (Figure S1).^{32,33} The mice were sacrificed on days 3, 7, 10, and 21 after the first injection of STZ by cervical dislocation. The thymic glands and spleens were subsequently removed and transferred into glass jars containing Hanks' Balanced Salt Solution (HBSS; Sigma-Aldrich), and PDLNs were removed and transferred into Eppendorf tubes containing medium RPMI1640 (Sigma-Aldrich) supplemented with antibiotics and put on ice. All animals were used in accordance to international guidelines with free access to food and water and a 12 hours dark and light cycle. Blood glucose concentrations were measured on days 0, 3, 7, 10, 14, 17, and 21 using a blood glucose meter (Medisense). Blood samples were obtained from the tail vein of non-fasted mice for measuring the blood glucose concentration.

2.2 | Single cell suspension from thymic glands, PDLNs, and spleens

Single cell suspensions from thymic glands, PDLNs, and spleens were prepared as described earlier.^{32,34,35} Briefly; the thymic glands and spleens were mechanically disrupted in HBSS with a pair of forceps. The cell suspensions were centrifuged (Beckman Coulter) at 1500 rpm at 4°C for 5 minutes and the erythrocytes were lysed by suspension of the pellets in 4 ml 0.2 mol/L of NH_4Cl (Sigma-Aldrich). The cell suspensions were then incubated at room temperature for 10 minutes, followed by a centrifugation. The supernatants were discarded and the pellets were washed twice with HBSS.

The PDLNs were put on a 200 μ m cell strainer and mechanically disrupted with a small forceps onto a Falcon tube. The forceps and the cell strainer were regularly rinsed with RPMI 1640 in order to release cells stuck in the cell strainer. The cell suspensions were then centrifuged (Beckman Coulter) at 1500 rpm at 4°C for 5 minutes.

2.3 | Flow cytometry

Isolated cells from thymic glands, PDLNs, and spleens were stained with the following antibodies: anti-CD11b (M1/70; BioLegend), anti-CD11c (N418; BioLegend), anti-PDCA-1 (129c1; BioLegend), anti-Ly6G (1A8; BioLegend), anti-CD8 α (53-6.7; BD BioSciences), anti-B220 (RA3-6B2; BioLegend), anti-CD19 (1D3; BD BioSciences), anti-CD19 (6D5; BioLegend), anti-CD5 (53-7.3; BD BioSciences), anti-CD21 (7E9; BioLegend),

anti-CD3 (17A2; BioLegend), anti-NK1.1 (PK136; BioLegend), anti-NKp46 (29A1.4; BioLegend), and anti-IFN- γ (XMG1.2; BioLegend). Dilutions, catalogue numbers, and RRIDs of antibodies are given in Table S1. The stained cells were analyzed by a LSR Fortessa at the BioVis core facility, Uppsala University, Uppsala, Sweden. Single stained, unstained, and Fluorescence Minus One controls were applied as negative controls to identify any spread of the fluorochromes into the channel of interest and to properly gate the fluorochromes. The data were analyzed by Flowlogic software (Inivai Technologies). Gating strategies for flow cytometric analysis for different cell types are shown in Figures S2-S6.

2.4 | Statistical analysis

The software GraphPad Prism was used for the statistical analysis and unpaired *t* tests were used for comparison between the groups. A value of $P < .05$ was considered as statistically significant. Further information about statistical analysis is given in the figure legends.

3 | RESULTS

3.1 | MLDSTZ and development of diabetes

MLDSTZ-treated mice had increased blood glucose starting at day 7 and developed hyperglycemia gradually from day 10 while vehicle-treated mice remained normoglycemic during the whole period of the study (Figure 1). These data are consistent with our previous studies.³²

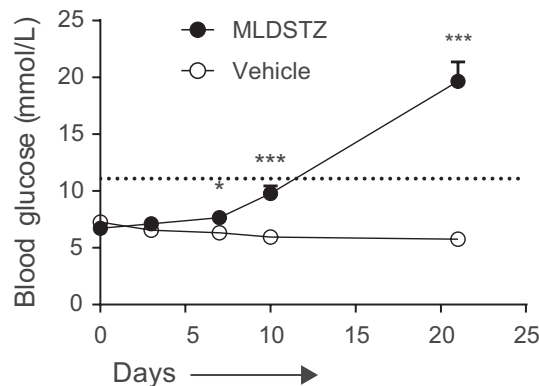


FIGURE 1 Male CD-1 mice were injected i.p. with STZ (40 mg/kg/day) or 200 μ L saline, for 5 consecutive days. The blood glucose concentrations were monitored in vehicle or MLDSTZ-treated mice on days 0, 3, 7, 10, and 21 after the first injection of STZ. Unpaired *t* tests were performed for comparisons between vehicle and MLDSTZ-treated groups on corresponding days. For most groups, the error bars are shorter than the height of symbols, thus they are not visible. * and *** denote $P < .05$ and $P < .001$, respectively. MLDSTZ, multiple low-dose streptozotocin

3.2 | Proportions of DCs and pDCs are increased in MLDSTZ-treated mice

We determined the proportions of CD11b⁺ CD11c⁻, CD11b⁻ CD11c⁺, and CD11b⁺ CD11c⁺ DCs using flow cytometry. The proportion of CD11b⁺ CD11c⁻ macrophages, that is, DCs was increased on day 10 in PDLNs of vehicle-treated mice

(Figure 2A). The proportion of CD11b⁻ CD11c⁺ DCs was increased on day 3 in thymic glands and PDLNs, and on day 10 in both PDLNs and spleens of MLDSTZ-treated mice (Figure 2B). We also observed increased proportion of CD11b⁺ CD11c⁺ DCs on day 10 in spleens of MLDSTZ mice (Figure 2C). Next, we examined the proportion of pDCs (CD11b⁻CD11c⁺B220⁺PDCA-1⁺ cells) as it has been reported that these cells are

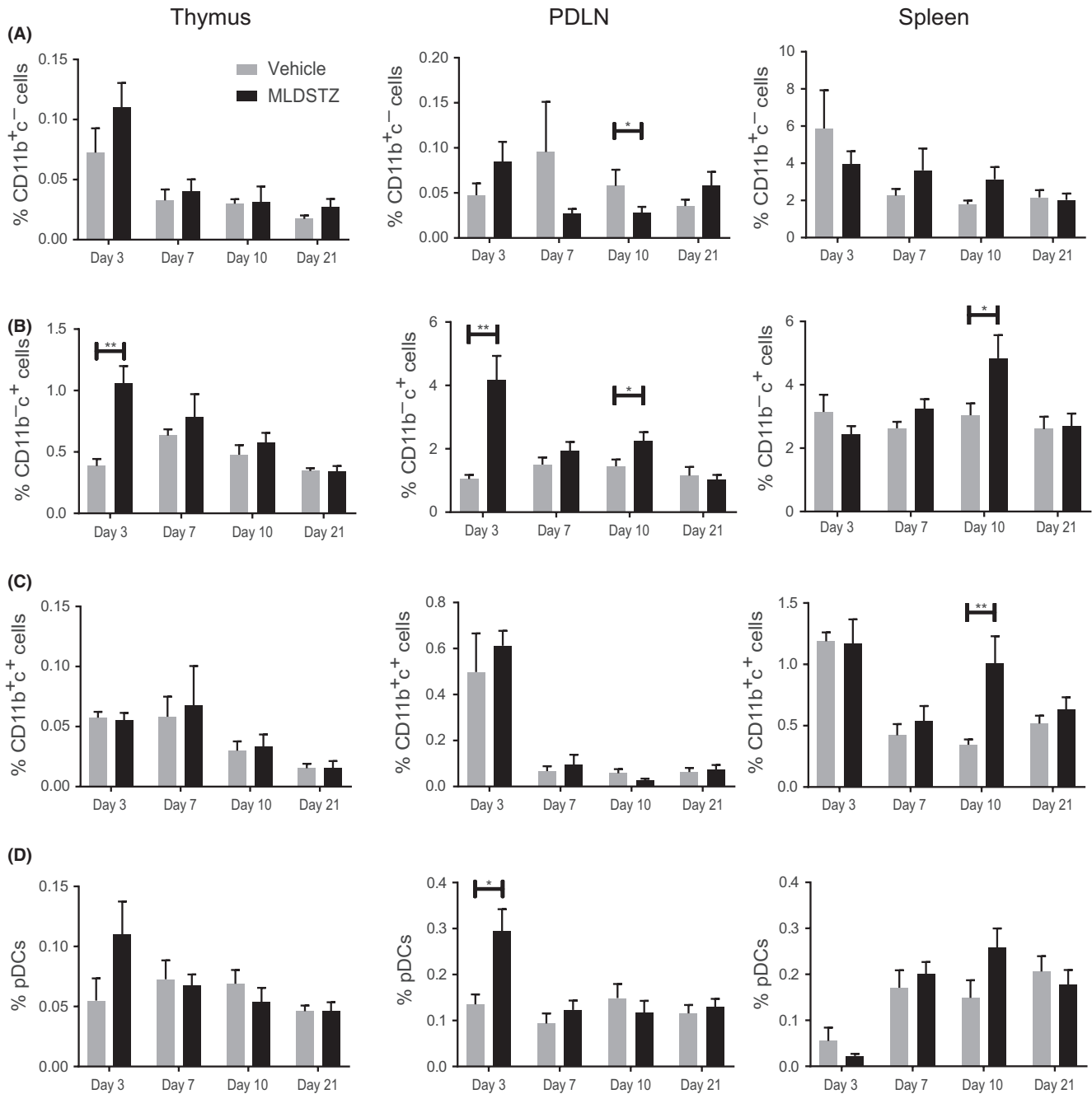


FIGURE 2 The percentages of DCs and pDCs are increased in MLDSTZ-treated mice. The percentages of (A) CD11b⁺ CD11c⁻ macrophages or DCs, (B) CD11b⁻ CD11c⁺, (C) CD11b⁺ CD11c⁺ DCs, and (D) CD11b⁻CD11c⁺B220⁺PDCA-1⁺ pDCs were analyzed using flow cytometry in thymic glands, PDLNs, and spleens of vehicle and MLDSTZ on days 3, 7, 10, and 21 after the first injection of STZ. Results are expressed as means ± SEM (n = 3-6 mice/group). Unpaired *t* tests were performed for comparison between vehicle and MLDSTZ-treated mice at each time point. * and ** denote *P* < .05 and *P* < .01, respectively. DCs, dendritic cells; MLDSTZ, multiple low-dose streptozotocin; pDCs, plasmacytoid DCs; PDLNs, pancreatic-draining lymph nodes

up-regulated in the early development of T1D in both NOD mice and T1D patients.^{15,36} Indeed, we found that the proportions of pDCs were increased on day 3 in PDLNs of MLDSTZ, (Figure 2D). Altogether, our data illustrate that the DCs and pDCs are increased at the early stage of T1D development in MLDSTZ mice.

3.3 | The proportion of neutrophils is decreased on day 21 in PDLNs of MLDSTZ-treated mice

It has been reported by Diana et al that neutrophils are increased in pre-diabetic NOD mice at the age of 2-3 weeks.¹⁵

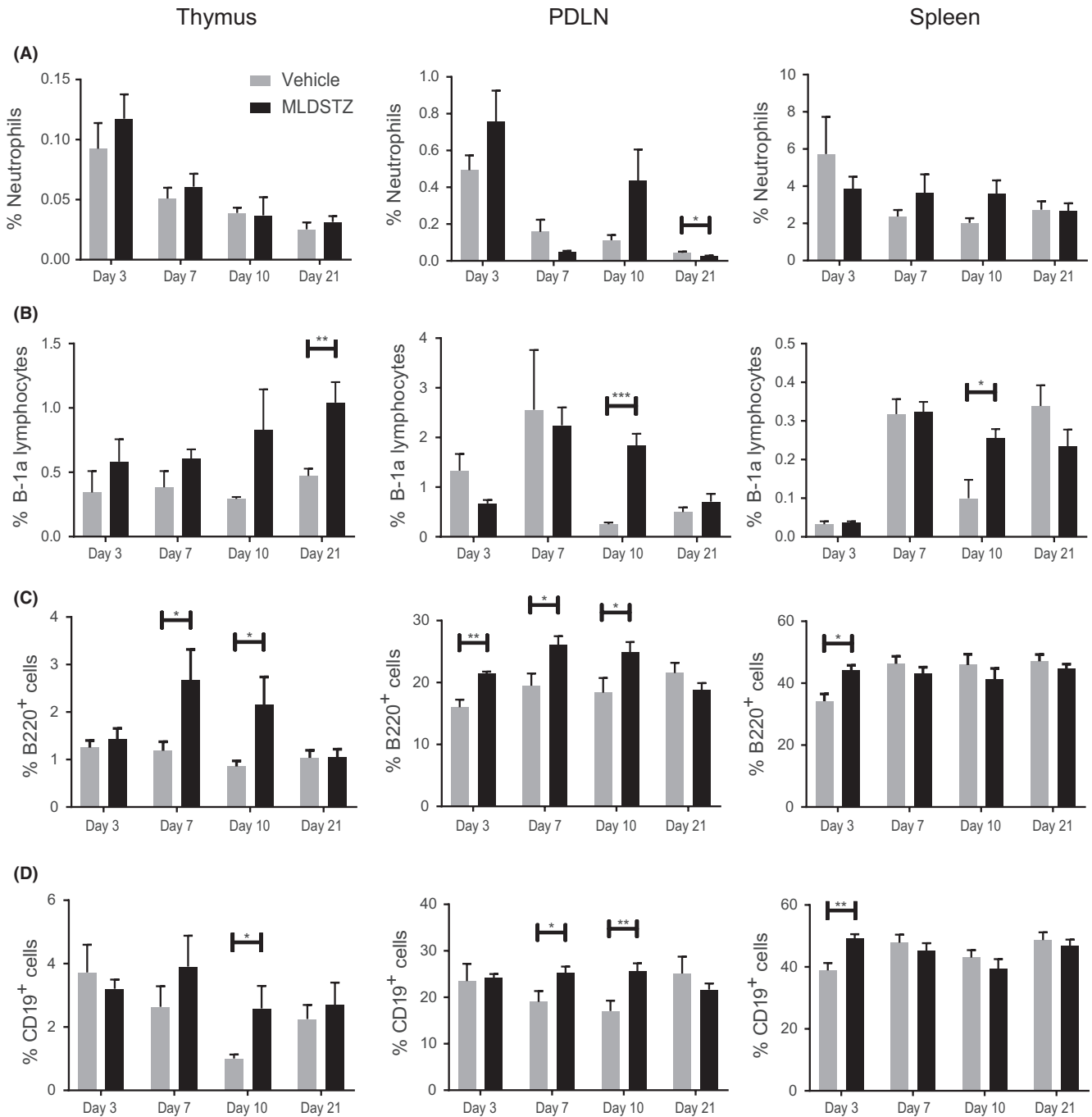


FIGURE 3 The percentages of B-1a lymphocytes and B lymphocytes are increased in MLDSTZ-treated mice. The percentages of (A) Ly6G⁺ neutrophils, (B) CD19⁺ CD5⁺ CD21⁻ B-1a lymphocytes, (C) B220⁺ B lymphocytes, and (D) CD19⁺ B lymphocytes were analyzed using flow cytometry in thymic glands, PDLNs, and spleens of vehicle and MLDSTZ on days 3, 7, 10, and 21 after the first injection of STZ. Results are expressed as means \pm SEM (n = 3-6 mice/group). Unpaired *t* tests were performed for comparison between vehicle and MLDSTZ-treated mice at each time point. *, ** and *** denote $P < .05$, $P < .01$, and $P < .001$, respectively. MLDSTZ, multiple low-dose streptozotocin; PDLNs, pancreatic-draining lymph nodes

To investigate the role of neutrophils in MLDSTZ model, we analyzed the proportion of Ly6G⁺ neutrophils on days 3, 7, 10, and 21 after the first injection of STZ in thymic glands, PDLNs, and spleen. The proportion of Ly6G⁺ cells was not altered on days 3, 7, 10, and 21 in thymic glands and spleens of MLDSTZ mice compared to vehicle-treated mice (Figure 3A). However, the proportion of Ly6G⁺ cells was decreased on day 21 but not on days 3, 7, and 10 in PDLNs of MLDSTZ mice compared to vehicle-treated mice (Figure 3A). Thus, our results show that the neutrophils are altered in the development of T1D in MLDSTZ mice.

3.4 | Proportions of B-1a lymphocytes and B cells are altered in MLDSTZ mice

Subsequently, we determined the proportion B-1a lymphocytes (CD19⁺ CD5⁺ CD21⁻ cells). The proportions of B-1a

lymphocytes were increased on day 10 in both PDLNs and spleens, and on day 21 in thymic glands of MLDSTZ mice (Figure 3B). B220⁺ B lymphocytes were increased on day 3 in both PDLNs and spleens of MLDSTZ mice, and were increased on day 7 and day 10 in thymic glands and PDLNs (Figure 3C). CD19⁺ B lymphocytes were increased on day 10 in thymic glands, on day 7 and 10 in PDLNs and on day 3 in spleens of MLDSTZ mice (Figure 3D). Thus, our results indicate an alteration of B-lymphocyte kinetics in MLDSTZ mice.

3.5 | Proportions of CD8⁺ DCs are increased in MLDSTZ-treated mice

The cross presentation by DCs play an important role to further activate T cells in autoimmune diseases^{37,38} and cancer.^{39,40} Cross-presenting DCs can be further characterized

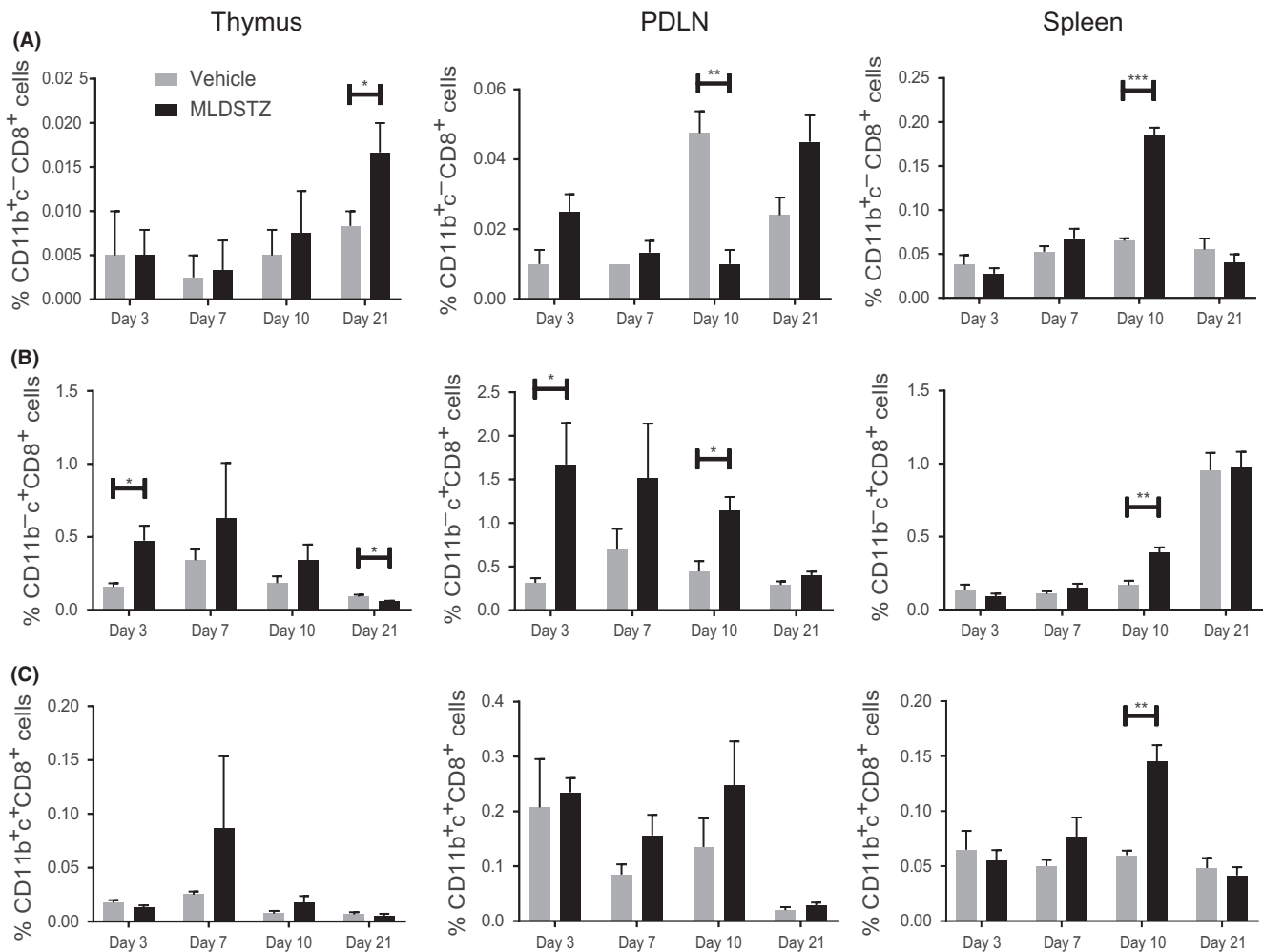


FIGURE 4 The percentages of CD8⁺DCs are increased in MLDSTZ-treated mice. The percentages of (A) CD11b⁺ CD11c⁻ CD8⁺, (B) CD11b⁻ CD11c⁺ CD8⁺, and (C) CD11b⁺ CD11c⁺ CD8⁺ were determined using flow cytometry in thymic glands, PDLNs, and spleens of vehicle, and MLDSTZ on days 3, 7, 10, and 21 after the first injection of STZ. Results are expressed as means ± SEM (n = 3-6 mice/group). Unpaired *t* tests were performed for comparison between vehicle and MLDSTZ-treated mice at each time point. *, **, and *** denote *P* < .05, *P* < .01, and *P* < .001, respectively. MLDSTZ, multiple low-dose streptozotocin; PDLNs, pancreatic-draining lymph nodes

by using a CD8 α marker together with DCs markers such as CD11b and CD11c. Therefore, we examined the proportion of CD11b⁺ CD11c⁻ CD8⁺, CD11b⁻ CD11c⁺ CD8⁺, and CD11b⁺ CD11c⁺ CD8⁺ DCs. The proportions of CD11b⁺ CD11c⁻ CD8⁺ DCs were increased on day 10 in PDLNs of vehicle but decreased in spleen of MLDSTZ-treated mice (Figure 4A). The proportions of CD11b⁻ CD11c⁺ CD8⁺ DCs were increased on day 3 in thymic glands and PDLNs, and on day 10 in both PDLNs and spleens of MLDSTZ treated mice (Figure 4B). The proportion of CD11b⁺ CD11c⁺ CD8⁺ cells was increased on day 10 in spleens of MLDSTZ-treated mice compared to vehicle-treated mice (Figure 4C). Taken together, our data illustrate that the cross-presenting CD8⁺ DCs are increased in MLDSTZ induced T1D.

3.6 | Proportions of IFN- γ ⁺ cells among CD3⁻NK1.1⁺ NKp46⁺ cells are increased in MLDSTZ mice

Next, we determined the proportions of NK cells by detecting the surface marker NK1.1 and the NK cell-specific activating receptor NKp46. The proportions of CD3⁻NK1.1⁺ cells were decreased on day 10 in thymic glands and spleens, and on day 21 in PDLNs of MLDSTZ mice (Figure 5A). In addition, we found that the proportions of CD3⁻NK1.1⁺NKp46⁺ cells were decreased on day 10 in thymic glands and spleens of MLDSTZ mice (Figure 5B). However, the proportions of IFN- γ ⁺ cells among CD3⁻NK1.1⁺NKp46⁺ cells were increased on day 10 in PDLNs and spleens of MLDSTZ mice (Figure 5C).

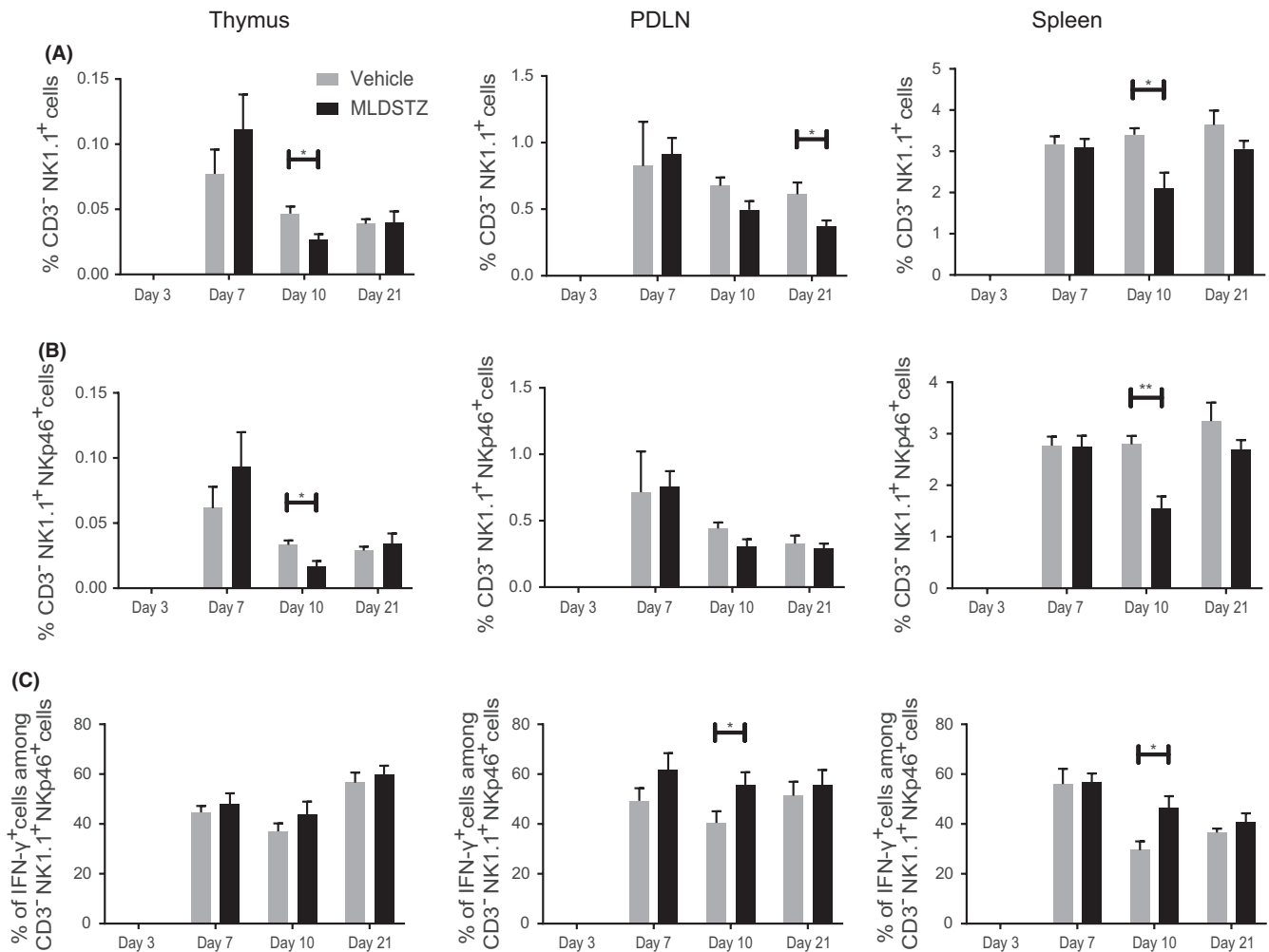


FIGURE 5 The percentages of NK cells are decreased but IFN- γ ⁺ cells among NK cells are increased in MLDSTZ-treated mice. The percentages of (A) CD3⁻NK1.1⁺ and (B) CD3⁻NK1.1⁺NKp46⁺ NK cells were determined using flow cytometry in thymic glands, PDLNs, and spleens of vehicle and MLDSTZ on days 7, 10, and 21 after the first injection of STZ. Results are expressed as means \pm SEM (n = 6 mice/group). Unpaired *t* tests were performed for comparisons between vehicle and MLDSTZ-treated mice at each time point. * and ** denote *P* < .05 and *P* < .01, respectively. MLDSTZ, multiple low-dose streptozotocin; NK, natural killer

3.7 | Proportion of Th1 and cytotoxic T cells are altered in MLDSTZ mice

Earlier we have shown that CD4⁺CD25⁻ T helper (Th) cells are first decreased on day 7 but increased on day 10 and onwards in PDLNs of MLDSTZ mice.³² T1D is considered to be a Th1 disease.^{2,41} Consequently, in the present study we examined the proportion of IFN- γ ⁺ cells among CD4⁺CD25⁻ Th cells, which are considered to be Th1 cells. We found that the proportion of Th1 cells was increased on day 21 in PDLNs, and on days 7, 10, and 21 in spleens of MLDSTZ mice (Figure 6A). Next, we determined the proportion of CD8⁺ cells, that is, cytotoxic T (Tc) cells since several reports have shown that CD8⁺ Tc cells kill the insulin-producing β cells in T1D. The proportion of CD8⁺ cells was increased on day 7 in thymic glands and on days 7 and 10 in PDLNs of MLDSTZ mice (Figure 6B).

4 | DISCUSSION

In the present study, we aimed to investigate the kinetics of innate immune cell responses at the early stage of T1D in the MLDSTZ-induced mouse model of T1D. We used in the present study the MLDSTZ mouse model since it has been reported that STZ treatment induces β -cell damage

that further causes the secretion of self-DNA.⁴²⁻⁴⁴ This further leads to the activation of immune responses.^{15,42-44} Herein, the thymic glands, PDLNs, and spleens were studied to elucidate responses of innate immune cells in central immune organs (thymus), localized immune organs (PDLNs), and systemic immune organs (spleen), respectively. The CD11b⁻ CD11c⁺ DCs, CD11b⁻ CD11c⁺ CD8⁺ DCs, pDCs, B220⁺, and CD19⁺ cells were the first cell types to increase on day 3 after the first injection of STZ, followed by other innate immune cells, which were increased on day 7 or 10.

The proportion of Ly6G⁺ cells was decreased in PDLNs on day 21 in MLDSTZ-treated mice. This finding differs compared to an earlier report, which showed that Ly6G⁺ cells are increased in NOD mice of 2-3 weeks old, when the mice were not diabetic and did not show any sign of insulinitis.¹⁵ Ly6G⁺ cells are considered as neutrophils that migrate to inflammatory sites in the early stage of inflammation. Moreover, Diana et al reported that neutrophils and B-1 lymphocytes cross talk through CRAMP, and that B-1a lymphocytes are increased at the same time point as the neutrophils.¹⁵ Our data are not in line with this since we found an increase in the proportion of B-1a lymphocytes in PDLNs and spleens of MLDSTZ on day 10. Neutrophils were reported to be decreased in the peripheral blood of T1D patients, indicating that they might migrate to local organs.¹⁶ Nevertheless, we did not find any alteration

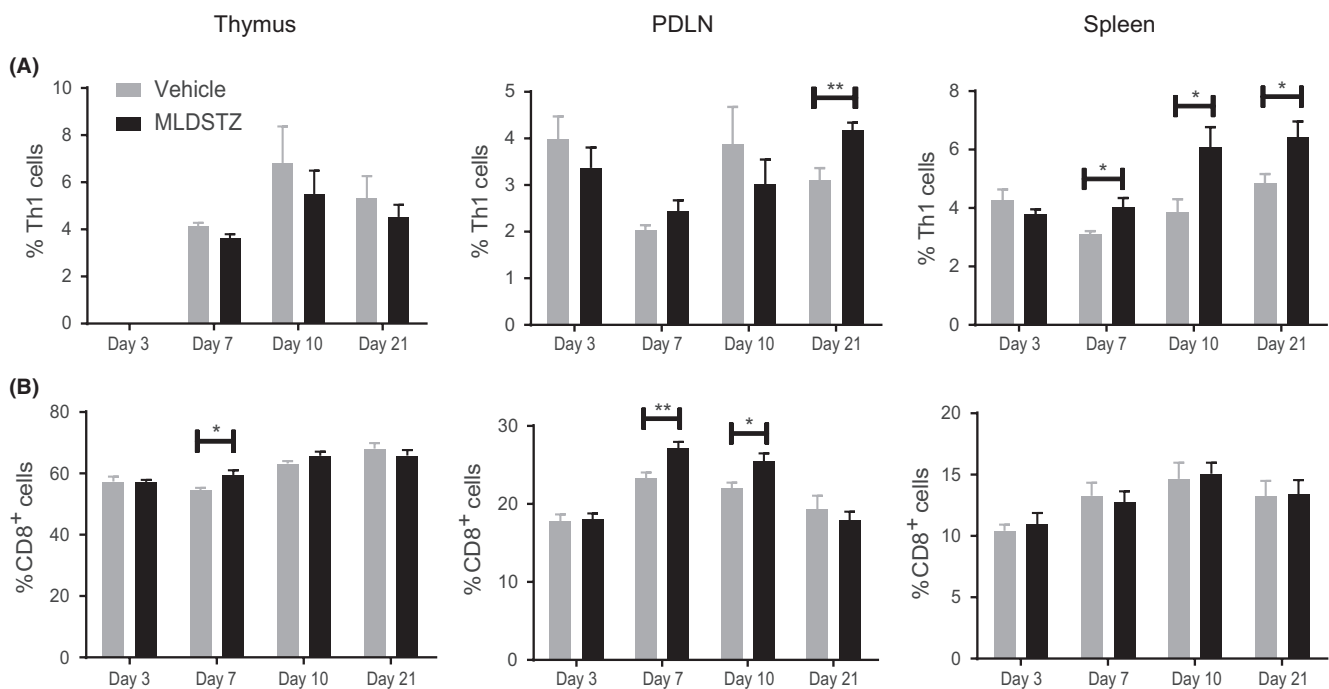


FIGURE 6 The percentages of Th1 and CD8⁺ T cells are increased in MLDSTZ-treated mice. The percentages of (A) CD4⁺ CD25⁻ IFN- γ ⁺ and (B) CD8⁺ were determined using flow cytometry in thymic glands, PDLNs, and spleens of vehicle and MLDSTZ on days 3, 7, 10 and 21 after the first injection of STZ. Results are expressed as means \pm SEM (n = 6 mice/group). Unpaired *t* tests were performed for comparisons between vehicle and MLDSTZ-treated mice at each time point. * and ** denote *P* < .05 and *P* < .01, respectively. MLDSTZ, multiple low-dose streptozotocin

of CD15^{low} neutrophils in the peripheral blood of T1D patients compare to healthy controls.^{29,45} In the present study, we found a decreased proportion of neutrophils in PDLNs of MLDSTZ mice. On the other hand, the response of B-1a lymphocytes were apparently later when compared to NOD mice since MLDSTZ mice were hyperglycemic and showed a moderate degree of insulinitis on day 10.³² However, regarding the role of neutrophils in autoimmunity, these cells may rather respond to B-1a lymphocytes due to self-antigens⁴⁶ than to pathogens, which may explain the perpetual response of neutrophils in the current study.

The proportions of CD11b⁻ CD11c⁺ DCs were increased on day 3 in thymic glands and PDLNs, and on day 10 in PDLNs and spleens in MLDSTZ-treated mice compared to vehicle. These data imply that the central and local immune response precede a systemic immune response. Early increase in CD11b⁻ CD11c⁺ DCs demonstrates that in the MLDSTZ mouse model of T1D, the autoimmune response may be led by DCs instead of neutrophils and B-1a lymphocytes. One could also argue that CD11b⁻ CD11c⁺ APCs may stimulate the B-1a lymphocytes, a notion that was further supported when we found increased proportion B-1a lymphocytes on day 10 in both PDLNs and spleens of MLDSTZ mice. In line with this hypothesis, a previous report has shown that B-1a lymphocytes migrate to secondary lymphoid organs where they reside until they encounter antigens.⁴⁷ Since the proportion of DCs was increased on day 10 in PDLNs and spleens, it shows that these DCs probably presented antigens to B-1a lymphocytes at this time point, which initiated the expansion of B-1a lymphocytes and neutrophils.

The proportion of CD11b⁺ CD11c⁺ cells was increased on day 7 and decreased on day 10 in PDLNs of MLDSTZ-treated mice, which may suggest a failure of innate immune tolerance. It has been reported that CD11b⁺ CD11c⁺ cells have suppressive function in autoimmune diseases.⁴⁸ It might be that CD11b⁺ CD11c⁺ cells have migrated to islets on day 10. Earlier Magnuson et al have shown that only CD11b⁺ CD11c⁺ myeloid cells can invade into islets of NOD.¹¹ The CD11b⁻ CD11c⁺ B220⁺ PDCA-1⁺ cells are most likely pDCs.^{49,50} The proportion of pDCs was increased on day 3 in PDLNs of MLDSTZ-treated mice, which is in line with human T1D as it has been reported that new-onset T1D patients have a higher proportion of pDCs compared to age-matched healthy controls.⁵¹

The proportion of B220⁺ cells was increased on day 3 in PDLNs and spleens of MLDSTZ-treated mice, and on day 7 and 10 in PDLNs. B220⁺ cells are considered to be a marker for both mature and immature B lymphocytes and our results illustrate that the expansion of B lymphocytes started at an early stage, possible due to the response of CD11b⁻ CD11c⁺ DCs and pDCs. Most of the CD19⁺ cells are mature B lymphocytes, and represent all subsets of B lymphocytes. Our results showed that the proportion was increased on day 3 in

spleens and on day 7 and 10 in PDLNs of MLDSTZ-treated mice, and this further are in accordance with the increased proportion of B-1a lymphocytes. B cells are the producers of antibodies, but they also work as antigen-presenting cells.⁵² It is possible that the increased B cells at the early stage is the result of antigen presentation by B cells, where they present self-antigens to T cells to initiate the immune attack to β cells. In accordance with this hypothesis, a study on NOD mice implicated that antigen presentation by B cells might contribute to the development of T1D.⁵³ B cell proportions remained increased in MLDSTZ mice until day 10, which may possibly be due to their ability to produce antibodies, although antibody production by B cells was suggested to be not involved in the development of T1D.⁵³ The proportion of B220⁺ cells was increased in thymus on day 3 and 10 in MLDSTZ-treated mice, while the proportion of CD19⁺ cells was also increased in thymus on day 10. There are normally a small number of B cells in thymus that promote the T-cell tolerance, but previous studies have shown that the B cells population seems to be increased in thymus of NOD mice.⁵⁴ It is therefore interesting that our results illustrate that the central immune response is similar in two different mouse models of T1D in terms of CD19⁺ B cell response.

The proportion of CD11b⁺ CD11c⁻ CD8⁺ was increased in spleens of MLDSTZ-treated mice on day 10. A previous study has shown that CD8⁺ T cells during certain circumstances can express CD11b in spleen in order to induce cytotoxic T cells,⁵⁵ while CD11b⁺ DCs normally do not express CD8. The cell type increased in this study could therefore be due to T cells rather than DCs. The proportion of CD11b⁻ CD11c⁺ CD8⁺ was increased on day 3 in thymus in MLDSTZ-treated mice. According to previous studies, CD8⁺ DCs are derived from the thymus and constitutes its own lineage.⁵⁶ Thus, the increase in thymus on day 3 is in line with hypothesis, and may also explain the increase in PDLNs and spleens on day 10 since they migrate from thymus to secondary lymphoid organs. CD8⁺ DCs are more active and may stimulate naive CD8⁺ T cells to cytotoxic T cells, but there are also reports suggesting that they rather have a suppressive function.⁵⁷ T cells that express all three markers CD11b, CD11c, and CD8 have been observed in virus-infected mice,⁵⁸ suggesting that the CD11b⁺ CD11c⁺ CD8⁺ cell type persist as an effector phenotype. However, in the present study we found that proportions of CD11b⁺ CD8⁺ and CD11b⁺ CD11c⁺ CD8⁺ cells were increased in MLDSTZ-treated mice, it is more likely that these cells were activated by the CD11b⁻ CD11c⁺ CD8⁺ cells and thus induced a pro-inflammatory response in the MLDSTZ-treated mice.⁴⁸

The role of NK cells in the development of T1D is still under debate. The ligands of NKp46 have been found on pancreatic β cells, and they facilitated NK cells degranulation in an NKp46-dependent manner.²⁸ Furthermore, the

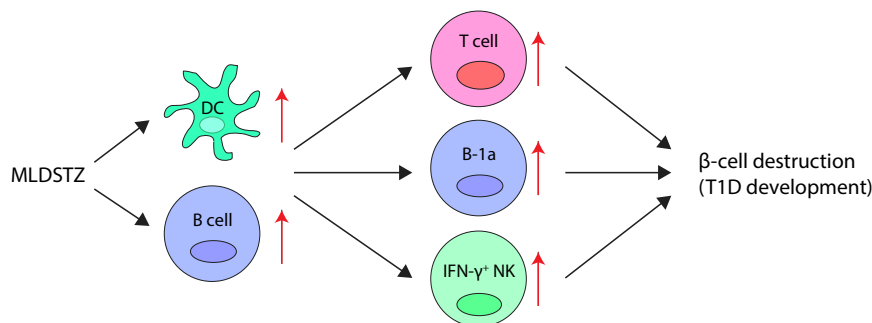


FIGURE 7 Tentative sequence of immune cell response in the early development of experimental type 1 diabetes. The first immune cells to increase in numbers from day 3 are antigen-presenting cells like DCs and B cells. Following that, Th1 cells and CD8 T cells are increased from day 7, and B-1a cells and IFN- γ^+ NK cells are increased from day 10, suggesting that the innate immune cells precede adaptive immune cell response in MLDSTZ mice. This sequence of immune response eventually leads to the development of T1D. MLDSTZ, multiple low-dose streptozotocin; DCs, dendritic cells; NK, natural killer

blockade of NKp46 prevented the development of T1D in NOD mice. On the other hand, NK cells also have been suggested to be protective in autoimmune diabetes.⁵⁹ NK cells can suppress β -cell-specific T cells and have been shown to produce TGF- β and IL-10.⁵⁹ Another study has shown that the activity of NK cells was decreased in the peripheral blood mononuclear cells of recently diagnosed T1D patients by investigating the activating receptors on NK cells.⁶⁰ In our current study, we observed the decreased proportions of NK cells from day 10 in MLDSTZ mice. In addition, we also found the increased proportions of IFN- γ^+ NK cells on day 10 in MLDSTZ mice. IFN- γ is considered as a pro-inflammatory cytokine that promotes the development of T1D, the decreased number of NK cells might be the result of potential immune tolerance. We speculate that NK cells might possess a protective property, which would explain why that the NK cell number was decreased in response of the increased IFN- γ^+ cells among NK cells. In accordance with this result, we have previously found that the proportion of CD56^{high} NK cells, which are considered to be IFN- γ -producing cells,⁶¹ are increased in LADA patients compared to healthy controls and T2D patients, but similar to T1D patients.²⁹

In this study, we have investigated the kinetics of innate and adaptive immune cells response during the early development of T1D in mouse model. We found that the proportions of DCs, pDCs, and B cells are increased at an early stage of T1D development in MLDSTZ-treated mice and that further mediates activation of other immune cells such as B1-a lymphocytes, Th1, and Tc cells, which might then migrate from secondary lymphoid organs to the pancreatic islets and contribute to insulin-producing β -cell damage (Figure 7). The possible protection from NK cells may be lost in MLDSTZ mice. This study reveals the kinetics of altered immune cell populations in an experimental mouse model of T1D. Our study suggests that more efforts should be devoted to study T1D early development.

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CONFLICT OF INTEREST

The authors have no financial conflict of interests.

AUTHOR CONTRIBUTIONS

KS conceived the study and design experiments. CS, KS, and ZL performed experiments, analyzed data, and wrote manuscript. MMC and MB performed experiment. LT and SS review the manuscript. KS and SS directed the study and obtained funding for the project.

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