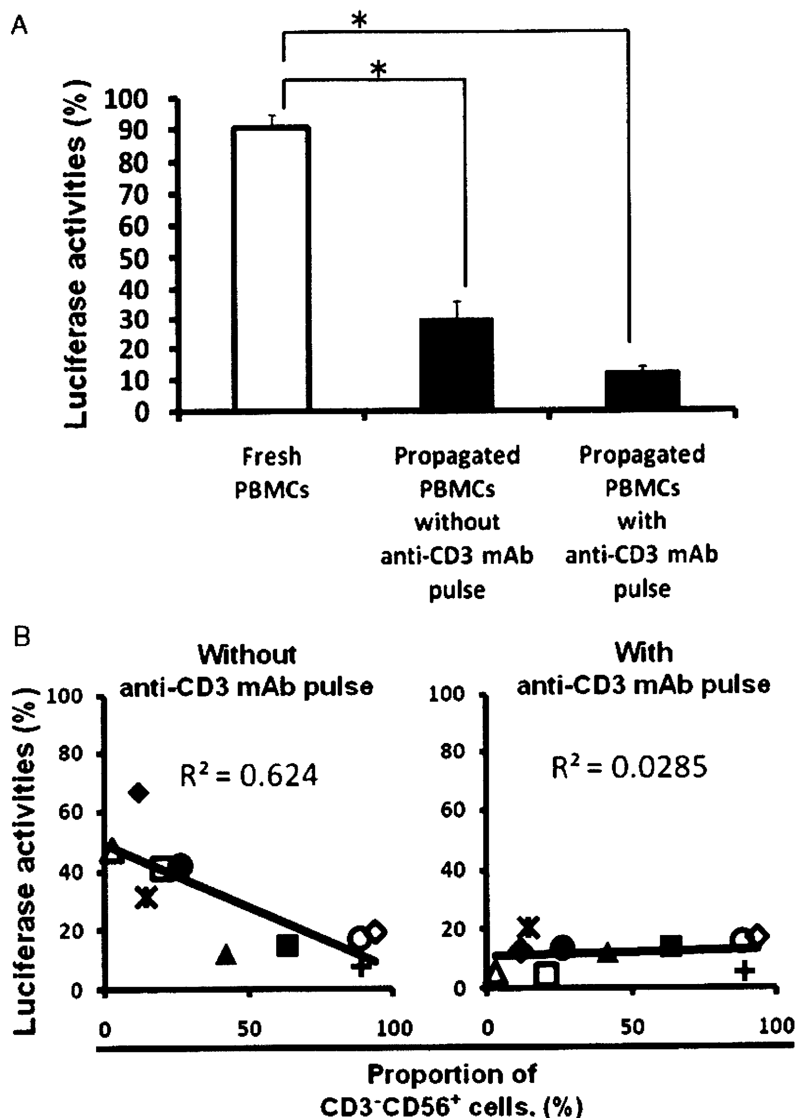

FIGURE 6. Propagated PBMCs exhibit marked anti-HCV activity. A, Propagated PBMCs cultured for 28 days in the presence of interleukin-2 and anti-CD3 mAb were incubated with HCV replicon-containing hepatic cells for 48 hours in transwell tissue culture plates (effector:target ratio = 10:1). One day before coculturing PBMCs with HCV replicon-containing cells, anti-CD3 mAb pulse (1 μg/mL) was added or not added to the culture of propagated PBMCs. Bar graphs indicate luciferase activity of HCV replicon-containing cells in the presence of effectors normalized to luciferase activity in the absence of effectors (% of luciferase activity; 5 individuals were used for freshly isolated PBMCs, and 10 individuals for propagated PBMCs). The difference in anti-HCV effect between the propagated PBMCs and the freshly isolated PBMCs was statistically significant (*$P<0.05$). B, Propagated PBMCs containing more $CD3^-CD56^+$ cells showed lower luciferase reporter activity without the anti-CD3 mAb pulse. All of the propagated PBMCs strongly inhibited luciferase reporter activity, regardless of the $CD3^-CD56^+/CD3^+CD56^+$ cell ratio after the anti-CD3 mAb pulse. Data were obtained from 10 volunteers. Each symbol indicates data from 1 individual. HCV indicates hepatitis C virus; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells.

induces significant cytotoxicity of LAK cells including the $CD56^+$ subsets against HCC (Figs. 3, 4). The contribution of TRAIL molecules to the cytotoxicity of NK cells against HCC was proved by the effect of a neutralizing anti-TRAIL mAb (Fig. 5).

In addition to anti-neoplastic effects, adoptive immunotherapy with LAK cells may lead to viral clearance. In fact, a reduction in hepatitis B virus (HBV) load has been described in patients undergoing treatment with LAK cells.[28] LAK cells might suppress HBV replication through the secretion of IFN-γ and tumor necrosis factor-α. Despite such an attractive approach, this therapy has never been applied to suppress HCV replication. In general, in the early phase of viral infection, the first line of host defense may be effective in removing the virus; however, recent reports have indicated that HCV effectively escapes the innate immune system comprising NK and natural killer T cells, resulting in persistent infection.[29,30] It has been also reported that cross-linking of CD81 on NK cells by the major envelope protein of HCV, HCV-E2, blocks NK cell activation, IFN-γ production, cytotoxic granule release, and proliferation.[29] Engagement of CD81 on NK cells

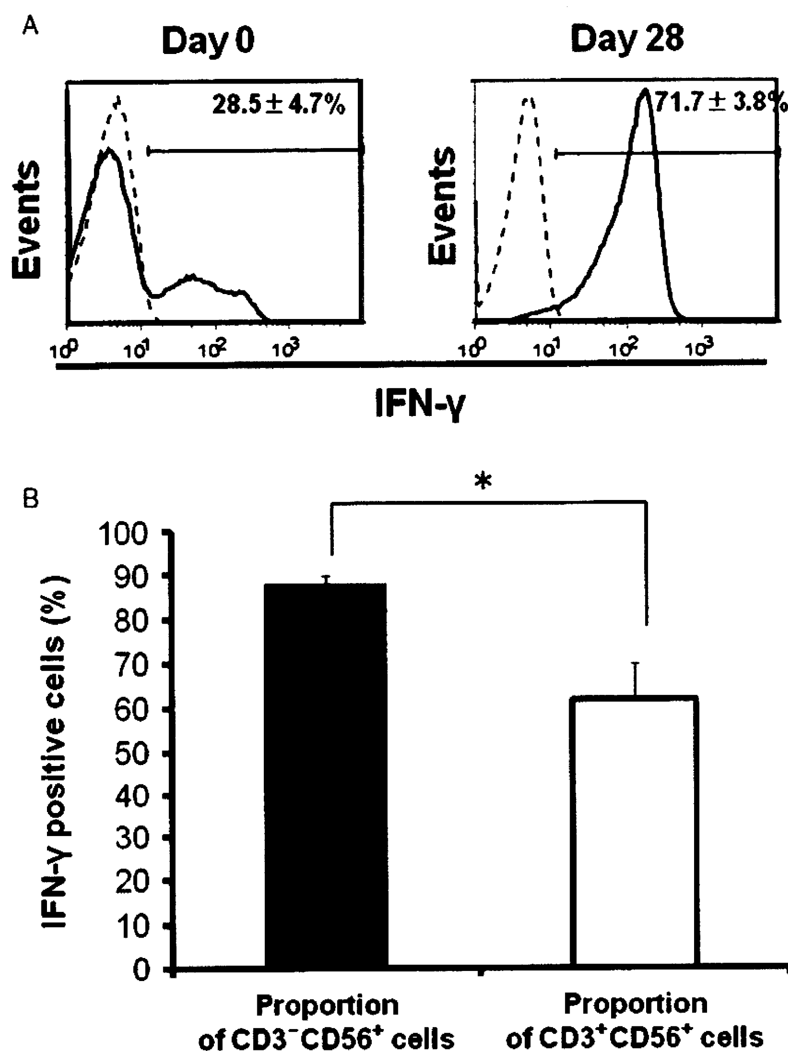

FIGURE 7. A, IFN-γ production by propagated PBMCs was evaluated on days 0 and 28 by a combination of cell-surface and cytoplasmic mAb staining and subsequent flow cytometry analysis. Histograms represent log fluorescence intensities obtained by staining whole propagated PBMCs for IFN-γ. Dotted lines represent negative control staining with isotype-matched mAb. Numbers indicate the percentage of cells positive for IFN-γ expression (mean ± SEM, n = 5 individuals). B, IFN-γ expression is higher in $CD3^-CD56^+$ cells than in $CD3^+CD56^+$ cells propagated for 28 days in the presence of interleukin-2 and anti-CD3 mAb (*$P<0.05$). The data were obtained after gating by staining $CD3^-CD56^+$ and $CD3^+CD56^+$ cells for IFN-γ after 28 days of culture. Data represent mean ± SEM (n = 5 individuals). IFN indicates Interferon; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells.

blocks tyrosine phosphorylation through a mechanism that is distinct from the negative signaling pathways associated with NK cell inhibitory receptors for major histocompatibility complex class I molecules.[30] These findings prove that HCV-E2-mediated inhibition of NK cells is an efficient HCV evasion strategy, which involves targeting the early antiviral activities of NK cells and allowing the virus to establish itself as a chronic infection. We earlier explored whether CD81 cross-linking-induced inhibitory effects occur even in IL-2-stimulated NK cells. CD81 cross-linking by a mAb specific for CD81 inhibited antitumor cytotoxicity and anti-HCV activity mediated by resting NK cells, but this manipulation did not alter both these activities of IL-2-stimulated NK cells.[9] This indicated that exposure to IL-2 before CD81 cross-linking abrogates subsequent inhibitory signals in NK cells and encourages us to study the possibility of adoptive immunotherapy with LAK cells to inhibit HCV replication. We have recently shown that $CD3^-CD56^+$ and $CD3^+CD56^+$ cells derived from liver resident lymphocytes show anti-HCV activity after short-term culture with IL-2 and anti-CD3 mAb through the secretion of IFN-γ.[9] Similarly, long-term cultivation in the presence of IL-2 and anti-CD3 mAb promotes the inhibitory effects of LAK cells from PBMCs on HCV replication. Although the role of NK cells in controlling HCV infection and replication has not been completely elucidated, a recent report has indicated that NK cells are not directly cytolytic towards HCV replicon-containing hepatic cells, but rather release IFN-γ, suppressing HCV RNA expression.[31] The role of IFN-γ in the expression of NK cell-mediated anti-HCV activity has been supported by the observation that NK cell-conditioned media have higher levels of signal transducer and activator of transcription 1, a nuclear factor essential in IFN-γ-mediated antiviral pathways. It has also been reported that

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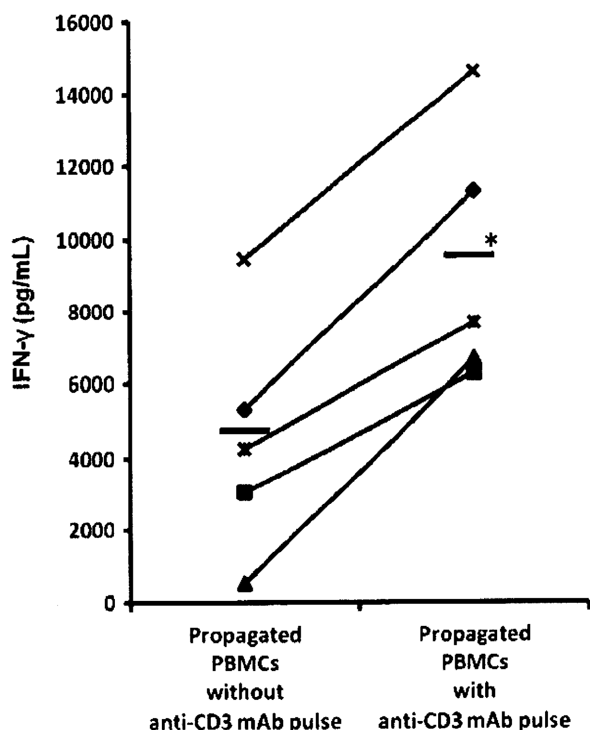

FIGURE 8. IFN-γ concentrations in culture supernatant increased with the addition of an anti-CD3 mAb pulse. PBMCs were propagated for 28 days in X-VIVO 15 medium supplemented with 5% autoserum in the presence of interleukin-2 and anti-CD3 mAb. One day before harvest, anti-CD3 mAb pulse (1 μg/mL) was added to the culture. IFN-γ was detected by enzyme-linked immunosorbent assay. Each symbol indicates IFN-γ culture supernatant concentration from each volunteer (n = 5 individuals). Horizontal line indicates the mean. IFN-γ expression was significantly different between the supernatants of nonstimulated and stimulated cells (*$P<0.05$). IFN indicates Interferon; mAb, monoclonal antibody.

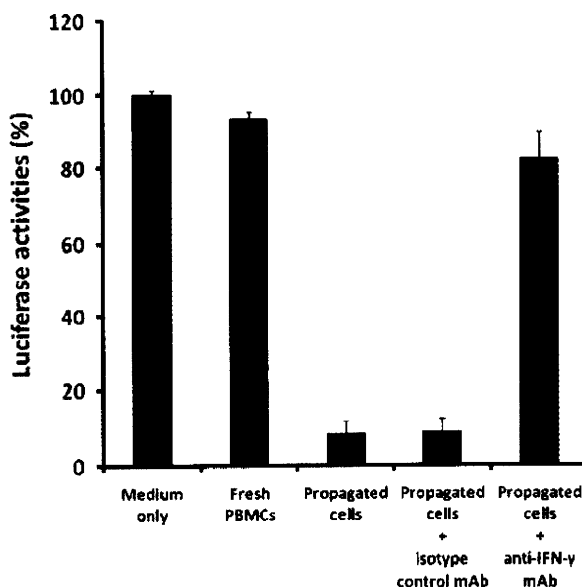

FIGURE 9. Blocking of IFN-γ with mAb in coculture with HCV replicon-containing hepatic cells abrogated the anti-HCV activity of the propagated PBMCs. HCV replicon-containing hepatic cells incubated with the control medium alone served as control. When HCV replicon-containing hepatic cells were incubated with freshly isolated PBMCs in transwell tissue culture plates (effector: target ratio = 10:1), the luciferase activities (indicating HCV replication) were not altered. However, when HCV replicon-containing hepatic cells were incubated with the PBMCs propagated with interleukin-2 and anti-CD3 mAb and additional anti-CD3 mAb pulse stimulation, the luciferase activities were significantly reduced. The propagated PBMCs were used as effector cells in assays of cytotoxicity against HCV replicon-containing hepatic cells in the presence or absence of anti-IFN-γ mAb (100 μg/mL) and isotype-matched control mAb (100 μg/mL). Blocking of IFN-γ with mAb elucidated the significant role played by IFN-γ in producing the anti-HCV effect. The bar graphs indicate the rate of luciferase activity of HCV replicon cells in the presence of effectors to that in the absence of effectors (% of luciferase activity) in each group. The data represent the mean ± SEM of 3 similar experiments. HCV indicates hepatitis C virus; IFN, Interferon; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells.

hepatocytes cultured in NK cell-conditioned media express higher levels of IFN-α/β, IFN regulatory factor 3, and IFN regulatory factor 7, confirming that NK cells suppress HCV infection of and replication in human hepatocytes via IFN.[32] Similar to recent studies, this study showed that propagated PBMCs consisting of CD56^{+} cells vigorously produce IFN-γ, thus suppressing HCV replication (Figs. 7, 8). However, we have confirmed that IFN-α/β production by propagated CD56^{+} cells was undetectable (data not shown).

Regarding preventing replication of other viruses, earlier studies have shown that IFNs are a group of inducible cytokines with a central role in innate antiviral immune responses because they establish an intracellular antiviral state that prevents viral replication.[33] Mice lacking either IFN-γ or its functional receptor are more susceptible to both viral and bacterial infections, indicating that IFN-γ plays an important role in antiviral and antibacterial responses.[34,35] It is possible that these propagated PBMCs can prevent the replication of other viruses through an IFN-γ-dependent mechanism. Further studies are required to address this possibility.

We have observed earlier that IL-2-stimulated NK cells were negligibly cytotoxic towards allogeneic and autologous lymphoblasts with 1 shared haplotype despite being strongly cytotoxic to HCC cells.[8] Therefore, we propose immunotherapy with propagated PBMCs of liver allografts derived from healthy donors with 1 shared haplotype or from the recipient patients themselves, to mount anti-HCC and anti-HCV responses in HCV-infected LT recipients. Living donor LTs are frequently performed between parent and child who share 1 haplotype of major histocompatibility complex. In this study, we confirmed that CD56^{+} cells from healthy donors expand well, indicating that our approach is realistic. However, it remains to be elucidated whether similar cells can be obtained from recipient patients for adoptive immunotherapy.

In conclusion, CD3^{-}CD56^{+} and CD3^{+}CD56^{+} cells obtained from the PBMCs show anti-HCV activity in addition to anti-HCC activity and would be used for adoptive immunotherapy in clinical setting.

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