cytometry. As shown in Fig. 3a, $CD8^+$ T cells dominated $CD4^+$ T cells in the observation period, and donor T cells, being fewer on day 4, rapidly increased to peak on day 7, and thereafter decreased rapidly or gradually. On day 4, the number of $CD8^+$ T cells in C57BL/6 → BDF1 recipients was significantly greater than in B6C3F1 → BDF1 recipients. On day 7, the number of $CD4^+$ and $CD8^+$ T cells in B6C3F1 → BDF1 recipients was significantly greater than that in C57BL/6 → BDF1 recipients. Regarding the kinetics of host T cells recruited to spleens, although 1 or 2 orders of magnitude lower than donor T cells, the number of host $CD4^+$ and $CD8^+$ T cells rapidly increased to peak on day 7, and thereafter decreased gradually (Fig. 3b). The numbers of $CD4^+$ and $CD8^+$ T cells on day 7 were significantly greater in B6C3F1 → BDF1 recipients than those in C57BL/6 → BDF1 recipients.

We next examined the CXCR3 expression status on donor T cells in recipient spleens. CXCR3 is a Th1-associated chemokine receptor, which plays an important role in the homing of donor T cells to GVHD-target organs [21, 22]. CXCR3 expression levels on donor T cells were highest on day 4, and thereafter decreased rapidly or gradually. Compared with B6C3F1 → BDF1 recipients, C57BL/6 → BDF1 recipients showed a significantly higher median fluorescent intensity of CXCR3 expression in $CD4^+$ T cells on days 4 and 7, and in $CD8^+$ T cells on days 7 and 11 (Fig. 3c). Regarding the expression of CCR5, another Th1-associated chemokine receptor, on donor T cells, the median fluorescent intensity of donor $CD4^+$ or $CD8^+$ T cells was also significantly higher in C57BL/6 → BDF1 recipients than in B6C3F1 → BDF1 recipients in the early transplantation days (data not shown). Thus, a relatively low CXCR3 or CCR5 expression on donor T cells proliferating in recipient spleen was considered to be associated with the occurrence of less severe GVHD in B6C3F1 → BDF1 recipients.

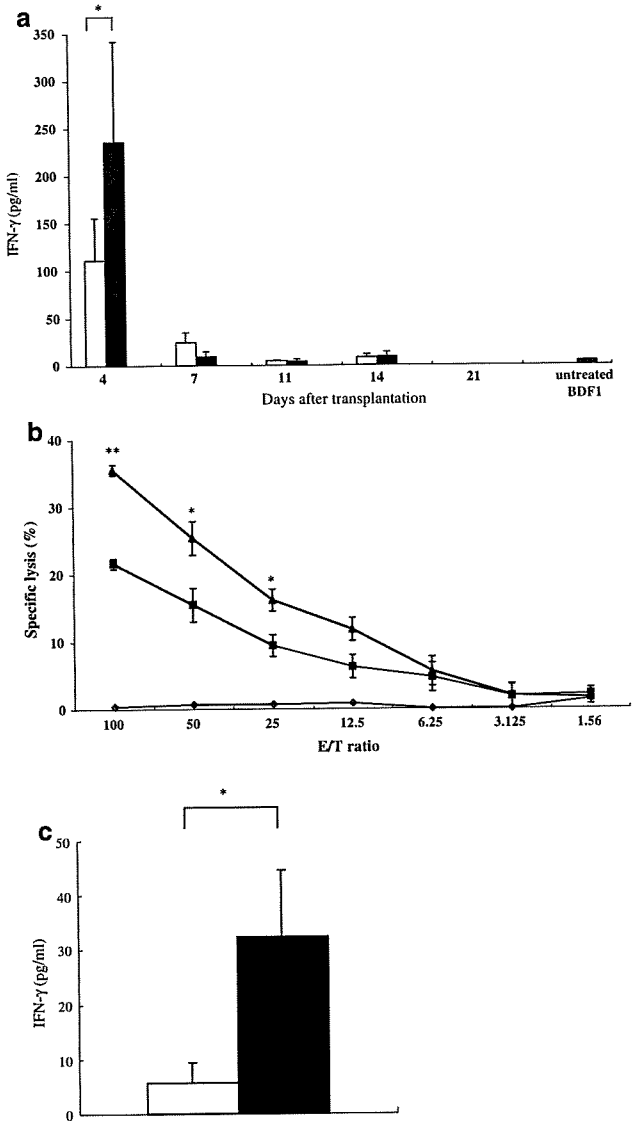
### 3.4 Stronger in vitro CTL activities and high IFN-γ expression in B6C3F1 → BDF1 recipients

To address the mechanism of stronger antileukemic activity in B6C3F1 → BDF1 recipients (Fig. 2), we next compared the production of inflammatory cytokines, TNF-α and IFN-γ, between B6C3F1 → BDF1 and C57BL/6 → BDF1 recipients. TNF-α is a well-known inflammatory cytokine to play a major role in inducing GVHD [23], while IFN-γ was recently reported to induce the separation of GVL effects from GVHD [11]. Serum TNF-α levels peaked on day 7, and thereafter decreased. There was no significant difference in serum TNF-α levels between the 2 groups (data not shown). On the other hand, serum IFN-γ levels were highest on day 4, and thereafter rapidly decreased (Fig. 4a). Serum IFN-γ levels on day 4 in B6C3F1 → BDF1 recipients were significantly higher than in C57BL/6 → BDF1 recipients.

To ensure powerful antileukemic effects in B6C3F1 → BDF1 recipients, we performed an in vitro cytotoxicity assay against P815 cells. Using, as responders, spleen cells that were recovered from the recipient mice on day 14 in the experiment in Fig. 2a, we compared B6C3F1 → BDF1 and C57BL/6 → BDF1 BMTs. Responder cells used for cytotoxicity assay of C57BL/6 → BDF1 BMT were composed of a mean of 72.6% $CD3^+$ cells (99.7% donor type; 18.0% $CD4^+$ cells and 82.0% $CD8^+$ cells) and a mean of 18.7% NK cells (88.2% donor type). Those of B6C3F1 → BDF1 BMT were composed of a mean of 71.2% $CD3^+$ cells (100% donor type; 22.3% $CD4^+$ cells and 77.7% $CD8^+$ cells) and a mean of 9.7% NK cells (55.4% donor type). There was no significant difference in the percentage of $CD4^+$ or $CD8^+$ cells between C57BL/6 → BDF1 and B6C3F1 → BDF1 spleen cells. Recipient NK cell numbers in B6C3F1 → BDF1 spleen were significantly greater (around twice) than those in C57BL/6 → BDF1 spleen. As shown in Fig. 4b, spleen cells from B6C3F1 → BDF1 recipients showed significantly stronger CTL activities than those from C57BL/6 → BDF1 recipients. In addition, spleen cells recovered from recipients on day 14 were cocultured with irradiated BDF1 spleen cells for 3 days, and we measured the IFN-γ concentration in the culture supernatant (Fig. 4c). Significantly higher IFN-γ production was observed in culture supernatants from B6C3F1 → BDF1 recipients than in those from C57BL/6 → BDF1 recipients. These results indicate that, compared with C57BL/6 → BDF1 recipients, B6C3F1 → BDF1 recipients have more powerful antileukemic effects, and also suggest that high IFN-γ production may have been involved in the induction of the powerful antileukemic effects.

### 3.5 Remarkable contribution of recipient immune cells to high IFN-γ production

To address the mechanism of high IFN-γ production in B6C3F1 → BDF1 recipients, we next intended to identify IFN-γ-secreting cells in recipient spleens. IFN-γ-secreting cells were calculated based on flow cytometry data using intracellular IFN-γ staining (Fig. 5a). In the analysis of whole mononuclear cells, on day 4, host-derived IFN-γ-secreting cells were significantly increased in B6C3F1 → BDF1 recipients than in C57BL/6 → BDF1 recipients but not donor-derived cells. On day 7, the number of IFN-γ-secreting cells in B6C3F1 → BDF1 recipients was significantly higher in both donor and host populations compared with in C57BL/6 → BDF1 recipients (Fig. 5b). In the analysis of each donor-derived lineage cell, there was no significant difference in the number




◀ **Fig. 4** Stronger in vitro CTL activity and higher IFN-γ expression in B6C3F1 → BDF1 recipients. **a** Serum IFN-γ concentration in recipient mice. B6C3F1 → BDF1 and C57BL/6 → BDF1 BMTs were performed as shown in Fig. 1. Serum IFN-γ concentration was consecutively measured by Bio-Plex (see "Materials and methods"). *Open bars* C57BL/6 → BDF1 recipients ($n = 8$), *closed bars* B6C3F1 → BDF1 recipients ($n = 8$), *gray bar* untreated BDF1 mice ($n = 3$). Serum IFN-γ levels on day 21 became undetectable in the 2 BMT groups. Data are expressed as the mean ± SD. $^*P < 0.05$. Representative data from 2 separate experiments are shown. **b** In vitro cytotoxicity assay for P815 cells was performed using spleen cells on day 14. To obtain responders, mice received TCD-BM ($5 \times 10^6$) and spleen ($2 \times 10^7$) cells with P815 cells ($1 \times 10^4$) after receiving TBI 9 Gy, as shown in Fig. 2a. Spleen cells were recovered from recipient mice on day 14, and directly checked for CTL activity against P815 cells by $^{51}$Cr release assay, as described in "Materials and methods". *Closed triangles* B6C3F1 → BDF1 recipients, *closed rectangles* C57BL/6 → BDF1 recipients, *closed diamonds* untreated BDF1 spleen cells. Values were calculated based on experiments using at least 4 samples. Data represent the mean ± SD of specific lysis percentage for P815 cells at a given E/T ratio. Representative results from 2 independent experiments are shown. **c** IFN-γ production in the culture supernatants of mixed lymphocyte culture. B6C3F1 → BDF1 and C57BL/6 → BDF1 BMTs were performed as shown in Fig. 1. Spleen cells were recovered from recipient mice on day 14, and were co-cultured with irradiated (20 Gy) BDF1 spleen cells for 3 days, and the culture supernatant was checked for IFN-γ concentration by Bio-Plex (see "Materials and methods"). *Open bar* C57BL/6 → BDF1 recipients, *closed bar* B6C3F1 → BDF1 recipients. Values were calculated based on experiments using 5 samples. Data are expressed as the mean ± SD. $^*P < 0.05$. Representative results from 2 independent experiments are shown

of IFN-γ-secreting cells between the 2 BMT recipients except for $CD8^+$ T cells on day 7 (Fig. 5c). When we calculated based on the donor T cell counts shown in Fig. 3a, the majority of $CD8^+$ T cells in the 2 BMT recipients on day 4 were IFN-γ-secreting cells, with IFN-γ-secreting cells rapidly decreasing to only 10–15% on day 7. In the analysis of each host-derived lineage cell, IFN-γ-secreting cells were significantly increased in $CD8^+$ T cells on day 7 and in NK cells on days 4 and 7 of B6C3F1 → BDF1 recipients compared with C57BL/6 → BDF1 recipients (Fig. 5d). In particular, the number of IFN-γ-secreting host NK cells on day 4 in B6C3F1 → BDF1 recipients was 4 times higher than in C57BL/6 → BDF1 recipients. These data strongly indicate that recipient immune cells, including T and NK cells, have highly contributed to high IFN-γ production in B6C3F1 → BDF1 recipients.

## 4 Discussion

In the present study, using MHC-haploidentical murine BMT models, we showed that, compared with C57BL/6 → BDF1 (homo-to-hetero-type BMT) recipients, B6C3F1 → BDF1 (hetero-to-hetero-type BMT) recipients showed stronger antileukemic effects with less severe GVHD (Figs. 1, 2). Using these models, we addressed the mechanism by which B6C3F1 → BDF1 BMT exerted stronger antileukemic effects with less severe GVHD than C57BL/6 → BDF1 BMT, because clarification of the mechanism is considered to be useful for the understandings of the separation of GVL effects from GVHD in MHC-haploidentical HSCT.

Significantly higher antileukemic activity of B6C3F1 → BDF1 recipients was also confirmed by in vitro cytotoxicity assay against P815 cells using spleen cells (Fig. 4b). We speculated that high IFN-γ production was involved in this higher antileukemic activity of B6C3F1 → BDF1 recipients. In fact, serum IFN-γ levels in the early transplant period (on day 4) were significantly higher in B6C3F1 → BDF1 recipients than in C57BL/6 → BDF1 recipients (Fig. 4a), and IFN-γ levels in the culture supernatant for MLC were significantly higher than in C57BL/6 → BDF1 recipients (Fig. 4c). We consider that this high IFN-γ production is not the effect but the cause of strong

allogeneic response. IFN-$\gamma$ has been shown to enhance Th1 polarization through the facilitation of production of IL-12 by dendritic cells (DC) [24] or through synergizing with T cell receptor signals to maximally induce T-bet [25]. Furthermore, we demonstrated that host immune cells, especially NK cells, recruited to the spleen, substantially contributed to high IFN-$\gamma$ production in B6C3F1 → BDF1 recipients (Fig. 5). The fact that host NK cells still remained in recipient spleens on day 14 and the fact that the number of the host NK cells was significantly greater in B6C3F1 → BDF1 spleens than in C57BL/6 → BDF1 spleens support that host NK cells were more involved in high IFN-$\gamma$ production in B6C3F1 → BDF1 recipients. In particular, high IFN-$\gamma$ production in spleen, a secondary lymphoid organ, is considered to be important for inducing strong antileukemic response. Although NK cells are largely excluded from lymph nodes under steady-state conditions, recruitment of NK cells to antigen-stimulated lymph nodes was recently reported to be important for providing an early source of IFN-$\gamma$ that is necessary for Th1 polarization [26]. NK cells are also shown to participate in adaptive immune responses by modulating DC function or by producing IFN-$\gamma$ [27].

Furthermore, IFN-$\gamma$ has been reported to enhance GVL effects mainly by the following 2 mechanisms: the augmentation of lymphohematopoietic GVH reactions [10–12, 28] and enhancement of the susceptibility of tumor cells to alloresponses. Although the mechanism by which IFN-$\gamma$ enhances lymphohematopoietic GVH reactions remains to be determined, this cytokine may mediate antihematopoietic activity that predominantly targets the recipient hematopoietic cells through its direct effect on hematopoietic cells [29]. IFN-$\gamma$ may enhance the sensitivity of tumor cells to cytotoxic donor T cells by upregulating surface expressions of Fas and Fas-L, or MHC on tumor cells [30–32], or its direct effect on tumor cells [33].

The analysis of chemokine receptors [21, 22] on donor T cells engrafted to recipient spleens revealed that $CD4^+$ or $CD8^+$ T cells from B6C3F1 → BDF1 recipients showed significantly lower CXCR3 expression levels, compared with those from C57BL/6 → BDF1 recipients (Fig. 3c). These donor T cells in B6C3F1 → BDF1 recipients, which also showed lower CCR5 expression (data not shown), were considered to have low ability to home to GVHD-target organs, which is a prerequisite for the induction of GVHD, despite highly expanding in the spleen. There was no significant difference in the serum levels of TNF-$\alpha$, a major inducer of GVHD [23], between the 2 groups.

Regarding GVHD-protective effects of IFN-$\gamma$, IFN-$\gamma$ inhibits T cell activation through various mechanisms, including the induction of apoptosis to alloreactive T cells [34], the inhibition of Th17 cells [35], the generation of CD4 regulatory T cells [36], and the activation of immunosuppressive mesenchymal stem cells [37, 38], and furthermore the direct interaction of IFN-$\gamma$ with recipient pulmonary parenchyma was recently reported to prevent idiopathic pneumonia syndrome in lethally irradiated mice after allogeneic HSCT [14].

In the experiment using another MHC-haploidentical BMT models, C3D2F1 ($H\text{-}2^{k/d}$) → B6C3F1 ($H\text{-}2^{b/k}$) and C3H ($H\text{-}2^{k}$) → B6C3F1, stronger antileukemic effects for EL4 cells ($H\text{-}2^{b}$) with less severe GVHD were observed in C3D2F1 → B6C3F1 recipients (hetero-to-hetero-type BMT) (data not shown). These findings suggest that separation of GVL effects from GVHD could be a generalized phenomenon observed mainly in hetero-to-hetero-type mismatched BMT; however, we need further experiments to confirm the hypothesis because the severity of GVHD is highly dependent on the strain combination in murine BMT models. Regarding the relationship between the findings in this animal study and our clinical data, we had 4 patients who underwent unmanipulated HLA-2-3 antigen-mismatched HSCT undergone in homo-to-hetero combination: 3 patients received myeloablative conditioning and the remaining 1 reduced-intensity conditioning. Two patients had no acute GVHD, 1 grade II GVHD, and the remaining early death. Among them, only 1 patient had a relapse. Homo-to-hetero combination does not seem to clinically develop severe GVHD, despite the experience of only 4 cases; however, the clinical outcome must have been influenced by intensive GVHD prophylaxis containing steroids in our HLA-haploidentical HSCT regimen [7, 8]. To obtain some clinical evidence, a large scale-study is needed, and we consider that the use of less intensified GVHD prophylaxis in our protocol may make the difference between homo-to-hetero and hetero-to-hetero combinations more evident in unmanipulated HLA-haploidentical transplant settings.

Our findings suggest that two-way in vivo MLC reaction occurs in spleens more strongly in hetero-to-hetero BMT recipients compared with homo-to-hetero BMT recipients, and that host immune cells, such as NK or T cells, produce IFN-$\gamma$ abundantly, which may be considered to have contributed to the separation of GVL from GVHD. Thus, the participation of host hematopoietic cells in the afferent phase of GVHD changes the balance between allogeneic GVH and GVL responses of donor T lymphocytes, and that appropriate utilization of host immune cells may enable greater separation of GVL from GVH reactions.

In conclusion, we showed, in MHC-haploidentical murine BMT models, that powerful antileukemic effects with less severe GVHD were associated with a high production of IFN-$\gamma$, and also data suggesting that host immune cells, including NK cells, played an important role in this IFN-$\gamma$ production.

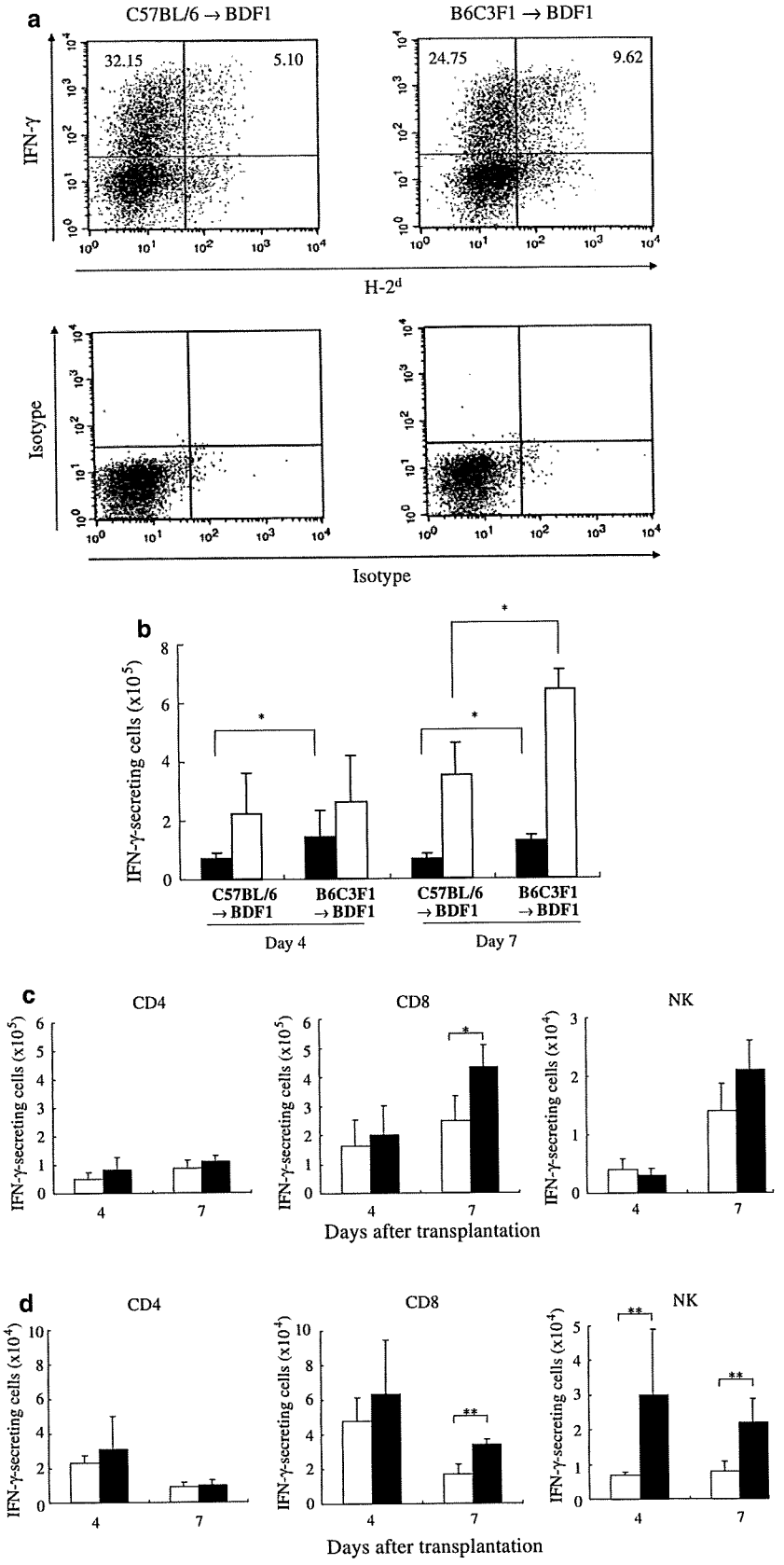



**Fig. 5** Contribution of recipient immune cells to high IFN-γ production in B6C3F1 → BDF1 recipients. Transplantation was performed as shown in Fig. 1. IFN-γ-secreting cells in recipient spleens were calculated based on flow cytometry data using intracellular IFN-γ staining. $*P < 0.05$, $**P < 0.01$. **a** Representative flow cytometry data using whole mononuclear cells recovered from spleens on day 4. The *upper panels* indicate the percentages of IFN-γ-secreting cells in the fraction of whole mononuclear cells. *Dot plots* in which isotype-matched control mAb was used are shown in the *lower panels*. A higher percentage of host-derived (H-$2^d$ positive) IFN-γ-secreting cells was observed in B6C3F1 → BDF1 recipients than in C57BL/6 → BDF1 recipients. **b** IFN-γ-secreting cells in the fraction of whole mononuclear cells retrieved from spleens on days 4 ($n = 7$) and 7 ($n = 4$). *Closed bars* host cells, *open bars* donor cells. **c** Donor-derived IFN-γ-secreting cells in each cell lineage. *Open bars* C57BL/6 → BDF1 recipients, *closed bars* B6C3F1 → BDF1 recipients. **d** Host-derived IFN-γ-secreting cells in each cell lineage. *Open bars* C57BL/6 → BDF1 recipients, *closed bars* B6C3F1 → BDF1 recipients. Regarding these IFN-γ-secreting cells, representative results from 2 independent experiments are shown

**Acknowledgments** We thank Kimiko Yamamoto, Hirotsugu Kubo and Hatsuka Seki for technical assistance and also Dr. Tsuyoshi Iwasaki for helpful comments.

**Conflict of interest statement** The authors have no financial conflicts of interest.

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