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Original Article

The dynamic impact of hydrodynamic gene transfer on the immune system

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Abstract: Hydrodynamic gene transfer (HGT) has been used as an effective and convenient way to achieve gene expressions *in vivo*. However, its time-dependent impact on the immune system is unknown. The aim of the current study is to investigate the dynamic changes of the immune parameters after HGT. Plasmids were delivered to BALB/c mice by HGT. Each group of mice was sacrificed on day 1, 2, 3, 4 and 5 after HGT. The immune cell subsets from spleens and livers were analyzed by flow cytometry. IFN-γ, IL-6 and TNF-α in the serum were quantitated by cytometric bead array. The mice without HGT injection were used as control group on day 0. Compared to the normal mice (day 0), the T lymphocyte infiltrations in the spleen and liver were increased starting from day 1 after HGT. T cells. NK cells and myeloid cells such as dendritic cells, neutrophils and macrophages were also significantly expanded and peaked around day 2-3. Both T cells and NK cells were greatly activated. Serum levels of IFN-γ and IL-6 increased and peaked on day 1 after HGT. Most of the increased immune parameters returned to normal levels after day 4. However, the activated T cells remained at a high level, especially in the liver. In conclusion, HGT significantly increased the immune cell infiltration in the spleen and liver and activated T cells and NK cells. The immune response induced by HGT should be taken into consideration when evaluating the functions of the over-expressed genes using this strategy.

Keywords: Hydrodynamic gene transfer, T cell, NK cell, cytokine

Introduction

Hydrodynamic gene transfer (HGT) is a method developed for *in vivo* gene delivery and can achieve high levels of foreign gene expression [1-3]. It can increase endothelia permeability and facilitate intracellular delivery of nucleic acids through a rapid tail vein injection of a large volume of DNA solution into a mouse to build high intravascular pressure. This method not only diminishes the need for repeated injections of large amounts of recombinant proteins and the associated toxicities but also allows for sustained delivery [4-6].

Since HGT generates more gene products than the previously used methods of plasmid DNA injection, it has been widely used for expression of various proteins in animals to assess the function of genes [7-11] or to study the activity of DNA sequence regulating gene expression [12-15]. Compared to the conventional bacterial or yeast system, HGT avoids the problem of misfolding and improper glycosylation of the protein besides the advantage of convenience. In the immunology field, HGT has become a useful tool for introduction of antigen coding sequences [16, 17] or cytokine genes [18-26] to evaluate the function of the gene products for immune modulation. It has been reported that HGT could promote both humoral- and cell-based immunity in animals in a gene dependent manner [2, 16]. However, one study suggested that the side effects due to the delivery method should also be considered besides nucleotide-related side effects because they

found HGT induced inflammatory cytokine productions, and high volumes injection would induce tissue damage [27].

Which cell subsets of the immune system are affected by HGT and how long the influence of HGT can sustain in the murine model are not clear. Although a control group with empty vector is always included in the HGT experiments, the function of the gene studied could be related to the local tissue environment induced by HGT, especially when the immune-related function was studied. In the current study, we examined the percentages and numbers of the immune cell subsets in spleens and livers after HGT and revealed significant increases of the immune cell infiltrations and activation of T cells and NK cells during the first four days after HGT. Serum levels of IFN-γ and IL-6 were also increased by HGT. Most of the immune parameters returned to close to normal levels by day 4 after HGT except the activated T cells.

Materials and methods

Mice

Female BALB/c mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Mice were maintained under specific pathogen-free (SPF) conditions, and experiments were performed when the mice were about 6-8 wk of age. All animal protocols were approved by the Institutional Laboratory Animal Care and Use Committee at Soochow University.

Hydrodynamic gene transfer

The pcDNA3.1 (Invitrogen, Carlsbad, CA) plasmid was purified by Maxi-prep Kit (Axygen, Union City, CA) for HGT. The BALB/c mice hydrodynamically injected i.v. with 80 μg of the plasmid in a total of 2 ml PBS solution within 5 s.

Cell preparation

BALB/c mice were sacrificed and single cell suspensions were prepared from spleen and liver. Hepatic mononuclear cells were isolated by 5 ml 40% Percoll (GE Healthcare, Piscataway, NJ) and centrifuged at 2000 rpm for 20 min.

Flow cytometric analysis

Anti-mouse CD16/CD32 FcR block, CD69-FITC, CD4-PE, CD8-PerCP-cy5.5, CD11b-PE, CD19-APC, CD3-APC, CD43-APC, NKG2D-FITC, NKP46-PE, NKP46-APC and DX-5-PerCP-cy5.5, were purchased from BD Biosciences (San Jose, CA). CD44-APC, CD62L-FITC, NKG2D-PE, CD11c-FITC, Gr-1-PerCP-cy5.5 and CD27 were purchased from BioLegend (San Diego, CA). All stainings were performed in FACS buffer (1% BSA, and 0.1% NaN_3) in the presence of purified anti-CD16/32 at saturation to block unspecific staining for 30 min at 4°C. The flow cytometric results were analyzed with FACSCalibur (BD Biosciences, San Jose, CA) using CellQuest software.

Histology

To evaluate immune cell infiltration and pathological changes of the organs by HGT, histological examinations of spleen, liver, lung and kidney were assessed using hematoxylin and eosin (H&E) staining. Samples were fixed in formalin (10%) and embedded in paraffin prior to sectioning. The procedures of the staining were performed as described previously [28]. All slides were read by an expert pathologist in a blinded fashion.

Serum cytokine assay

Serum was collected from the mice after HGT and assayed to determine the concentrations of IFN-γ, TNF-α and IL-6 with Flow Cytomix kits (eBioscience, San Diego, CA) according to the manufacturer's instructions. The test samples were analyzed by flow cytometry. For each analysis, up to 50,000 events were acquired. The mean concentration of each cytokine was expressed as pg/ml.

Statistical analysis

Statistical differences between two groups were determined by unpaired student t test. A P value <0.05 was considered statistically significant.

Results

The T lymphocyte infiltration was increased in spleen and liver by HGT

The tissue morphology and immune cell infiltrations of spleen, liver, lung and kidney were histological examined by H&E staining day 2 after HGT. As shown in **Figure 1A**, the morphology of the tissues were not affected by HGT, suggest-

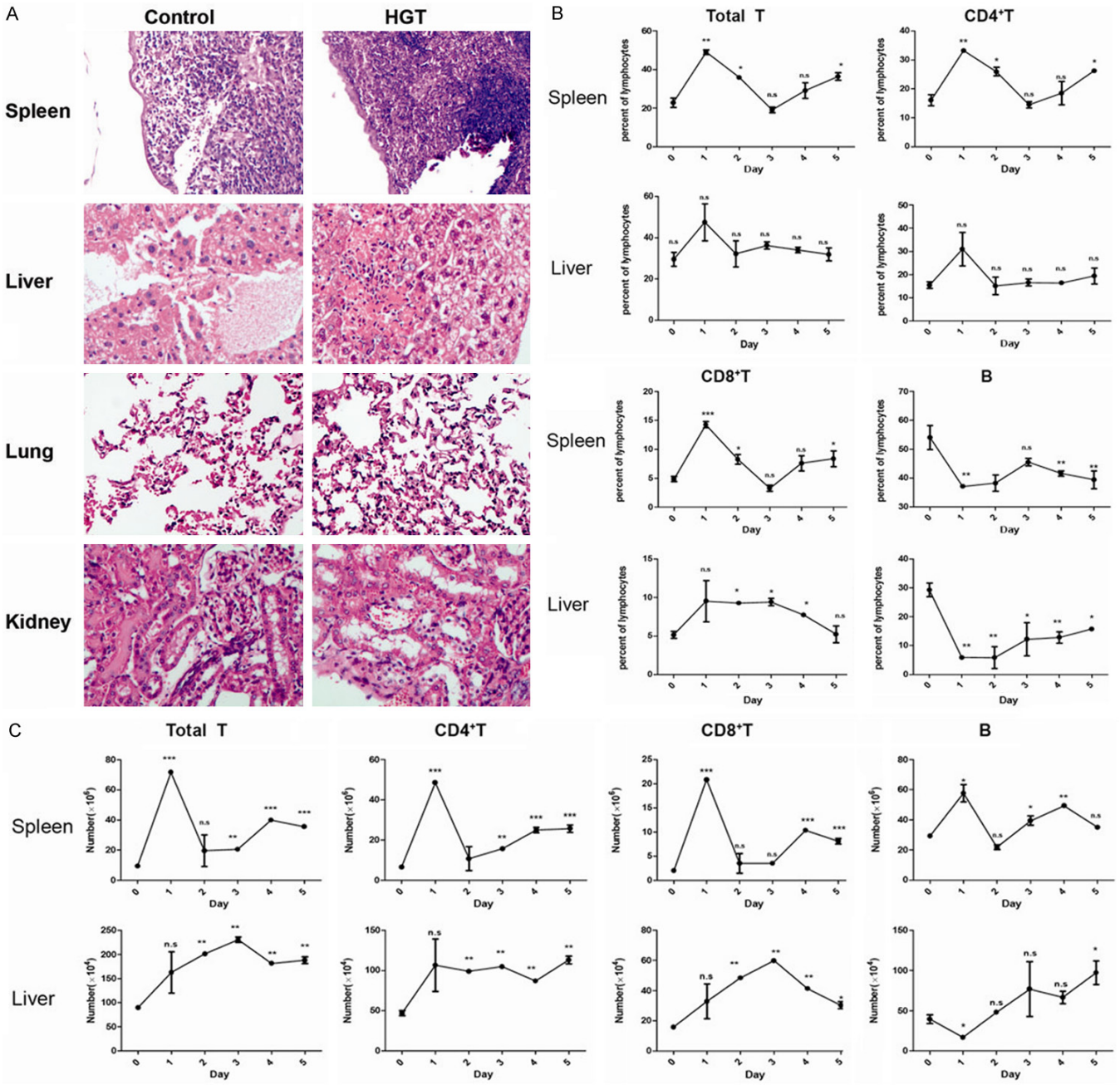
A
Control
HGT
Spleen
Liver
Lung
Kidney
B
Total T
CD4+T
CD8+T
B
Spleen
Liver
percent of lymphocytes
Day
C
Total T
CD4+T
CD8+T
B
Spleen
Liver
Number(×10^6)
Number(×10^4)
Day

Figure 1. Histology analysis of different organs and T cell percents and numbers in the spleen and liver after HGT. A. HE staining were performed with sections of spleen, liver, lung and kidney (original magnification 200×). B. The percentages of total T, $CD4^+T$, $CD8^+T$ and B cells in both spleen and liver. C. The numbers of total T, $CD4^+T$, $CD8^+T$ and B cells in both spleen and liver. Values are presented as means ± SD. The data shown are the representative of three experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

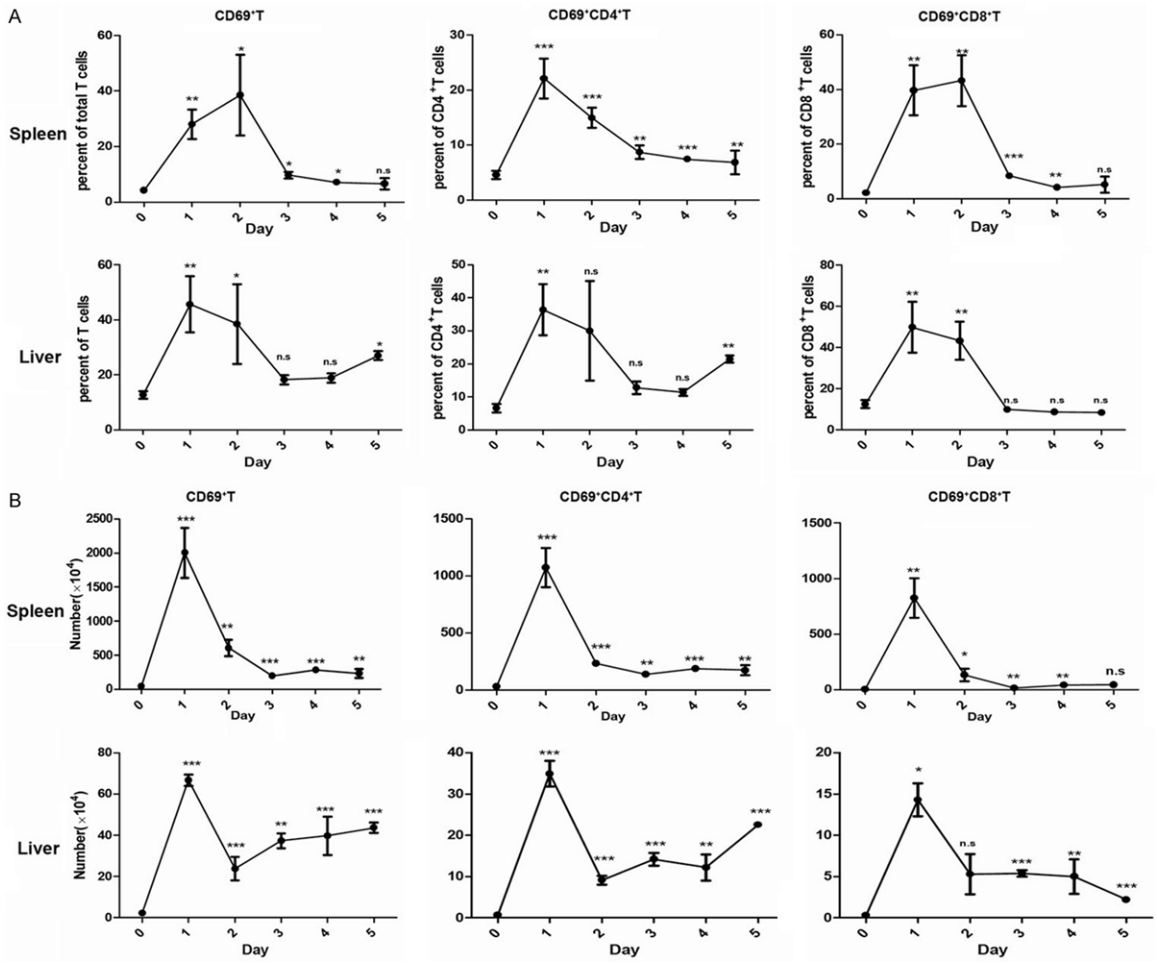

Figure 2. The CD69 expression on T, $CD4^+T$ and $CD8^+T$ cells after HGT. A. The percentages of $CD69^+T$, $CD69^+CD4^+T$, and $CD69^+CD8^+T$ cells in both spleen and liver. B. The absolute numbers of $CD69^+T$, $CD69^+CD4^+T$, and $CD69^+CD8^+T$ cells in both spleen and liver. Values are presented as means ± SD. The data shown are the representative of three experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

ing hydrodynamic injection did not cause obvious tissue damages, especially in the liver. However, immune cell infiltrations were significantly increased in the spleen and liver after HGT compared with those of control mice.

To determine the effect of HGT on the lymphocyte infiltration, we examined the percent (**Figure 1B**) and number (**Figure 1C**) of total T cells, $CD4^+T$ cells, $CD8^+T$ cells and B cells in the spleen and liver. The percent and number of total T cells, $CD4^+T$ cells, and $CD8^+T$ cells increased significantly in the spleen and peaked on day 1 after HGT. Their percentages also increased in the liver on day 1, but did not reach statistical significance. The number of total T cells, $CD4^+T$ cells, $CD8^+T$ cells in the liver increased gradually and stayed at relatively high levels until day 5 after HGT. On the other hand, the percent of B cells in both spleen and liver decreased significantly. The number of B cells slightly increased in the spleen, but remained unchanged in the liver. Therefore, these results demonstrated that HGT signifi-

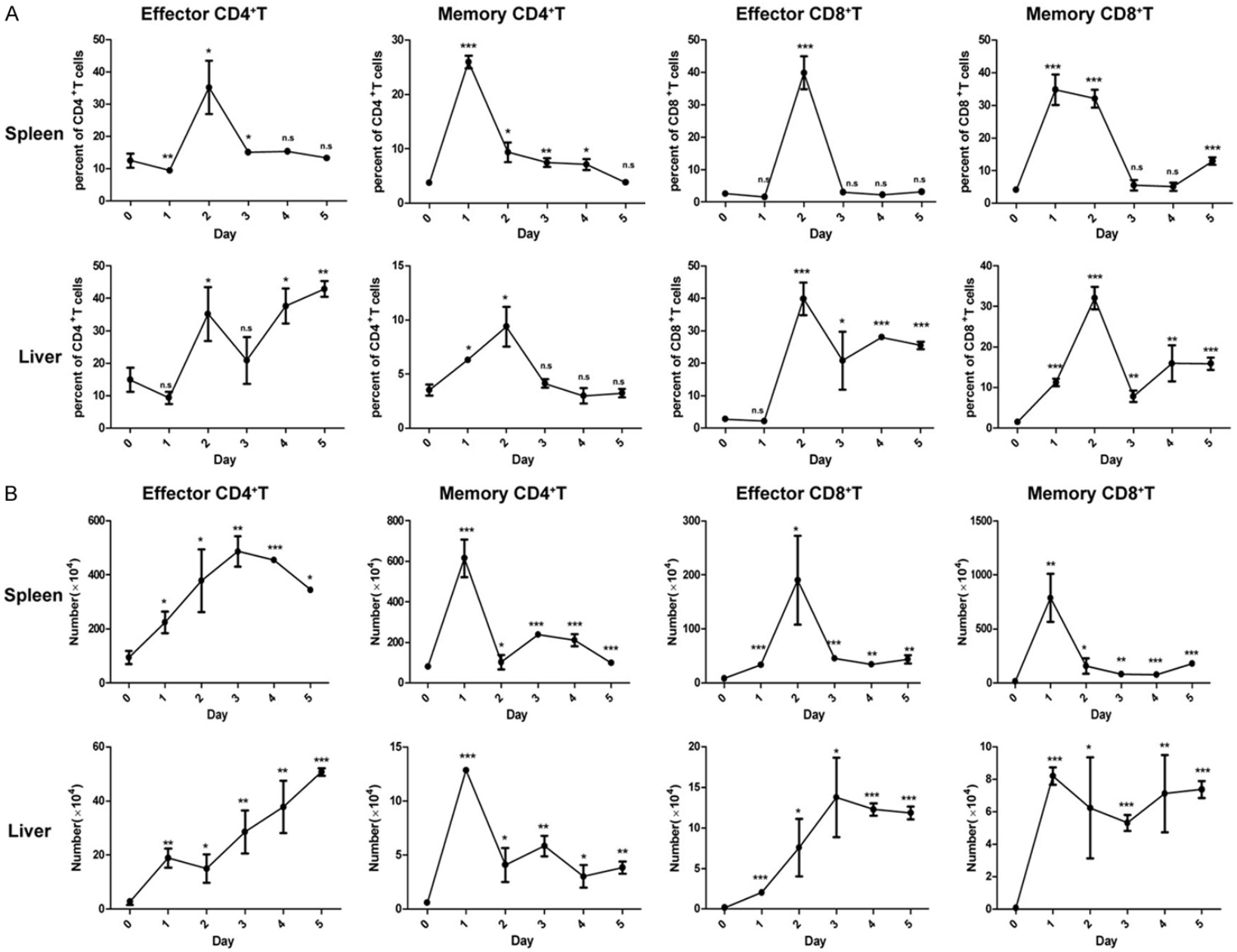
A
Effector CD4+T
Memory CD4+T
Effector CD8+T
Memory CD8+T
Spleen
Liver
percent of CD4+T cells
percent of CD8+T cells
Day
B
Number(×10^4)

Figure 3. The effects of HGT on effector and memory T cell percentages and numbers in both spleen and liver. A. The percentages of spleen and liver effector ($CD62L^-CD44^{hi}$) and memory ($CD62L^+CD44^{hi}$) T cells. B. The absolute numbers of spleen and liver effector ($CD62L^-CD44^{hi}$) and memory ($CD62L^+CD44^{hi}$) T cells. Values are presented as means ± SD. The data shown are the representative of three experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

cantly increased T lymphocyte infiltration in both spleen and liver.

The T lymphocytes were activated in spleen and liver by HGT

In order to evaluate the activation status of T lymphocytes of spleen and liver from the BALB/c mice that received HGT, the percent and number of $CD69^+$T cells were analyzed by flow cytometry (**Figure 2**). The percentages of $CD69^+$T cells, $CD69^+CD4^+$T cells, and $CD69^+CD8^+$T cells in both spleen and liver increased significantly by HGT and peaked around day 1-2 (**Figure 2A**). The numbers of $CD69^+$T cells, $CD69^+CD4^+$T cells, and $CD69^+CD8^+$T cells in the spleen and liver also markedly increased on day 1 after HGT (**Figure 2B**).

HGT also augmented the percentages of effector $CD4^+$T, $CD8^+$T cells ($CD44^{hi}CD62L^-$) and memory $CD4^+$T, $CD8^+$T cells ($CD44^{hi}CD62L^+$) in spleen and liver (**Figure 3A**). The dynamic changes are somewhat different. The percent of effector T cells increased dramatically in both spleen and liver on day 2 after HGT, while the percent of memory cells markedly increased on day 1 in the spleen, but gradually increased and peaked on day 2 in the liver. The numbers of effector $CD4^+$T, $CD8^+$T cells and memory $CD4^+$T, $CD8^+$T cells also increased in both spleen and liver after HGT (**Figure 3B**). The number of effector $CD8^+$T, memory $CD4^+$T and $CD8^+$T cells returned to relatively low levels in the spleen after day 2, while the number of effector $CD4^+$T cells stayed high even on day 5 after HGT. The number of memory $CD4^+$T cells in the liver dropped dramatically after day 2 of HGT. However, the number of effector $CD4^+$T cells, effector and memory $CD8^+$T cells remained high even after day 5. Taken together, HGT significantly activated $CD4^+$ and $CD8^+$T cells in both spleen and liver and they stayed activated 5 days after HGT.

Increased NK cell numbers and activation in spleen and liver by HGT

We also examined the percent and number of NK cells after HGT in both spleen and liver. The percent and number of NK cells dramatically increased in the spleen on day 3 after HGT (**Figure 4A**). The percent and number of NK cells also increased in the liver mainly on day 2-3 after HGT.

To further dissect the phenotypic characteristics of the NK cells, the expressions of NKG2D, NKp46 and CD43 on NK cells were analyzed by flow cytometry (**Figure 4B** and **4C**). The expressions of the activation markers, NKG2D and NKp46 on the NK cells were significantly upregulated in the spleen on day 3 after HGT. The NKG2D and NKp46 expressions on NK cells also increased in the liver around day 2-3 after HGT. The expressions of the maturation marker CD43 were also increased and peaked on day 3 in the spleen and day 2-3 in the liver. These results demonstrated that HGT significantly increased NK cell numbers and activated NK cells in spleen and liver around day 2-3.

Increased myeloid cells in spleen and liver and serum cytokine levels by HGT

To explore the effect of HGT on the myeloid cells, we examined the percent and number of dendritic cells (DCs), macrophages and neutrophils in spleen and liver (**Figure 5**). The percentages of DCs, macrophages and neutrophils in the spleen were not significantly changed by HGT, while the percent of DCs increased on day 2 and the percent of macrophages increased on day 1 in the liver (**Figure 5A**). On the other hand, the numbers of DCs, macrophages and neutrophils increased significantly in both spleen and liver mostly around day 2-4 after HGT (**Figure 5B**).

Serum levels of IFN-γ and IL-6 significantly increased after HGT (**Figure 6A** and **6B**) especially on day 1 after HGT. The serum levels of TNF-α also increased on day 1-2 but did not reach statistical significance (**Figure 6C**). Serum cytokine levels gradually returned back to the controls level on day 5. These results demon-

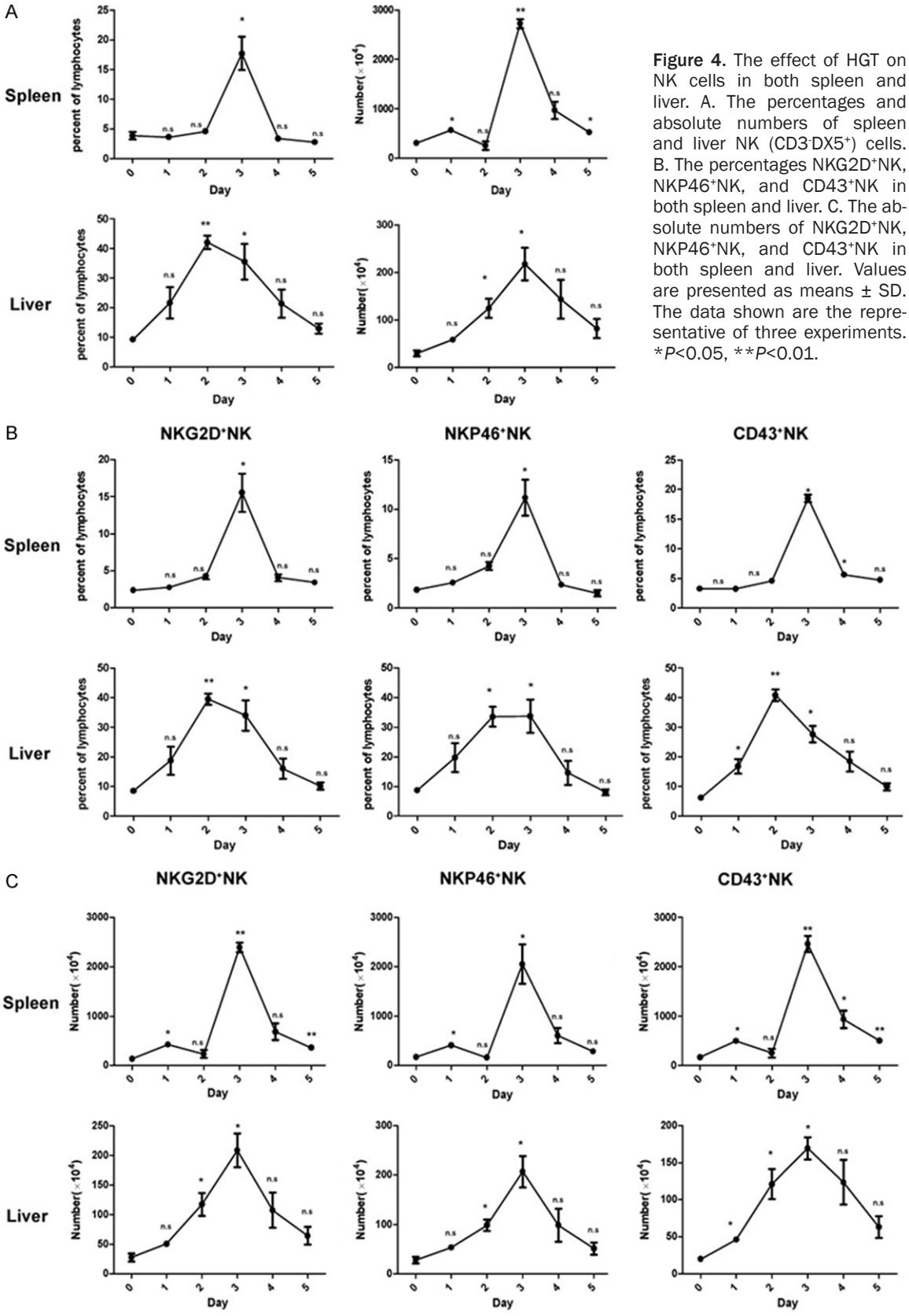

Figure 4. The effect of HGT on NK cells in both spleen and liver. A. The percentages and absolute numbers of spleen and liver NK ($CD3^-DX5^+$) cells. B. The percentages $NKG2D^+NK$, $NKP46^+NK$, and $CD43^+NK$ in both spleen and liver. C. The absolute numbers of $NKG2D^+NK$, $NKP46^+NK$, and $CD43^+NK$ in both spleen and liver. Values are presented as means ± SD. The data shown are the representative of three experiments. **P*<0.05, ***P*<0.01.

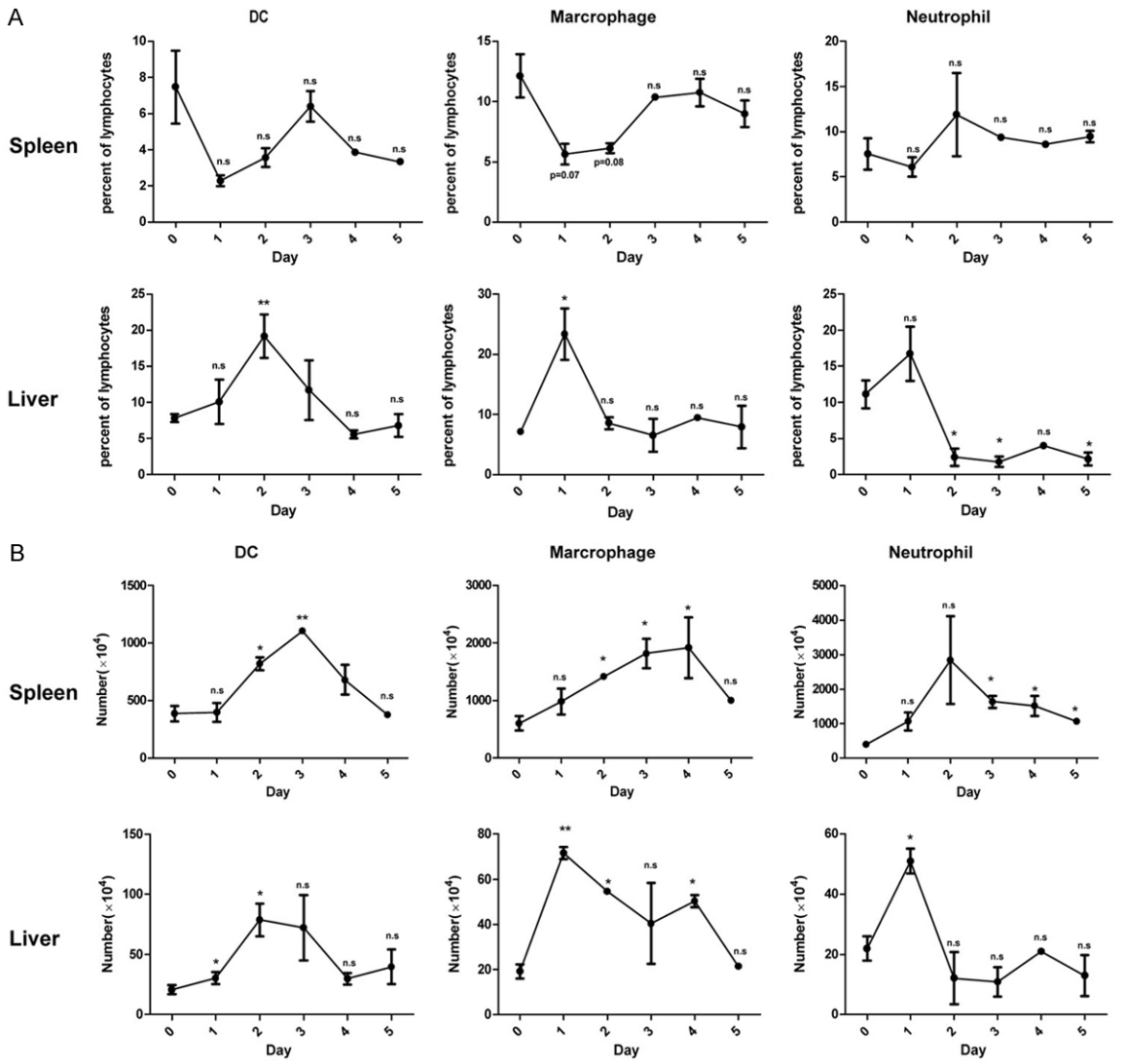

Figure 5. The effect of HGT on myeloid cells in both spleen and liver. A. The percentages of DCs, macrophages and neutrophils in spleen and liver. B. The absolute numbers of DCs, macrophages and neutrophils in spleen and liver. Values are presented as means ± SD. The data shown are the representative of three experiments. **P*<0.05, ***P*<0.01.

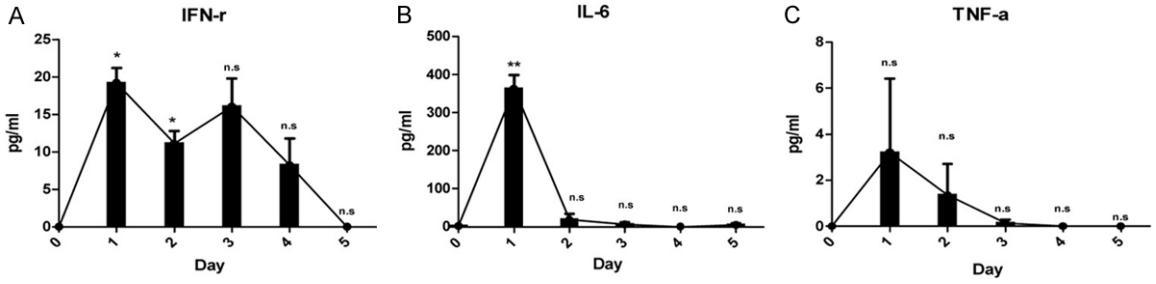

Figure 6. The effect of HGT on serum cytokine levels. Serum (A) IFN-γ, (B) IL-6 and (C) TNF-α concentrations were de tected by Flowcytomix kit on different time points after HGT. Values are presented as means ± SD. The data shown are the representative of three experiments. **P*<0.05, ***P*<0.01.

strated that HGT could increase serum IFN-γ and IL-6 levels.

Discussion

HGT has become a common method to assess the involvement of cytokine genes in immune modulation. Barao et al. found that hydrodynamic human IL-15 cDNA delivery resulted in high levels of hIL-15 protein in the serum that lasted for several days, and this hIL-15 induced a significant increase of mature donor-derived NK cells within the bone marrow, spleens, and livers of the bone marrow transplanted mice [21]. It is also reported that hydrodynamics-based delivery of an IL-1 receptor II fusion gene might ameliorate rat autoimmune myocarditis by inhibiting IL-1 and Th17 cell polarization [21]. It seems HGT is able to promote or suppress immune response in a gene dependent manner. However, some studies also found the treatment of hydrodynamic injection but not the target siRNA induced inflammatory cytokine production, and high volume injection would induce tissue damage [30]. Therefore, the effect of HGT on immune system has to be taken into consideration during analyzing the data. To our knowledge, this is the first study dynamically analyzing the effect of HGT on the immune cell subsets in both spleen and liver as well as serum cytokine levels.

It is known that the high blood pressure across the liver would cause the liver to significantly expand and induce a structural deformation during HGT [29, 30]. It is also reported that there is a transient increase of liver enzyme and other blood composition in hydrodynamically treated animals [31]. Although the expanded liver could return to its original size within 30 minutes [30], the increased concentration of the blood composition will not be returned to normal level until 72 hours after injection [31]. Although histology did not show obvious tissue damage by HGT (**Figure 1A**), these transient changes could very well cause inflammatory responses and immune cell infiltration in the organs. Most of the marked changes in immune cell subsets happened on day 1-3 after HGT, some of them returned to control level by day 5. However, the activated T cells, especially the effector and memory T cells remained in high numbers in the liver even on day 5 after HGT. These results suggest that the immune system is not back to normal after five days post HGT. Some of the immune regulatory roles of the studied gene could be pre-conditioned by HGT, especially when the T cell functions were focused on.

Acknowledgements

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Disclosure of conflict of interest

None.

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