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Inflammation-induced CD69$^+$ Kupffer cell feedback inhibits T cell proliferation via membrane-bound TGF-β1

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Kupffer cells, tissue-resident macrophage lineage cell, are enriched in vertebrate liver. The mouse F4/80$^+$ Kupffer cells have been subclassified into two subpopulations according to their phenotype and function: CD68$^+$ subpopulation with potent reactive oxygen species (ROS) production and phagocytic capacities, and CD11b$^+$ subpopulation with a potent capacity to produce T helper 1 cytokines. In addition, CD11b$^+$ Kupffer cells/macrophages may be migrated from the bone marrow or spleen, especially in inflammatory conditions of the liver. For analyzing diverse Kupffer cell subsets, we infected mice with *Listeria monocytogenes* and analyzed the phenotype variations of hepatic Kupffer cells. During *L.monocytogenes* infection, hepatic CD69$^+$ Kupffer cells were significantly induced and expanded, and CD69$^+$ Kupffer cells expressed higher level of CD11b, and particularly high level of membrane-bound TGF-β1 (mTGF-β1) but lower level of F4/80. We also found that clodronate liposome administration did not eliminate hepatic CD69$^+$ Kupffer cell subset. We consider the hepatic CD69$^+$ Kupffer cell population corresponds to CD11b$^+$ Kupffer cells, the bone marrow-derived population. Hepatic CD69$^+$ Kupffer cells suppressed Ag-nonspecific and OVA-specific CD4 T cell proliferation through mTGF-β1 both *in vitro* and *in vivo*, meanwhile, they did not interfere with activation of CD4 T cells. Thus, we have identified a new subset of inflammation-induced CD69$^+$ Kupffer cells which can feedback inhibit CD4 T cell response via cell surface TGF-β1 at the late stage of immune response against infection. CD69$^+$ Kupffer cells may contribute to protect host from pathological injure by preventing overactivation of immune response.

Kupffer cell, immune regulation, CD69, TGF-β1, *Listeria monocytogenes*

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INTRODUCTION

Kupffer cells, an abundant population of macrophages that reside in the sinusoids of the liver, play a critical role in both liver homeostasis and immunoregualtion, performing essential tissue-specific functions as well as protecting the organism from infection (Crispe, 2009; Parker and Picut, 2005; Jenne and Kubes, 2013). Kupffer cells constitute the first innate immune cell population of the body to deal with bacteria, bacterial endotoxins and microbial debris derived from the gastrointestinal tract and transported to the liver via the portal vein (Vollmar and Menger, 2009; Zhang et al., 2009). Together with the sinusoidal endothelial cells, natural killer T cells, dendritic cells and soluble compounds such as complement factors and acute phase proteins, Kupffer cells, an important component of innate immunity, make rapid response to potentially dangerous factors. This is extremely critical, since there are lots of pathogen-derived products coming from venous portal blood, such as lipopolysaccharide, and pathogens from the gut, which need to be eliminated

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from the circulation to avoid systemic immune activation. The fact that Kupffer cells constitute 80%–90% of tissue macrophages present in the body suggest a predominant role of the liver in systemic as well as regional defense.

Besides their barrier (Vollmar and Menger, 2009) and janitor function (Sitia et al., 2011; Bellone et al., 1997), Kupffer cells have been demonstrated to play an important role in the response to pathogens, including viruses. Until now, it has been discovered several possible anti-viral roles of Kupffer cells, including binding and/or uptake of virus inducing immune recognition and the production of pro-inflammatory factors resulting in inhibition of viral replication in hepatocytes, activation of neighboring cells, and attraction, activation, and interaction with other immune cells, which will further increase the anti-viral and inflammatory response. These immune activating roles of Kupffer cells are beneficial to combat viruse infection in the early stage after infection, but may also contribute to tissue damage and the development of fibrosis and cirrhosis during chronic viral hepatitis (Zhao et al., 2014). Furthermore, Kupffer cells also have immune regulatory functions, either through direct virus-Kupffer cells interaction, or as a component of the complex tolerogenic liver environment. These qualities may counteract the development of effective anti-viral immunity and support viral persistence during viruse-infection and promoting related disease pathogenesis (Thimme et al., 2001; Day et al., 2002; Lauer et al., 2002; Rehermann and Nascimbeni, 2005; Boonstra et al., 2008; Jager et al., 2016).

The different phenotypes of Kupffer cells attribute to their specialized function. Kupffer cells were identified in the early 1970s as peroxidase-positive cells with cytoplasm containing numerous granules and vacuoles, and occasional tubular, vermiform invaginations (Fahimi, 1970; Klockars and Reitamo, 1975; Crofton et al., 1978; Widmann et al., 1972). At present, human Kupffer cells are identified by immunohistochemistry or flow cytometry using antibodies directed against CD68, CD14, and CD16 (Tu et al., 2008; Brown et al., 2001; Baldus et al., 1998). However, it is important to aware that these markers are not unique for human Kupffer cells, but are also expressed on monocytes, which are also documented as a source of precursor cells for Kupffer cells, and/or dendritic cells (Gregori et al., 2010). Rat Kupffer cells are commonly identified by antibodies against CD68 or CD163 (ED1 and ED2, respectively) (Dijkstra et al., 1985), and mouse Kupffer cells by the F4/80 marker (Austyn and Gordon, 1981). However, also the rat and mouse markers are not characteristic for Kupffer cells, but are shared with other myeloid cells.

The identification of Kupffer cells that exists under pathological conditions is even more difficulty than under steady state conditions. In pathological process, cellular infiltrates are observed consisting of inflammatory monocytes and/or dendritic cells that share certain surface markers with Kupffer cells. In rat studies, large and small Kupffer cells were proved to be present in a distinct area within the liver, i.e., in the peri-portal, and peri-venous and mid-zonal area, respectively, and two subpopulations of Kupffer cells have been isolated from rat liver tissue: $ED1^+ED2^-$ and $ED1^+ED2^+$ cells (Bouwens et al., 1986; Kono et al., 2002; Sleyster and Knook, 1982; Dixon et al., 1986). Similarly, some studies have suggested that there are two subpopulations of mouse Kupffer cells: $F4/80^+CD68^+$ and $F4/80^+CD11b^+$ cells isolated from mouse liver tissue (Kinoshita et al., 2010; Movita et al., 2012). There is another possibility that these subpopulations are actually distinct differentiation phases rather than distinct Kupffer cells subpopulations, or maybe they only represent the different stages of infiltrating inflammatory monocytes instead of resident tissue macrophages.

In this study, we identified a new subset of $CD69^+$ Kupffer cells in the mouse models with *L. monocytogenes* infection. These $CD69^+$ Kupffer cells express extremely higher level of membrane-bound TGF-β1 (mTGF-β1), and produced similar levels of IFN-γ, IL-12 with $CD69^-$ Kupffer cells. Interestingly, these $CD69^+$ Kupffer cells could inhibit CD4 T cell immune response *in vitro* and *in vivo* through mTGF-β1, but not via traditional manner like production of soluble molecules. The inflammation-induced $CD69^+$ Kupffer cells may play an important role in the termination of the immune response at the late stage of immune response against infection when pathogens have been eliminated, thus protecting host from immunopathological damage by controlling immune response. Our data provide new insight to the immunobiology and function of hepatic Kupffer cells.

RESULTS

Expansion of $CD69^+$ Kupffer cells in response to intracellular bacterial infection

For analyzing diverse hepatic Kupffer cell subsets, we infected mice with *L. monocytogenes* and analyzed the phenotype variations of hepatic Kupffer cells. Among the various phenotype markers, the expression of CD69 on hepatic Kupffer cells attracted our attention. $CD69^+$ Kupffer cells contained 15%–17% of liver isolation of mononuclear cell (MNC) (Figure 1A) and 70%–80% of liver Kupffer cells (data not shown), and most of the $CD69^+$ Kupffer cells (~84.7%) expressed CD11b (Figure 2), on day 4 and 6 after injection of bacteria. Through the kinetics of CD69 expression on liver Kupffer cells after *L. monocytogenes* infection, we found that the CD69 expression dramatically increased on day 4 and 6, and then declined rapidly to ~4.1% on day 8 and to the normal level after infection (Figure 1B).

To confirm the validity of this mouse model, we detected the bacterial burdens in the liver at different times after *L. monocytogenes* infection. The bacterial titers peaked on day 2 of infection, then declined, and completely eliminated on day 10 (Figure 1C). These data indicate that *L. monocytogenes*

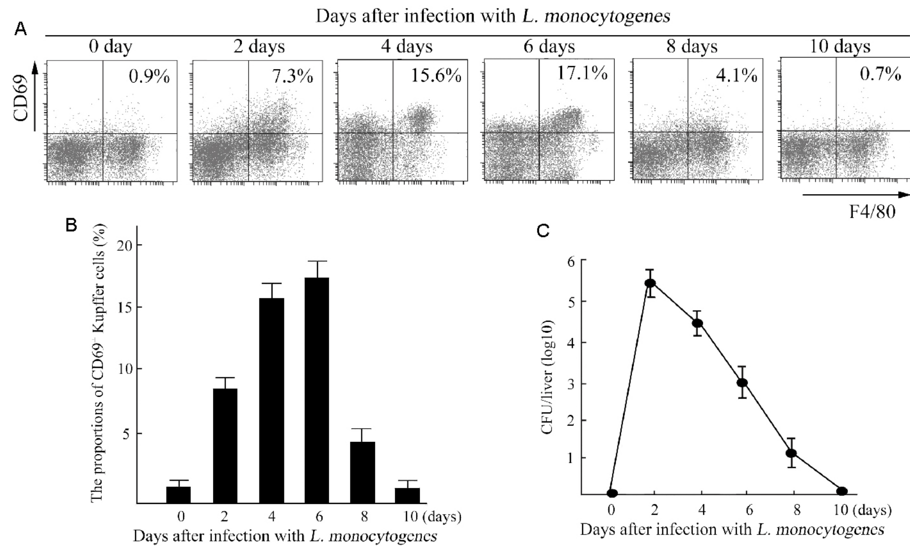

Figure 1 Expression of CD69 on hepatic Kupffer cells during *L. monocytogenes* infection. A, Flow cytometry analysis of the CD69 expression on hepatic F4/80$^+$ Kupffer cells from *L.monocytogenes*-infected mice after different days. B, Statistical analysis of CD69$^+$ cells in total hepatic MNC (%). C, Titers of *L.monocytogenes* in the liver of the mice different days after *L.monocytogenes* infection. The dot plots represent one of five independent experiments with similar results. Means±SE (n=5; eight mice per group).

infection-induced and/or -expanded CD69$^+$ Kupffer cells existed in the mid- and late stages of infection and occupied high proportion in total hepatic Kupffer cells.

Liver Kupffer cells have been proposed to be heterogeneous macrophage lineage cells. F4/80 is a representative surface marker of mouse mononuclear phagocytes. CD11b, a C3b receptor, is expressed on the surface of monocytes/macrophages, granulocytes, and NK cells. CD68 (macrosialin) is also used as a marker of macrophages, including Kupffer cells. This antigen is predominantly localized in the cytosol of macrophages but is expressed on the cell surface upon activation. The present studies suggested that mouse F4/80$^+$ Kupffer cells may be subclassified into two major subsets according to their phenotype and function: the CD68$^+$ subset with phagocytic capacities, and the CD11b$^+$ subset, with a potent capacity to produce cytokines and chemokines. In addition, CD11b$^+$ Kupffer cells/macrophages may be migrated from the bone marrow or spleen, especially in inflammatory conditions of the liver. In our experiments, large amounts of CD69$^+$ Kupffer cells express high levels of CD11b (~84.7%) and low levels of CD68 (~5.2%), meanwhile, it was found that the proportion of hepatic CD69$^+$ Kupffer cell subset do not change significantly with clodronate liposome administration (Figure 2). Together with above data, the results demonstrate that CD69$^+$CD11b$^+$ Kupffer cells, derived from circulating monocytes, are inducible and significantly expanded in response to infections.

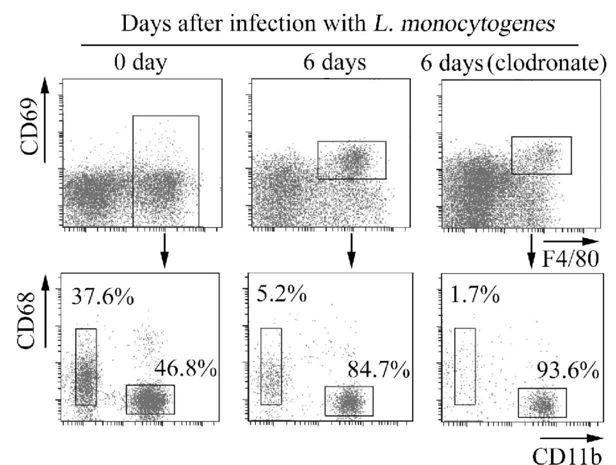

Figure 2 Hepatic CD69$^+$ Kupffer cells during *L.monocytogenes* infection belong to CD11b$^+$ Kupffer cells/macrophages. Mice were i.p. injected with 200 μL of clodronate liposomes 24 h before *L.monocytogenes* infection. Flow cytometry analysis of the CD68 and CD11b expression on hepatic F4/80$^+$ Kupffer cells cells from *L.monocytogenes*-infected mice zero day and six days with or without administration of clodronate liposomes. The dot plots represent one of five independent experiments with similar results.

Distinct phenotype and cytokine profile of CD69$^+$ Kupffer cells

The maximal proportion of CD69$^+$ Kupffer cells in hepatic Kupffer cells is induced on day 6 after *L. monocytogenes* infection, so we analyzed hepatic Kupffer cells on day 6 after infection in the following experiments for phenotypic and functional investigation. First, we analyzed the phenotype of CD69$^+$ Kupffer cells, comparing with CD69$^-$ Kupffer cells. We found that most of CD69$^+$ Kupffer cells (~93.5%) expressed relatively high levels of mTGF-β1, while only few CD69$^-$ Kupffer cells (~0.7%) expressed mTGF-β1 (Figure 3A).

Kupffer cells always display their roles through secretion of different cytokines, so we analyzed the cytokine profile of

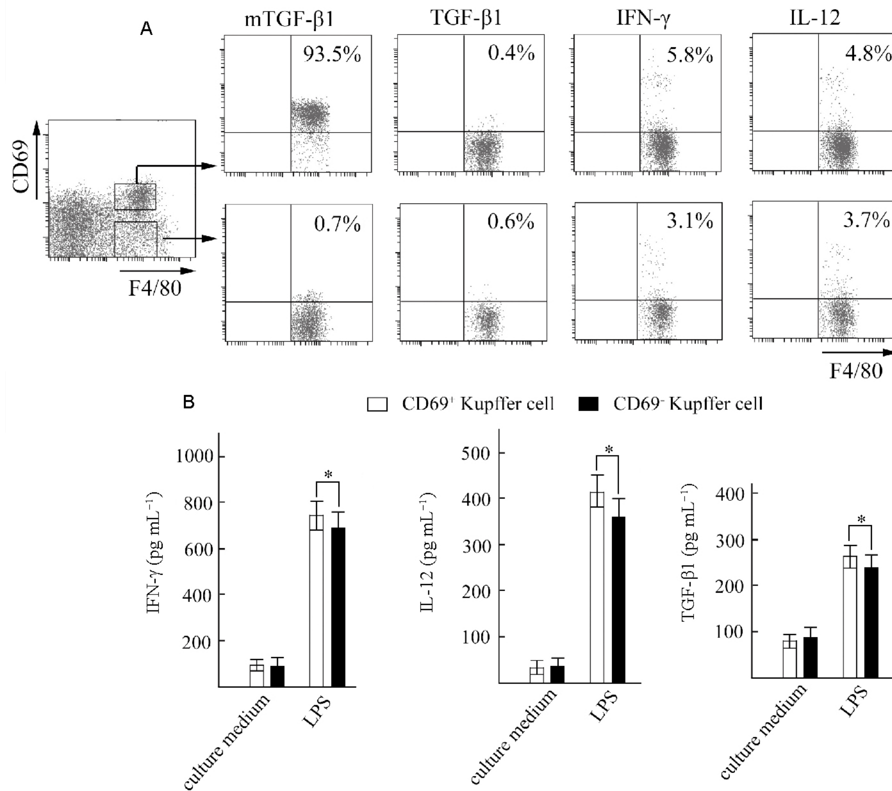

Figure 3 Distinct phenotype and cytokine profile of CD69$^+$ Kupffer cells. A, Flow cytometry analysis of hepatic CD69$^+$ or CD69$^-$ Kupffer cells (day 6) stained with anti-TGF-β1 PE, anti-IFN-γ PE and anti-IL-12 p40 PE respectively. The dot plots represent one of five independent experiments with similar results. B, Hepatic CD69$^+$ or CD69$^-$ Kupffer cells (day 6) were purified and stimulated with LPS for 24 h in RPMI-1640. Supernatants were collected for IFN-γ, IL-12 and TGF-β1 detection by ELISA. Means±SE (n=4; five mice per group). *, P>0.05.

CD69$^+$ Kupffer cells. The supernatants from purified hepatic CD69$^+$ or CD69$^-$ Kupffer cells stimulated with LPS for 24 h were collected, and IFN-γ, IL-12 or TGF-β1 concentrations were determined by ELISA. As expected, CD69$^+$ and CD69$^-$ Kupffer cells produced relatively high levels of IFN-γ and IL-12 after stimulation, and secreted low level of TGF-β1, while there was no significant difference between these two subsets (Figure 3). These data show that CD69$^+$ Kupffer cells might exhibit their functions through the other untraditional manner besides being potent cytokine-producers.

CD69$^+$ Kupffer cells inhibit CD4 T cell proliferation *in vitro* but not interfere with activation of proliferating CD4 T cells

As studied above, CD69$^+$ Kupffer cells occupy the maximal proportion in hepatic Kupffer cells on day 4–6 after *L. monocytogenes* infection. This is the mid- and late stage of infection when the *L. monocytogenes* are almost eliminated. Usually, the immune response at the late stage of this acute infection model should be negatively regulated, so as to avoid immunopathological damage to selftissue. Meanwhile, these cells express high level of the potent immunosuppressive molecule mTGF-β1, so we hypothesize that CD69$^+$ Kupffer cells may play immunosuppressive functions through mTGF-β1. To testify this hypothesis, we detected whether these cells could inhibit CD4 T cell response *in vitro*. Splenic CD4 T cells, isolated from normal mice, proliferated after cultured for five days in the presence of anti-CD3 and anti-CD28 mAbs. Obviously, when cocultured with hepatic CD69$^+$ Kupffer cells purified from *L. monocytogenes*-infected mice on day 6, the proliferation of CD4 T cells was suppressed, while the proliferation was not affected when cocultured with CD69$^-$ Kupffer cells (Figure 4A). So, we proved that hepatic CD69$^+$ Kupffer cells, but not CD69$^-$ Kupffer cells, can inhibit Ag-nonspecific CD4 T cell proliferation.

We also detected whether CD69$^+$ Kupffer cells could suppress Ag-specific CD4 T cell proliferation. Splenic OVA-specific CD4 T cells massively proliferated after cocultured with mDCs for five days in the presence of $OVA_{323–339}$ (Figure 4B). Accordingly, when cocultured with hepatic purified CD69$^+$ Kupffer cells, the proliferation of CD4 T cells was significantly suppressed, while the proliferation was not affected when cocultured with CD69$^-$ Kupffer cells (Figure 4B), suggesting that CD69$^+$ Kupffer cells also can suppress Ag-specific CD4 T cell proliferation.

Then, we analyzed whether CD69$^+$ Kupffer cells could affect activation of CD4 T cells by intracellular staining and

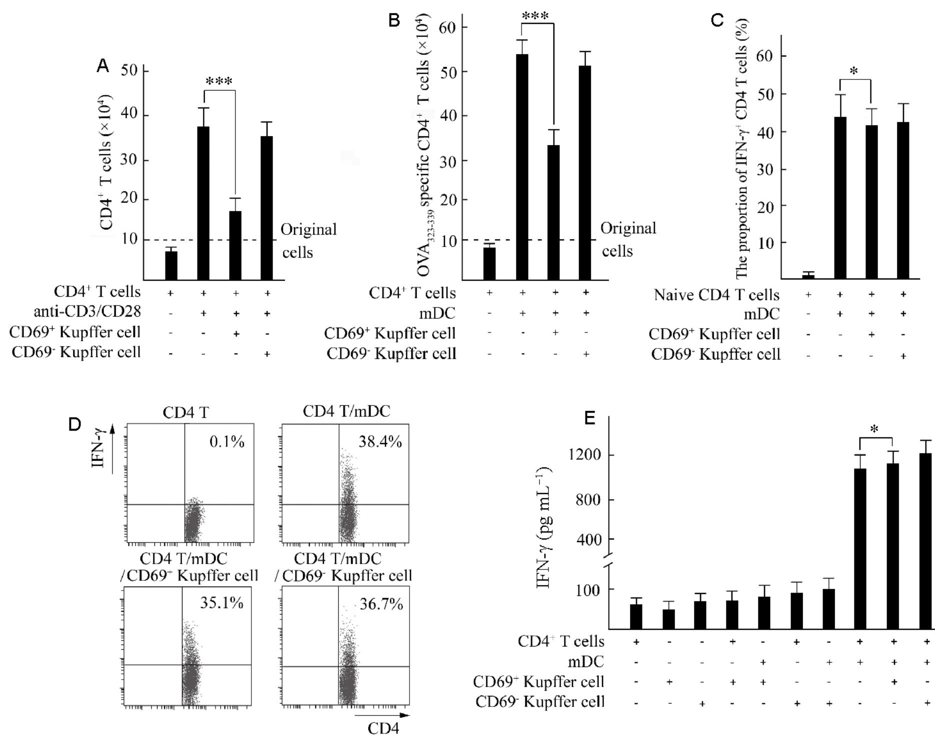

Figure 4 CD69+ Kupffer cells inhibit CD4 T cell proliferation *in vitro*. A, The suppressive effect of CD69+ Kupffer cells on Ag-nonspecific CD4 T cell proliferation. Purified CD4 T cells from normal C57BL/6 mice were cultured for five days in the presence of anti-CD3 mAb and anti-CD28 mAb, together with or without purified CD69+ or CD69− Kupffer cells. The total number of live CD4 T cells in each well was measured by flow cytometry. B, The suppressive effect of CD69+ Kupffer cells on OVA-specific CD4 T cell proliferation. Purified CD4 T cells from normal (DO11.10×C57BL/6) F_1 hybrid mice were cocultured with mature dendritic cells (mDCs) for five days in the presence of $OVA_{323–339}$, together with or without purified CD69+ or CD69− Kupffer cells. The total number of live KJ1-26+CD4+ T cells in each well was measured by flow cytometry. C−E, CD69+ Kupffer cells don't affect the activation of CD4 T cells. After proliferation for five days described above in B, the intracellular IFN-γ expression of CD4 T cells was detected by flow cytometry. The proportion of IFN-γ+ in total CD4 T cells (C) and the representable dot plots (D) are shown. Supernatants were collected for IFN-γ detection by enzyme linked immunosorbent assay (ELISA) (E). Means±SE (*n*=4; five mice per group). *, $P>0.05$; ***, $P<0.01$.

ELISA assay of IFN-γ expression. As shown in Figure 4C and D, adding hepatic purified CD69+ Kupffer cells into the coculture system didn't affect the proportion of IFN-γ+ CD4 T cells induced by $OVA_{323–339}$-pulsed mDCs, which was further supported by ELISA (Figure 4E). Therefore, the data show that hepatic CD69+ Kupffer cells could suppress Ag-nonspecific and OVA-specific CD4 T cell proliferation, but did not interfere with activation of proliferating CD4 T cells.

CD69+ Kupffer cells inhibit CD4 T cell proliferation *in vivo*

To confirm the inhibition function of hepatic CD69+ Kupffer cells on CD4 T cell proliferation *in vivo*, we adoptively transferred $OVA_{323–339}$-loaded mDCs, together with or without hepatic purified CD69+ Kupffer cells or CD69− Kupffer cells, into the mice pre-injected with OVA-specific CD4 T cells. Consistent with the previous results *in vitro*, the transferred hepatic CD69+ Kupffer cells distinctly reduced the frequency of KJ1-26+CD4+ T cells in the liver, while hepatic CD69− Kupffer cells could not (Figure 5A and B), suggesting that CD69+ Kupffer cells inhibit CD4 T cell proliferation *in vivo*.

CD69+ Kupffer cells suppress T cell proliferation via membrane-bound TGF-β1

Finally, we analyzed the underlying mechanisms for the inhibition function of hepatic CD69+ Kupffer cells on CD4 T cells. To determine whether soluble factor produced by CD69+ Kupffer cells or cell-cell contact is responsible for the suppressive effect, we incubated CD69+ Kupffer cells and OVA peptide/mDCs/T cells in the transwell system (0.4 μm), or incubated the fixed CD69+ Kupffer cells with OVA peptide/mDCs/T cells. As shown in Figure 6, CD69+ Kupffer cells lost their suppressive ability in the transwell system, while the fixed CD69+ Kupffer cells still played the suppressive effect, demonstrating that the inhibition of T cell proliferation depends on cell-cell contact, but not via soluble molecules.

Considering that CD69+ Kupffer cells expressed high level of mTGF-β1, we detected whether mTGF-β1 mediated the suppressive ability of CD69+ Kupffer cells. Using neutralizing anti-TGF-β1 mAb, we found that neutralization of TGF-β1 in the coculture system restored the OVA-specific CD4 T cell proliferation in the presence of hepatic CD69+ Kupffer cells (Figure 6). Consequently, the above data

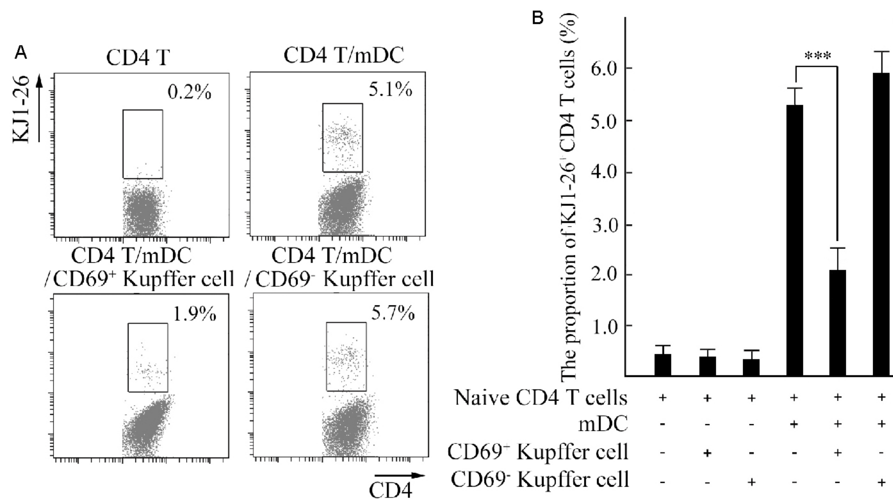

Figure 5 CD69$^+$ Kupffer cells inhibit CD4 T cell proliferation *in vivo*. A and B, $OVA_{323–339}$-pulsed mDCs and purified CD4 T cells from normal (DO11.10×C57BL/6) F_1 hybrid mice were transferred, together with or without purified CD69$^+$ or CD69$^-$ Kupffer cells, into recipient mice. After five days, the hepatic MNCs were stained with CD4-FITC, KJ1-26-PE and 7AAD for analysis by flow cytometry. The representable dot plots (A) and the proportions of KJ1-26$^+$ in total CD4 T cells from liver (B) are shown. Means±SE (n=4; eight mice per group). ***, P<0.01.

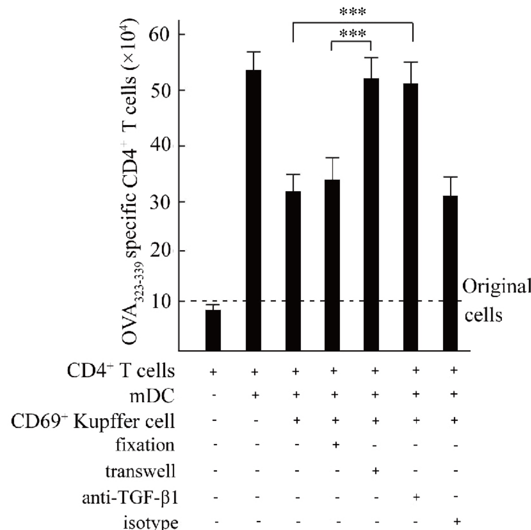

Figure 6 CD69$^+$ Kupffer cells inhibit CD4 and CD8 T cell proliferation through mTGF-β1. Purified CD4 T cells from normal (DO11.10×C57BL/6) F_1 hybrid mice were cocultured with mDCs for five days in the presence of $OVA_{323–339}$, together with or without purified CD69$^+$ Kupffer cells. The total number of live KJ1-26$^+$CD4$^+$ T cells in each well was measured by flow cytometry. In some experiments, 0.4-μm Transwells were used to separate mDCs/T cells from CD69$^+$ Kupffer cells in the coculture system (transwell), or neutralizing mAb against mouse TGF-β1 was added to the coculture system. Fixation, coculture system of mDCs/T cells with CD69$^+$ Kupffer cells which were fixed before being added into the coculture system; and isotype, isotype control for anti-TGF-β1 mAb. Means±SE (n=4; five mice per group). ***, P<0.01.

demonstrate that hepatic CD69$^+$ Kupffer cells inhibit T cell proliferation via their expression of mTGF-β1.

DISCUSSION

It is well known that Kupffer cells have been demonstrated to play a critical role in innate immunity against bacteria, parasites, yeasts, and viruses, because they can produce large kinds of cytokines rapidly after infection with microbial pathogens. Kupffer cells not only act as innate effector cells but also as regulator of innate and adaptive immunity during the microbial infection by secreting immunosuppressive cytokines. Kupffer cells are constantly exposed to pathogen derived products from the gut. To prevent excessive inflammation and pathology of the liver, continuous activation of Kupffer cells is avoided as these cells become refractory to subsequent endotoxin challenge, a phenomenon known as endotoxin-tolerance (Biswas and Lopez-Collazo, 2009; Thomson and Knolle, 2010). This contributes to the well-described tolerogenic microenvironment in the liver. Besides modulation of TLR-signaling pathways, also expression of anti-inflammatory mediators, such as IL-10 and TGF-β, and other soluble and membrane-bound inhibitory molecules are underlying the intrahepatic tolerance (Thomson and Knolle, 2010; Roth et al., 1998; You et al., 2008; Mengshol et al., 2010).

In the present study, we analyzed the variation of phenotypes and functions of Kupffer cells in the liver of the mice infected with *L. monocytogenes*, a model organism that is widely used to dissect the mechanisms of innate and adaptive immune response to infection (Williams et al., 2012). Among the various phenotype markers, we found the expression of CD69 may be used to study the subpopulations and function of Kupffer cell. After infection of *L. monocytogenes*, the CD69 expression of hepatic Kupffer cells dramatically upregulated, with peak expression on day 4−6 of infection, and then suddenly declined to the normal level along with diminishment of bacteria. To further analyze the relationship between CD69 expression and functions of Kupffer cells, we classified two subsets of Kupffer cells, including CD69$^+$ Kupffer

cells and CD69⁻ Kupffer cells. We found that CD69⁺ Kupffer cells expressed relatively higher levels of mTGF-β1, and produced large amounts of IFN-γ and IL-12, and low level of TGF-β1 simultaneously. The most obvious difference between CD69⁺ and CD69⁻ Kupffer cells is the expression of mTGF-β1, a potent immunosuppressive molecule. Together with the functional data that hepatic CD69⁺ Kupffer cells, but not CD69⁻ Kupffer cells, suppressed CD4 T cell proliferation *in vitro* and *in vivo* through mTGF-β1, we identified CD69⁺mTGF-β1⁺ Kupffer cells subset as regulatory Kupffer cells, induced by inflammation.

Previous studies have reported that virus components affect the production of immunoregulatory cytokines, and consequently promote the tolerogenic microenvironment of the liver. On this point, it has been demonstrated that hepatitis B virus (HBV) particles preferably induced TGF-β production by rat Kupffer cells, but not pro-inflammatory cytokines. It is an important role of TGF-β played in maintaining tolerance towards self-antigens that selectively support the differentiation of FoxP3⁺ regulatory T cells (Oertelt et al., 2006; Gandhi et al., 2007). Increased intrahepatic IL-10 levels may inhibit pro-inflammatory cytokine production by intrahepatic cells, blockage of Kupffer cells-NK cell interaction (Lassen et al., 2010) and antigen presentation to T cells and their activation (Lassen et al., 2010). Kupffer cells express membrane-bound inhibitory ligands that could facilitate a tolerogenic milieu in the liver. Such as, under steady state conditions, Kupffer cells are reported to express PD-L1, which is a ligand for PD-1 and known to suppress T cell function by inhibiting proliferation and cell division (Iwai et al., 2003). Now, our identification of regulatory CD69⁺ Kupffer cells provides new evidence for the presence of particular Kupffer cells subset responsible for the regulatory properties of Kupffer cells previously reported. Unexpectedly, we proved here that CD69⁺ Kupffer cells regulatory Kupffer cells inhibited T cell response through mTGF-β1, but not soluble factors, suggesting that Kupffer cells also could take advantage of cell surface TGF-β1 to show their various functions other than various cytokines they produced. Therefore, our data not only enrich the family of regulatory Kupffer cells, but also help us have better understanding of the immune regulation function of Kupffer cells.

In our work, the CD69 expression on hepatic Kupffer cells was remarkably up-regulated during process of infection. In the late stage of infection, 80%–90% hepatic Kupffer cells were CD69 positive, and CD69⁺ Kupffer cells not only were activated cells induced by inflammation but also could inhibit CD4 T cell proliferation through mTGF-β1 in a cell contact-dependent manner. CD69 belongs to the C-lectin type superfamily, which appears to be the earliest inducible cell surface glycoprotein induced during lymphoid activation, is involved in lymphocyte proliferation and functions as a signal-transmitting receptor in lymphocytes, including natural killer (NK) cells, and platelets. Previous studies have also shown that CD69, an activating marker of T cells, is persistently expressed in chronic inflammation. Engagement of CD69 can activate NK and T cells, resulting in the up-regulated cytotoxic activity and pro-inflammatory cytokines secretion (Sancho et al., 2005).Owing to the absence of a known ligand, the biological function and underlying mechanisms of CD69 have not been extensively studied. In recent years, CD69 has been reported as the negative regulating factor for the T cell response because CD69-deficient mice develop more severe T cell-mediated autoimmune diseases, meanwhile, CD69 deficiency could promote the antitumor response (Radulovic and Niess, 2015; Wieland and Shipkova, 2016). Furthermore, CD69 engagement can not only induce apoptosis in monocytes and eosinophils, and also suppress IL-1 receptor- or CD3-mediated T cell proliferation (Ramírez et al., 1996; Walsh et al., 1996; Cosulich et al., 1987). The subset of CD4⁺CD69⁺ T cells, detected in peripheral lymphoid tissues of a murine lupus model, is demonstrated being anergic, with impaired ability to produce pro-inflammatory cytokines. CXCR4/CXCL12 play critical role in the promotion of tumor growth and metastasis. It is shown that the proportion of CD4⁺CD69⁺CXCR4⁺ T cells increases in the tumor, by analyzing the CXCR4 expression on the tumor-infiltrating immune cells isolated from lung adenocarcinoma. 30% of these CD4⁺CD69⁺CXCR4⁺ T cells express high levels of CD25 and Foxp3. However, there was no functional study of these T cells as inhibitor of T cell response and the biological significance and underlying mechanisms of CD69 for the function of these T cells was not studied intensively. Consistent with the above studies, CD69 expressed on Kupffer cells during *L. monocytogenes* infection not only plays as an activation marker, but also triggers inhibitory signal in CD4 T cell proliferation. And the underlying mechanisms for how pathogen infection induces generation and expansion of CD69⁺ Kupffer cells and how mTGF-β1 is induced on the surface of CD69⁺ Kupffer cells remain to be elucidated in the future.

Liver is not only a lymphoid organ, but also an immune tolerogenic organ. The liver tolerance may mediate local and systemic tolerance to self and foreign antigens. There are lots of specialized hepatic infiltrated cells attributed to immune tolerance, including DCs, liver sinusoidal endothelial cells, hepatic stellate cells as well as CD4⁺CD25⁺Foxp3⁺ Tregs, all of these cells express anti-inflammatory fectors and/or inhibitory cell surface ligands such as IL-10, Il-4, TGF-β1, PD-L1 and CTLA-4. However, the precise mechanisms for liver tolerance have not been totally elucidated yet. Kupffer cells are most abundant in the liver, so Kupffer cells are probably involved in the tolerance effect of liver. In this study, we found that ~90% hepatic Kupffer cells express CD69 at the mid- and late stage of infection, so we consider that regulatory CD69⁺ Kupffer cells, the activated cell subset induced

by inflammation, contribute to the tolerogenic properties of liver through expressing mTGF-β1 simultaneously. Furthermore, our results have determined that CD69+ Kupffer cells suppress CD4 T cell proliferation without influence activation of CD4 T cells, suggesting that CD69+ Kupffer cells can control the immune response to the appropriate level by limiting the number of CD4 T cells but still maintain the activation of effector CD4 T cell functions, favoring the elimination of invading bacteria.

TGF-β1 has long been considered as one of the most important immunosuppressive cytokines (Li et al., 2006). For example, TGF-β1 not only plays important roles in inhibitory mechanisms of Tregs, but also is essential to the inducement of iTreg cells and Treg maintenance in general (Vignali et al., 2008; Chen et al., 2003). In addition to soluble TGF-β1, mTGF-β1 also can mediate suppression function expressed on Tregs in a cell-cell contact-dependent manner (Nakamura et al., 2001, Green et al., 2003). It is found that human tumor-derived exosomes enhance the inhibitory functions of Tregs by mTGF-β1 (Clayton et al., 2007). What's more, in anti-tumor immunity, tumor apoptotic bodies inhibit CTL response by inducing CD8 T cell anergy and generation of regulatory T cell 1 (Tr1) response through mTGF-β1 (Xie et al., 2009). Our group previously showed that cancer-expanded myeloid-derived suppressor cells (MDSCs) induced anergy of NK cells through mTGF-β1 (Li et al., 2009), tumor-induced CD4+CD69+CD25−Foxp3− Tregs suppress CD4 T cell proliferation through mTGF-β1 (Han et al., 2009; Han et al., 2014), and apoptotic cells attenuated fulminant hepatitis by priming Kupffer cells to produce IL-10 through mTGF-β1 (Zhang et al., 2011), pathogen induced hepatic CD11b+ regulatory NKT cells inhibit CD4 and CD8 T cell response *in vitro* and *in vivo* through mTGF-β1 (Han et al., 2015). Together with this study in which inflammation-induced CD69+ Kupffer cells which can feedback inhibit CD4 T cell response via mTGF-β1, we can conclude that mTGF-β1 may be the important, shared common mechanism for mediating negative regulation of immune response by many kinds of regulatory immune cells.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai) and used at the age of 6–8 weeks. The OVA-specific TCR transgenic DO11.10 mice were purchased from The Jackson Laboratory and bred in specific pathogen-free conditions. All experimental manipulations were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Second Military Medical University, Shanghai, China.

Reagents

The Abs for flow cytometry, including Abs against F4/80 (BM8), CD68 (FA-11), CD11b (M1/70), CD69 (H1.2F3), TGF-β1 (2Ar2), TCR DO11.10 (KJ1-26), and IL-12/IL-23 p40 (C15.6), IFN-γ (XMG1.2), and activating anti-CD3 (145-2C11) and anti-CD28 (37.51) mAbs were from BD PharMingen (USA). Anti-TGF-β1 Ab (1D11) and its isotype control mouse IgG1 mAb (11711), and all ELISA kits were from R&D Systems (USA). Lipopolysaccharide (LPS), 7-AAD, clodronate liposomes and PBS liposomes, and $OVA_{323–339}$ were from Sigma-Aldrich (USA).

Bacterial infections and quantification

L. monocytogenes strain (rLM6) was kindly provided by Dr. Hao Shen (University of Pennsylvania School of Medicine, Philadelphia, PA). Bacteria were grown overnight in brain-heart infusion broth. Aliquots were thawed and bacterial titers were determined by plating serial dilutions on brain-heart infusion broth agar plates. For infection, aliquots were thawed and appropriately diluted in PBS, and 1.5×10^4 bacteria were injected in a volume of 200 μL PBS into the lateral tail veins of mice. Bacteria in the liver were measured at the different time after infection by homogenization and lysis of tissues in 5 mL of 0.05% (v/v) Triton X-100, followed by plating onto brain-heart infusion plates. Total colonies per liver were counted after incubation overnight at 37°C (Han et al., 2015).

Depletion of liver Kupffer cells by clodronate liposomes

For depletion of liver Kupffer cells, mice were i.p. injected with 200 μL of clodronate liposomes (Sigma Chemical Co., USA) 24 h before *L. monocytogenes* infection (Kinoshita et al., 2010).

MNCs from liver

Liver specimens were minced thoroughly with scissors and digested with collagenase IV for 0.5 h, and then the single-cell suspension was filtered through a 40-μm cell strainer (BD Falcon, USA). MNCs were purified by centrifugation through a Percoll gradient. Cells were collected, washed, and resuspended in 33% Percoll (Sigma-Aldrich). Then the cell suspension was gently overlaid onto 67% Percoll and centrifuged for 20 min at 750×*g*. MNCs were collected from the interface, washed twice with PBS, and re-suspended in 1640.

Flow cytometry

Before staining with fluorescent Abs, cells were incubated for 15 min with Ab against CD16/32 for blockade of Fc receptors. Fluorsecent Abs and their respective isotype controls (1 μg 10^6 $cells^{-1}$ 100 $μL^{-1}$) were then added, and cells were incubated for 30 min at 4°C. The cells were washed with PBS

containing 0.1% NaN_3 and 0.5% BSA, and resuspended in 200 μL PBS. Cell phenotype was analyzed by flow cytometry with a FACS LSRFortessa (BD Biosciences, USA), and data were analyzed with FACSDiva software.

For intracellular staining of IFN-γ or IL-12 p40, cells were stimulated with 100 ng mL^{-1} LPS for 24 h, and brefeldin A (10 μg mL^{-1}) was included for the last 6 h of incubation. Cells were collected and then fixed and permeabilized with the Cytofix/Cytoperm kit according to the manufacturer's instructions (eBioscience, USA). The cells were washed with permeabilization buffer and labeled with PE anti-IFN-γ mAb, PE anti- IL-12 p40 mAb or its isotype control. Flow cytometry was done with a FASC LSRFortessa.

Isolation and purification of hepatic CD69$^+$ Kupffer cells and CD69$^-$ Kupffer cells

Hepatic MNCs were prepared from *L. monocytogenes* infected mice (day 6). Cells were stained with anti-CD69 PE and anti-F4/80 APC, and then CD69$^+$F4/80$^+$ cells (CD69$^+$ Kupffer cells) and CD69$^-$F4/80$^+$ cells (CD69$^-$ Kupffer cells) were sorted respectively by MoFlo high-speed cell sorter (Dako, Denmark). The purity of each population was confirmed by FACS to be >97%.

Preparation of mDCs from mouse bone marrow

DCs were prepared from mouse bone marrow progenitors by culturing in 10 ng mL^{-1} recombinant mouse GM-CSF and 1 ng mL^{-1} recombinant mouse IL-4 (PeproTec, USA) as described previously. Briefly, nonadherent cells were gently removed on day 3 of culture, and the remaining adherent cells were cultured for another 4–5 days in the presence of 10 ng mL^{-1} LPS (Sigma-Aldrich). Then cells were positively isolated from floating cells with CD11c magnetic microbeads (Miltenyi Biotec, USA) and used for mDCs (Han et al., 2015).

Detection of cytokines by ELISA

For detection of IFN-γ, IL-12p40 or TGF-β1 secretion, cell supernatants were collected, and the cytokine concentrations were determined by ELISA kits (R&D).

Assay for Ag-nonspecific CD4 T cell proliferation *in vitro*

Splenic CD4 T cells from normal C57BL/6 mice were purified by magnetic-activated cell sorting (MACS) for use as responders, and then cultured for five days in the presence of anti-CD3 mAb (10 μg mL^{-1}) and anti-CD28 mAb (2 μg mL^{-1}), together with or without purified CD69$^+$ or CD69$^-$ Kupffer cells at the ratio of 10:1 (T cells/Kupffer cells) (1×10^5 T cells in 200 μL $well^{-1}$). Then cells were harvested, stained with anti-CD4-FITC and 7-AAD, resuspended in 200 μL^{-1} PBS, and cellular data were acquired for 70 s with FACS LSRFortessa (1×10^5 PE-labeled beads were added to each well as an internal control before Ab labeling). The number of CD4$^+$7AAD$^-$ cells and control bead events acquired were analyzed, and the total cells in each well were calculated according to the formula: total no.=(no. live CD4 or CD8/no. beads)$\times10^5$.

Assay for OVA-specific CD4 T cell proliferation *in vitro*

Splenic CD4 T cells from normal (DO11.10×C57BL/6) F_1 hybrid mice were purified by MACS, and then cocultured with mDCs for five days at the ratio of 10:1 (T cells/mDCs) (1×10^5 T cells in 200 μl $well^{-1}$) in the presence of $OVA_{323-339}$, together with or without purified CD69$^+$ or CD69$^-$ Kupffer cells. The ratios of mDCs and Kupffer cells were 1:1. In some experiments, 0.4-μm Transwells were used to separate Kupffer cells from T cells/mDCs in the coculture system, or Kupffer cells were fixed with 1% glutaraldehyde before adding to the coculture system. For the blocking assay, 10 μg mL^{-1} anti-TGF-β1 mAb was added to the coculture system. After five days, the cells were collected and stained with anti-CD4-FITC, KJ1-26-PE and 7-AAD, and cellular data were acquired for 70 s with FACS LSRFortessa. The total numbers of CD4$^+$KJ1-26$^+$7-AAD$^-$ cells were calculated as described above (Han et al., 2015).

Assay for OVA-specific T cell proliferation *in vivo* after adoptive transfer of CD69$^+$ Kupffer cells

OVA-specific TCR-transgenic splenic CD4 T cells (5×10^6) from normal (DO11.10×C57BL/6) F_1 hybrid mice were injected i.p. into (BALB/c×C57BL/6) F_1 hybrid mice. After 24 h, 5×10^6 $OVA_{323-339}$-pulsed mDCs, together with or without the same number of purified CD69$^+$ or CD69$^-$ Kupffer cells, were transferred i.p. into the mice. After five days, hepatic MNCs were prepared, and the cells were stained with anti-CD4-FITC, KJ1-26-PE and 7-AAD, and then analyzed by flow cytometry. The ratios of KJ1-26$^+$ cells in total CD4 T cells were calculated (Han et al., 2015).

Statistical analysis

Data were analyzed for statistical significance using Student's *t* test. Statistical significance was determined as $P<0.05$.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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