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Differential effectiveness of anti-CD8 treatment on ongoing graft-versus-host reactions in mice

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Abstract: Analysis of T cell subsets in the spleen during graft-versus-host (GVH) reactions in a fully allogeneic mouse strain combination demonstrated that first $CD4^+$ T cells become activated, and initiate the GVH reaction. Subsequently, $CD8^+$ T cells become involved. Here we show that anti-CD8 treatment on day +3 resulted in a significant increase in survival, while early treatment (day −1 or day +1) did not. Acute GVH reactions were induced (day 0) in lethally irradiated (C57BL/6 × CBA/J)F1 ($H\text{-}2^{b/k}$) mice by intravenous injection of BALB/c ($H\text{-}2^d$) spleen and lymph node cells (3.6×10^7) within 24 h after irradiation. Mice were treated intraperitoneally with a single optimally depleting dose of rat anti-CD8 (YTS 169.4) or untreated. Symptoms of GVHD became obvious 6 days after reconstitution, and mortality started at day 8. The mutual influence of $CD4^+$ and $CD8^+$ T cells in the development of GVHD becomes apparent from our data, and demonstrates that GVHD lethality can be caused by $CD8^+$ T cells as well as by $CD4^+$ T cells.

Introduction

As became clear from allogeneic bone marrow transplantation in animals, mature T cells in the marrow inoculum can cause graft-versus-host disease (GVHD).[1] When these T cells recognize foreign histocompatibility antigens expressed on host tissue, a systemic cellular immune response develops. Both $CD4^+$ and $CD8^+$ T cells are involved in GVHD in mice and humans, depending on the MHC or non-MHC differences between donor and recipient. We formerly demonstrated that in the fully allogeneic BALB/c–(C57BL × CBA)F1 strain combination transplantation of purified $CD4^+$ T cells induced lethal GVHD, whereas purified $CD8^+$ T cells did not, suggesting a major role for the $CD4^+$ T cell subset.[2]

Flow cytometric analysis of T cell subsets in the spleen of the (C57BL × CBA)F1 recipients early after reconstitution demonstrated a strong increase in the number of $CD4^+$ T cells peaking on day 4 or 5. Also the number of $CD8^+$ T cells increased strongly, but consistently peaked 1–2 days later. $CD8^+$ T cells then became the predominant T cell subset. Since the first signs of acute GVHD became obvious 6 days after reconstitution, we concluded that in this mouse strain combination subsequent waves of $CD4^+$ and $CD8^+$ T cells preceded the development of clinically overt GVHD.[3]

Anti-CD4 treatment given 1 day before reconstitution was able to prevent the development of lethal GVHD, whereas anti-CD8 treatment was not. Flow cytometric analysis of the spleen of the recipient mice after allogeneic reconstitution and anti-CD4 monoclonal antibody (mAb) treatment revealed that not only was the number of $CD4^+$ T cells reduced, but also the number of $CD8^+$ T cells.[4] These data suggest that by virtue of their interleukin 2 (IL-2) production[7] the activated $CD4^+$ T cells likely account for the activation and proliferation of the $CD8^+$ T cells. These results do not reveal the role of the $CD8^+$ T cells in the clinical symptoms of GVHD, although our finding that a strong increase in the number of $CD8^+$ T cells preceded the development of overt GVHD suggests that they are important.[3]

Objective

In this study we further investigated whether our assumption that $CD8^+$ T cells do play an important role in the effector phase of graft-versus-host disease (GVHD) could be affirmed. To this end, we postponed anti-CD8 treatment to days of ongo-

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ing GVHD and determined the effect on survival as well as on kinetics of T cell subset populations in the spleen by flow cytometry.

Materials, methods and experimental design

GVH reactions were induced in lethally irradiated (10 Gy) (C57BL/6 × CBA/J)F1 (H-$2^{b/k}$) mice by intravenous injection (day 0) of a mixture of 3×10^7 BALB/c (H-2^d) spleen cells with 6×10^6 BALB/c lymph node cells within 24 h after irradiation. Mice were treated with one optimally depleting dose of 200 μg or even 1 mg of highly purified rat anti-CD8 IgG2b mAb (YTS 169.4)[5,6] 4 h after irradiation, but before the reconstitution (day −1), or on day +1, +3, +5 or +7. Anti-CD4 treatment was performed with 1 mg of YTS 191.1,[6] an IgG2b mAb. Mice were examined daily for the development of signs of acute GVHD. Mortality started at day 8. Irradiated control mice reconstituted with syngeneic cells survived >250 days without signs of disease. Radiation controls died between days 10 and 22.

Flow cytometric analyses of the recipient spleen cells were performed 4, 5 and 7 days after induction of GVHD. On these days, cell suspensions of recipient spleens were prepared in buffered salt solution. Nucleated cell concentrations were determined with a Coulter Counter model ZB1. From these spleen cell suspensions (10^7 nucleated cells/ml) 25 μl was used for staining. The background staining was determined by staining with FITC (fluorescein isothiocyanate)-labelled rabbit anti-rat $F(ab')_2$ fragments alone. $CD3^+$, $CD8^+$ and $CD4^+$ T cells were determined by direct staining with FITC-labelled KT3, 53-6.72, and H129.19, respectively. Cells were also stained with a phycoerythrin-labelled Thy-1.2 mAb (30H12). Forward and side scatter settings were gated on leucocyte populations excluding red cells and debris. A total of 4000 cells was analyzed for each determination.

Results

Survival after anti-CD8 treatment (200 μg) on day −1, +1 or +3

Postponing anti-CD8 treatment of recipients to day 3 after reconstitution, which is the start of the massive proliferation of $CD4^+$ and $CD8^+$ T cells, resulted in a significantly improved survival as compared to treatment on day −1 or day +1 and to untreated mice (Figure 1). However, also after postponed anti-CD8 treatment all mice eventually died.

Flow cytometric analysis of T cell subsets in the spleen after treatment on day −1

After anti-CD8 treatment on day −1, low numbers of $CD8^+$ T cells were found in the spleen on day 4 as well as on days 5 and 7 compared to untreated mice (Figure 2). When mice were treated on day +3 similar results were obtained (Figure 3). Moreover, more $CD4^+$ T cells were found on days 4, 5 and 7 compared to untreated mice (Figure 2). In Figure 2 the indicated numbers reflect the additional $CD4^+$ T cells above the control. On day 7 this increase in $CD4^+$ T cells was statistically significant from untreated mice ($P < 0.05$).

By comparing Figures 2 and 3, a difference in kinetics of $CD4^+$ T cells was observed at several time points. The increase in $CD4^+$ T cells occurred much more quickly in the experiment depicted in Figure 3. Already on day 4 a statistically significant increase in $CD4^+$ T cells (7×10^6) was found as compared to untreated mice.

Flow cytometric analysis of T cell subsets in the spleen after treatment on day +3

After anti-CD8 treatment on day +3 very low numbers of $CD8^+$ T cells were found on days 4 and 7 (Figure 3). Moreover, fewer $CD4^+$ T cells were found on days 4 and 7 as compared to treatment on day −1. Half the number and 0.7 times the number of $CD4^+$ T cells ($P < 0.05$) were found on days 4 and 7, respectively, after treatment on day +3 compared to

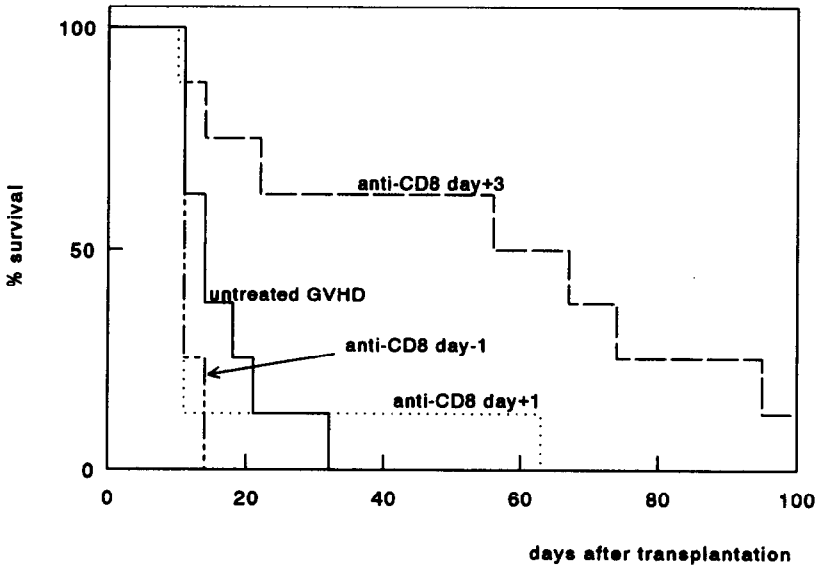

Figure 1 The effect of the moment of anti-CD8 treatment on the survival of lethally irradiated (10 Gy) (C57BL/6 × CBA)F1 (H-$2^{b/k}$) recipients reconstituted with a mixture of 3×10^7 BALB/c (H-2^d) spleen cells and 6×10^6 BALB/c lymph node cells within 24 h after irradiation. Mice were untreated or treated with a single dose (i.p.) of anti-CD8 IgG2b 1 day before reconstitution, or at day +1 or +3 (each group: $n = 8$).

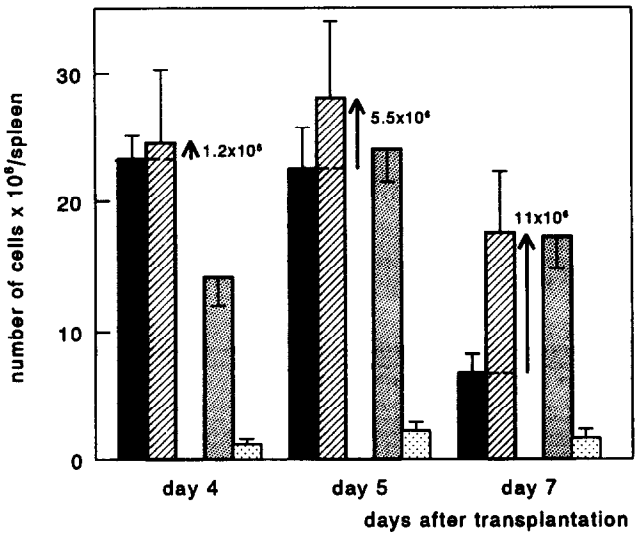

Figure 2 FACScan analysis of CD4+ (■) and CD8+ T cells (▨) from spleen cell suspensions of (C57BL/6 × CBA)F1 mice 4, 5 and 7 days after GVHD induction (day 0) by injection of a mixture of 3×10^7 spleen cells and 6×10^6 lymph node cells of BALB/c mice (H-2^d). Similarly CD4+ (▩) and CD8+ (▩) T cells were analyzed after treatment with a single dose of 200 μg of anti-CD8 IgG2b (day −1). At day 4, 5 and 7 we found 1.2, 5.5 and 11×10^6, respectively, more CD4+ T cells in anti-CD8 treated mice than in untreated mice. On day 7 this increased number of CD4+ T cells was significantly different from untreated mice ($P < 0.05$) ($n = 4$ for each day and each treatment).

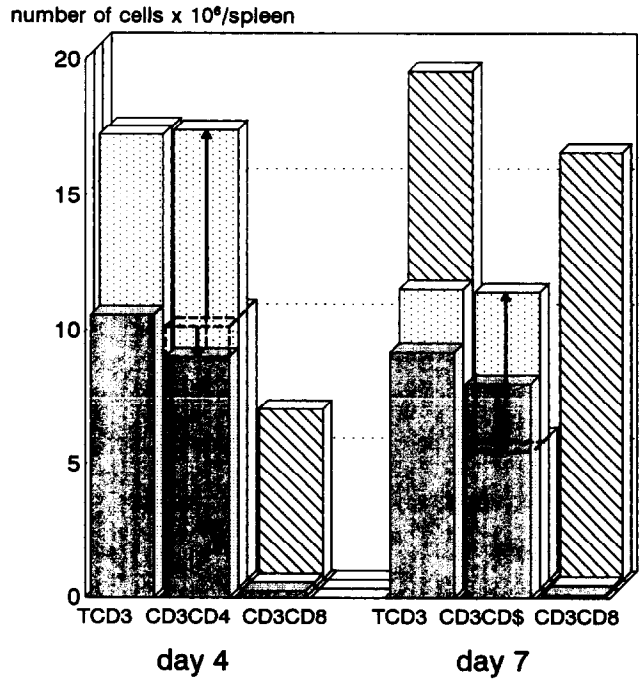

Figure 3 (▨) FACScan analysis of spleen cell suspensions of (C57BL/6 × CBA)F1 mice 4 and 7 days after GVHD induction (day 0) by injection of a mixture of 3×10^7 BALB/c spleen cells and 6×10^6 BALB/c lymph node cells. Similar recipients were treated with a single dose of 200 μg of anti-CD8 IgG2b on day −1 or (▩) on day +3 (■). On days 4 and 7 we found 7 and 5.7×10^6, respectively, more CD4+ T cells in anti-CD8 day −1 treated mice compared to untreated mice. For anti-CD8 day +3 treated mice we found on day 4 a comparable number and, on day 7, 2.6×10^6 more CD4+ T cells compared to untreated mice. These differences were all statistically significant ($P < 0.05$) ($n = 4$ for each day and each treatment).

treatment on day −1. The number of CD4+ T cells on day 4 was comparable with that of untreated mice. A slight but statistically significant increase in the number of CD4+ T cells ($P < 0.05$) was seen on day 7 compared to untreated mice.

Survival after treatment with a single high dose (1 mg) of anti-CD8 or anti-CD4 mAb on days −1, +3, +5 or +7

Treatment of mice on days −1, +3, +5 or +7 with 1 mg of anti-CD8 mAb demonstrated that only after treatment on day +3 was there a significant improvement in survival (Figure 4a). Moreover, treatment with this high dose of anti-CD8 mAb resulted in a 60% survival at 100 days.

Treatment of mice with a single high dose (1 mg) of anti-CD4 mAb on day −1 or +3 resulted in survival of all mice. Treatment on day +5 or +7 did not have any effect on survival (Figure 4b).

Discussion

This is a first study describing the kinetics of CD4+ and CD8+ T cells during the development of GVHD and the effect of anti-CD8 and anti-CD4 treatment on these kinetics in relation to the progress of the disease. We demonstrate that anti-CD8 treatment of recipients on day 3 after reconstitution resulted in a significantly improved survival as compared to treatment on day −1 or day +1 and to untreated mice (Figure 1). Treatment postponed to day +5 or +7 after transplantation, however, had no effect on the development of the disease (Figure 4a). Eventually all mice died after postponed anti-CD8 treatment when a dose of 200 μg was used. This finding confirms our hypothesis that CD8+ T cells play an essential role in the effector phase, and that a beneficial effect on survival can only be observed when the CD8+ T cells are depleted at the start of the effector phase.

Three possibilities can explain the improved survival after anti-CD8 treatment at day 3. Firstly, it is possible that the CD8+ T cells have a different role in the induction and effector phase of GVHD. Depletion of CD8+ T cells, therefore, could have a different effect on survival dependent on the moment of anti-CD8 treatment. Secondly, if a few CD8+ T cells 'escape' the depleting effect of the mAb or if the mAb has a short half-life, early injection of the mAb cannot completely prevent the development of effector CD8+ T cells at a later stage. Injection of anti-CD8 mAb 4 days later (day +3), on the other hand, would be expected to result in lower numbers of CD8+ T cells in the effector phase of GVHD. This would be consistent with the observed improvement in survival (Figure 1). Flow cytometric analyses, however, demonstrated that only few CD8+ T cells escaped from the depleting mAb (Figure 2). Moreover, after treatment on day −1 an equally low number of CD8+ T cells was found on days 4, 5 and 7 (Figure 2) as in mice treated on day +3 (Figure 3).

A third explanation is focused on the involvement of the CD4+ T cell. We propose that cytokines produced by CD4+ T cells (e.g. IL-2)[7] are being consumed for activation and proliferation of CD4+ and CD8+ T cells in GVHD, but after anti-CD8 treatment just for CD4+ T cells alone. This is based on the data of Figure 2, showing more CD4+ T cells on days 4,

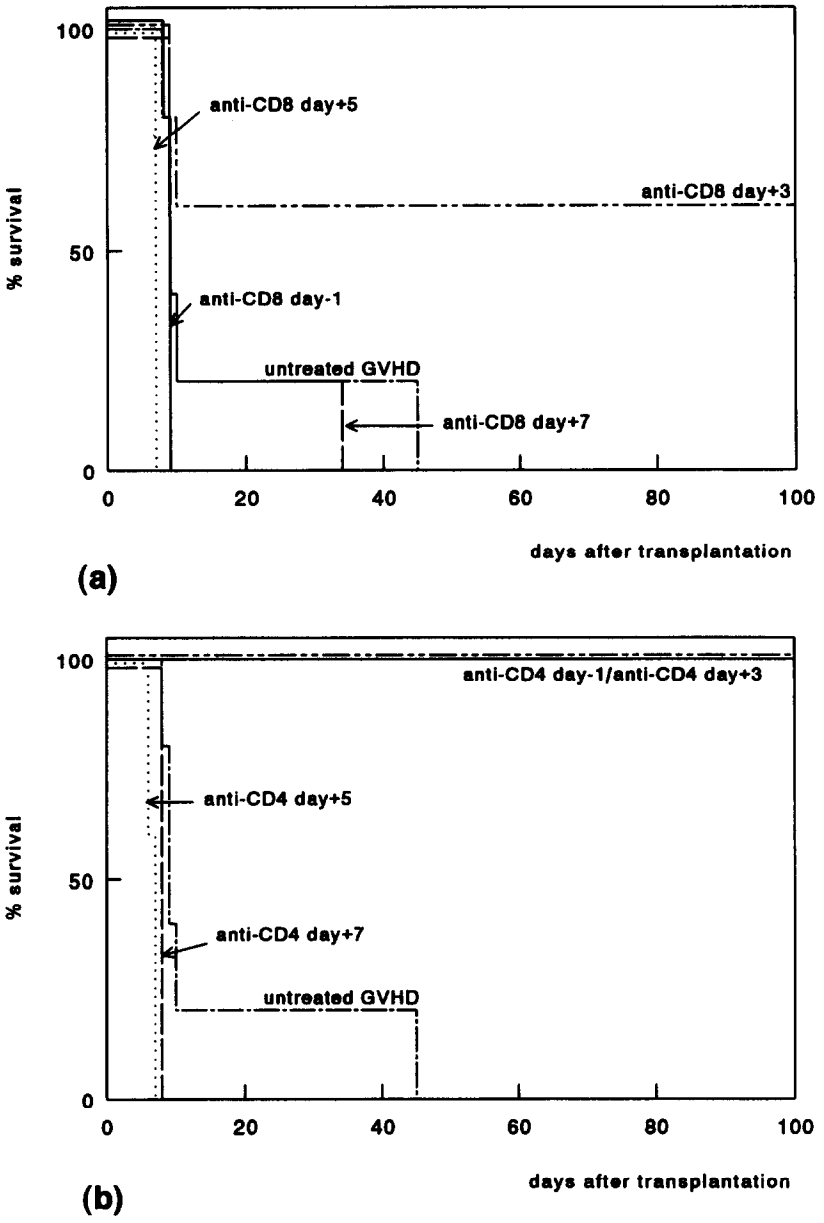

Figure 4 (a) Effect of the moment of anti-CD8 treatment and (b) effect of the moment of anti-CD4 treatment on survival. Lethally irradiated (C57BL/6 × CBA)F1 recipients were reconstituted with a mixture of 3×10^7 BALB/c spleen cells and 6×10^6 BALB/c lymph node cells within 24 h after irradiation. Mice were untreated or treated with a single dose (i.p.) of 1 mg of mAb 4 h after irradiation and thus 1 day before reconstitution, or on day +3, +5 or +7 (each group: $n = 5$).

5 and 7 (1.2, 5.5 and 11×10^6, respectively) after anti-CD8 treatment on day −1 than in untreated mice. It is likely that anti-CD8 treatment on day +3 may result in a lower number of $CD4^+$ T cells than treatment on day −1 or day +1 after reconstitution, since until then cytokines are being used for activation and proliferation of $CD4^+$ T cells as well as $CD8^+$ T cells. Experiments were performed to test this hypothesis. These showed that after treatment on day +3 the number of $CD4^+$ T cells was significantly lower than after treatment on day −1 (Figure 3). Moreover, compared to untreated mice, a slight increase in the number of $CD4^+$ T cells was seen on day 7. This increase of $CD4^+$ T cells in combination with the complete absence of $CD8^+$ T cells after treatment is probably insufficient to cause death at that particular moment but merely postpones GVH lethality (Figure 1).

In summary, $CD8^+$ T cells do not escape from the depleting effect of anti-CD8 mAb, at least not during the first 8 days after injection. Depleting the $CD8^+$ T cells by mAb injection shortly before reconstitution results in an increase of the number of $CD4^+$ T cells. Under these conditions, the $CD4^+$ T cells probably can take over the role of the $CD8^+$ T cell in the effector phase of GVHD, since no difference in survival was observed between anti-CD8 treated (day −1 or +1) and untreated mice. Anti-CD8 treatment on day +3 after reconstitution shortens the period for the $CD4^+$ T cells to expand. Thereby the role of the $CD4^+$ T cells in the effector phase is probably limited and the onset of deaths by GVHD is postponed. The question arises whether this postponed death is actually caused by a subsequent increase in $CD4^+$ T cells or an activation of $CD8^+$ T cells by $CD4^+$ T cells once the anti-CD8 mAb is cle-

ared from the circulation. As treatment with a single high dose (1 mg) of anti-CD8 mAb resulted in 60% survival at 100 days (Figure 4a), we suggest that the anti-CD8 mAb prevents the activation of $CD8^+$ T cells by $CD4^+$ T cells. Thus the development of clinical symptoms of GVHD is limited. Together these data show the mutual influence of $CD4^+$ and $CD8^+$ T cells in the development of GVHD, and demonstrate that GVHD lethality can be caused by $CD8^+$ T cells as well as by $CD4^+$ T cells.

References

1 Korngold R. Biology of graft-vs.-host disease. *Am J Pediatr Hematol Oncol* 1993; **15**: 18–27.

2 Knulst AC, Bril-Bazuin C, Benner R. Prevention of lethal graft-versus-host disease by a single low dose of anti-T cell monoclonal antibody to the allograft recipients. *Eur J Immunol* 1991; **21**: 103–107.

3 Knulst AC, Tibbe GJM, Noort WA, Benner R, Savelkoul HFJ. Prevention of lethal graft-versus-host disease in mice by monoclonal antibodies directed to T cells or their subsets. I. Evidence for the induction of a state of tolerance based on suppression. *Bone Marrow Transplant* 1994; **13**: 293–301.

4 Knulst AC, Noort WA, Tibbe GJM, Benner R, Savelkoul HFJ. Prevention of lethal graft-versus-host disease in mice by monoclonal antibodies directed to T cells or their subsets. II. Differential effectiveness of IgG2a and IgG2b isotypes of anti-CD3 and anti-CD4 moAb. *Bone Marrow Transplant* 1994; **14**: 535–43.

5 Savelkoul HFJ, Vossen ACTM, Breedland EG, Tibbe GJM. Semi-preparative purification and validation of monoclonal antibodies for immunotherapy in mice. *J Immunol Methods* 1994; **172**: 33–42.

6 Cobbold SP, Martin G, Qin S, Waldman H. Monoclonal antibodies to promote marrow engraftment and tissue graft tolerance. *Nature* 1986; **323**: 164–66.

7 Via CS. Kinetics of T cell activation in acute and chronic forms of murine graft-versus-host disease. *J Immunol* 1991; **146**: 2603–609.