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Inhibition of Allogenic T-Cell Cytotoxicity by Hepatic Stellate Cell via $CD4^+$ $CD25^+$ $Foxp3^+$ Regulatory T Cells In Vitro

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ABSTRACT

Background. The liver is considered to be an immune-privileged organ. Several types of liver cells have been implicated in the induction of immunologic tolerance. Hepatic stellate cells (HSCs) seem to participate in hepatic fibrosis and to display immunological properties.

Materials and results. In this study, HSCs isolated from C3H mice were highly positive for GFAP (98.4%) and α-SMA (95.4%). After stimulation by interferon-γ (IFN-γ), HSCs were more active in morphology with enhanced expression of $H2\text{-}K^K$, $I\text{-}A^K$, CD80, and CD54, similar to mature myelogenic dendritic cells (MDCs). Through allogeneic stimulation, C3H HSCs induced proliferation of both $CD8^+$ and $CD4^+$ T cells in B6 mice. However, the T cells activated by allogeneic HSCs produced less INF-γ, interleukin (IL)-4, IL-10, and IL-17, but large amount of transforming growth factor-β. These T cells expressed immunoregulatory rather than effector functions. Naïve T cells stimulated by allogeneic HSCs expressed Foxp3 compared with MDCs (8.67% vs 2.14%, $P < .05$). $CD8^+$ T cells activated by HSCs lost their allogeneic cytotoxicity, and $CD4^+$ T cells activated in this fashion suppressed the allogeneic cytotoxicity of $CD8^+$ T cells activated by MDCs.

Conclusion. HSCs seem to act as liver-resident antigen-presenting cells instructing the generation of $Foxp3^+$ regulatory T cells, a property suggestion of induction of immunologic tolerance.

THE LIVER is considered to be an immune-privileged organ with a special status in transplantation tolerance.[1] Recipients of human liver allografts require less immunosuppression than is necessary with other organs. Weaning of immunosuppression from liver transplant recipients has been reported in the literature.[2] In an experimental model of liver transplantation, allograft are accepted spontaneously across an MHC class I disparity without immunosuppression. Liver allografts can protect other cotransplanted organs such as skin or heart.[3] Clinically human liver allografts have been shown to protect other cotransplanted organs against rejection. Why is the liver special for immunotolerance? It is possible that some cells in the liver microenvironment have an immune regulatory role. Hepatic stellate cells (HSCs) are the major nonparenchymal elements, accounting for 5% to 8% of liver cells. Stellate cells are responsible for vitamin A metabolism and are involved in hepatic fibrogenesis and cirrhosis. The stellate cells also have special functions in immunity. In this study, we demonstrated inhibitory effects of HSCs on immune responses through their interactions with T cells. Murine HSCs activated by interferon (IFN)-γ displayed the

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antigen presenting cell (APC) functions and instructed $CD4^+CD25^+Foxp3^+$ regulatory T cells to suppress the allogeneic cytotoxicity of effector T cells.

MATERIALS AND METHODS

Male C3H ($H\text{-}2^k$, $I\text{-}A^k$) mice of 10 to 12 weeks old were used as donors and C57BL/6J (B6; $H\text{-}2^b$, $I\text{-}A^b$) mice as recipients. C3H HSCs were obtained as primary cultures from liver by two-step collagenase digestion followed by Percoll density gradient centrifugation.[3] Bone marrow-derived dendritic cells (MDC) from the same mice were used for comparisons. Enriched T cells were obtained from splenocytes by passage through nylon wool columns. Flow cytometry were employed to analyze the surface molecules and intracellular cytokine expression on DC or T cells after staining with flourescence-conjugated monoclonal antibodies. ELSA was used to measure cytokines released into the culture medium. Cell-mediated cytotoxicity was performed by the lactate dehydrogenase (LDH) assay (Roche). B6 T cells activated by C3H MDC or IFN-γ stimulated HSC through MLR for 5 days were applied as effectors. Using the $CD8^+$ or $CD4^+$ T cell isolation kit (Miltenyi Biotect, MACS), we isolated untouched $CD8^+$ or $CD4^+$ T cells separately as effectors or inhibitors. EL4 ($H\text{-}2^b$, syngeneic), R1.1 ($H\text{-}2^k$, allogeneic), and P815 ($H\text{-}2^d$, third party) cells were applied as targets at various effector: target cell ratios.

RESULTS

Primary cultured HSCs were highly positive for GFAP (98.4%) and α-SMA (95.4%). HSCs were cultured in uncoated plastic plates for 2 (quiescent) or 7 (activated) days. Further stimulation of the activated HSCs was achieved by adding IFN-γ (200 U/mL) for 3 days, yielding large cells with many cell processes in a star-shape phenotype (Fig 1). We compared them with CD-11 c-positive MDC, which are powerful APC able to induce effective immunity. HSCs cultured for 7 days expressed low levels of MHC class I and II, CD80, PDL-1 and the adhesion molecule CD54. However, further stimulation with IFN-γ for 3 days enhanced the expression of surface markers for APC. Similar to MDC, these IFN-γ stimulated HSCs activated not only $CD8^+$ but also $CD4^+$ T-cell proliferation via allogeneic stimulation in mixed lymphocyte reactions and CFSE proliferation assays.

To assay the properties of T cells activated by MDCs versus IFN-γ stimulated HSCs, we performed intracellular cytokine staining and measurements of releasing cytokines by ELISA (Fig 2). The B6 T cells activated by C3H MDC showed a higher percentage of IFN-γ secreting $CD8^+$ and $CD4^+$ T cells. High levels of INF-γ were measured in culture medium (299.82 ± 12.02 ρg/mL), suggesting effector functions and Th1 differentiation of B6 T cells after activated by C3H MDC. However, T cells activated by IFN-γ-stimulated HSCs produced less INF-γ, interleukin (IL)-4, IL-10, and IL-17 but greater amounts of transforming growth factor (TGF)-β (8.90 ± 0.29 ng/mL), indicating a loss of effector function with Th2 differentiation of T cells activated by IFN-γ stimulated HSCs versus T cells activated by MDC. High levels of TGF-β secretion by T cells suggest immunoregulatory functions of T cells activated by IFN-γ-stimulated HSCs. Naïve T cells expressed a higher percentage of $Foxp3^+$ expression after stimulation by allogeneic HSCs, compared with those stimulated by MDCs (8.67% vs 2.14%, $P < .05$) upon flow cytometry analysis, as well as Foxp3 mRNA expression by qPCR. This result implied that IFN-γ-stimulated HSCs may elicit Foxp3 regulatory T-cell expansion.

The allogeneic cytotoxicity of B6 $CD8^+$ T cells activated by C3H MDC (Fig 3A) was much greater compared with $CD8^+$ T cells activated by IFN-γ-stimulated C3H HSCs (Fig 3B) or naïve $CD8^+$ T cells (Fig 3C). $CD8^+$ T cells activated by IFN-γ-stimulated C3H HSCs lost their effector function and could not initiate cell-mediated cytotoxicity compared with $CD8^+$ T cells activated by allogeneic MDCs. Allogeneic cytotoxicity of B6 $CD8^+$ T cells activated by C3H MDC was enhanced when adding with B6 $CD4^+$ T cells activated by C3H MDC compared with naïve B6 $CD4^+$ T cells. However, the allogeneic cytotoxicity was dramatically reversed when we added B6 $CD4^+$ T cells activated by IFN-γ-stimulated C3H HSCs. These data implied that $CD4^+$ T cells activated by IFN-γ-stimulated HSCs suppressed cell-mediated cytotoxicity of $CD8^+$ T cells activated by allogeneic mature DCs.

DISCUSSION

Long-term acceptance of solid organ grafts requires either continuous immunosuppression or the acquisition of peripheral regulatory mechanisms. Regulatory T cells

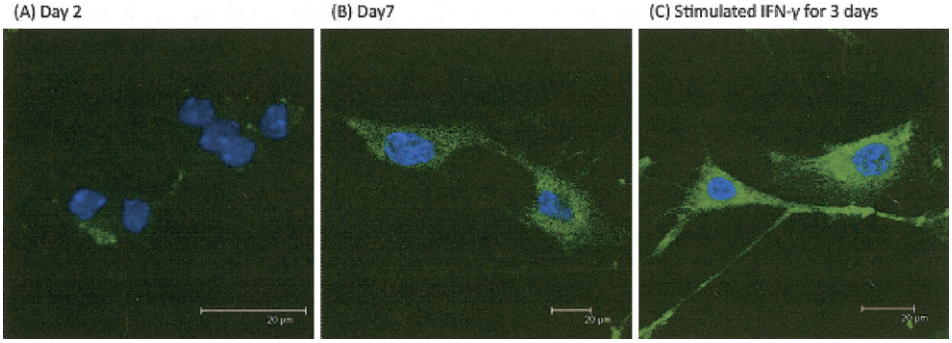

Fig 1. The morphology of hepatic stellate cell (HSC). HSCs were stained with anti-GFAP and analyzed by confocal microscopy. HSCs were cultured in uncoated plastic plates for (**A**) 2 days (quiescent), (**B**) 7 days (activated), and (**C**) further stimulated by interferon-γ for 3 days.