Recipient lymphocyte infusion in MHC-matched bone marrow chimeras induces a limited lymphohematopoietic host-*versus*-graft reactivity but a significant antileukemic effect mediated by CD8⁺ T cells and natural killer cells

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ABSTRACT

Background
Challenge of MHC-mismatched murine bone marrow chimeras with recipient-type lymphocytes (recipient lymphocyte infusion) produces antileukemic responses in association with rejection of donor chimerism. In contrast, MHC-matched chimeras resist eradication of donor chimerism by recipient lymphocyte infusion. Here, we investigated lymphohematopoietic host-*versus*-graft reactivity and antileukemic responses in the MHC-matched setting, which is reminiscent of the majority of clinical transplants.

Design and Methods
We challenged C3H→AKR radiation chimeras with AKR-type splenocytes (i.e. recipient lymphocyte infusion) and BW5147.3 leukemia cells. We studied the kinetics of chimerism using flowcytometry and the mechanisms involved in antileukemic effects using *in vivo* antibody-mediated depletion of CD8⁺ T and NK cells, and intracellular cytokine staining.

Results
Whereas control chimeras showed progressive evolution towards high-level donor T-cell chimerism, recipient lymphocyte infusion chimeras showed a limited reduction of donor chimerism with delayed onset and long-term preservation of lower-level mixed chimerism. Recipient lymphocyte infusion chimeras nevertheless showed a significant survival benefit after leukemia challenge. *In vivo* antibody-mediated depletion experiments showed that both CD8⁺ T cells and NK cells contribute to the antileukemic effect. Consistent with a role for NK cells, the proportion of IFN-γ producing NK cells in recipient lymphocyte infusion chimeras was significantly higher than in control chimeras.

Conclusions
In the MHC-matched setting, recipient lymphocyte infusion elicits lymphohematopoietic host-*versus*-graft reactivity that is limited but sufficient to provide an antileukemic effect, and this is dependent on CD8⁺ T cells and NK cells. The data indicate that NK cells are activated as a bystander phenomenon during lymphohematopoietic T-cell alloreactivity and thus support a novel type of NK involvement in anti-tumor responses after post-transplant adoptive cell therapy.

Key words: recipient lymphocyte infusion, antileukemic effect, CD8 T cells, NK cells.

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Introduction

Donor lymphocyte infusion (DLI) after allogeneic stem cell transplantation (alloHSCT) represents a successful strategy to induce or reinforce graft-*versus*-leukemia (GvL) responses. In the current understanding, donor T cells and recipient antigen presenting cells (APC) in the lymphohematopoietic compartment play a critical role in initiating graft-*versus*-leukemia responses, whereas it has been proposed that donor antigen presenting cells contribute by maintaining alloreactive and antitumor T-cell activity through cross-presentation of alloantigens and tumor antigens.[1-3] In clinical and experimental models, donor lymphocyte infusion induced a graft-*versus*-leukemia effect which is usually associated with conversion from mixed to full chimerism, and in those cases where donor lymphocyte infusion is used to prevent relapse, this is also the actual objective.[4-8] Lymphohematopoietic graft-*versus*-host T-cell reactivity is, therefore, considered critical. The major problem associated with induction of a graft-*versus*-leukemia effect by donor lymphocyte infusion is the high risk of graft-*versus*-host disease (GvHD). The overall incidence of acute GvHD is 19-60%, with grade III-IV GvHD affecting 6-35% of patients. Chronic GvHD occurs in 33-61% of patients, and the mortality rate attributable to this is in the range of 6-11%.[9]

Recent studies indicate that not only graft-*versus*-host, but also host-*versus*-graft lymphohematopoietic T-cell reactivity can participate in the effector phase of an antitumor response. The exploitation of antileukemic effects initiated and/or effectuated by recipient immune cells holds the invaluable advantage of not causing GvHD. In the clinic, 2 studies have reported on a group of patients in which loss of donor chimerism was still associated with a potent anti-tumor response.[10,11] Inspired by this clinical observation, the group of M. Sykes developed an MHC-mismatched mouse model where recipient lymphocyte infusion (RLI) induced an antileukemic effect.[12] This was associated with a strong lymphohematopoietic host-*versus*-graft reaction resulting in rapid loss of donor chimerism. The antileukemic effect was shown to be dependent on recipient $CD4^+$ T cells, recipient iNKT and RLI derived $CD8^+$ T cells.[13,14]

Interestingly, we had previously shown that in AKR→C3H MHC-matched bone marrow chimeras such a challenge with RLI does not result in a loss of donor chimerism.[15] This is in contrast with the strong lymphohematopoietic graft-*versus*-host response that is generally seen after donor lymphocyte infusion in these chimeras[16] and also with the pronounced lymphohematopoietic host-*versus*-graft response seen after RLI in the MHC-mismatched model.[12] Thus, in MHC-matched chimeras, lymphohematopoietic alloreactivity elicited by RLI follows a particular course.

Here, we aimed to explore in more detail how adoptive cell therapy with recipient lymphocytes influences lymphohematopoietic T-cell alloreactivity (and possibly antileukemic responses) in the MHC-matched setting, which is representative of the majority of clinical transplants.[17] We found that RLI resulted in a limited and delayed-onset lymphohematopoietic host-*versus*-graft response with long-term preservation of mixed chimerism; this was nevertheless associated with a significant antileukemic response involving $CD8^+$ T cells and NK cells.

Design and Methods

Bone marrow transplantation

AKR and C3H mice were obtained from Harlan BV (Horst, The Netherlands). Recipient mice were given 9.5 Gray total body irradiation on day-1 and 5×10^6 T-cell depleted AKR or C3H bone marrow (BM) cells on day 0.[16] At indicated time points, bone marrow chimeras received an intravenous (IV) infusion of 50×10^6 host-type AKR (RLI) or donor-type C3H (DLI) splenocytes. For leukemia survival studies, mice were challenged one week after donor lymphocyte infusion or RLI with 5×10^6 BW5147.3 leukemia cells (AKR mouse lymphoma; ATCC, Rockville, MD, USA).[16] All experiments were approved by the Ethical Committee for Animal Science of the K.U.Leuven.

In vivo *cell depletion*

Anti-asialoGM1 (Wako, Germany) Ab was administered via intraperitoneal (IP) injection (20 μL per mouse) twice weekly from day 16 after allogeneic bone marrow transplantation (BMT) to deplete NK cells. RLI donor mice were given 2 doses of anti-asialoGM1 Ab at day-3 and day-1 before sacrifice.

YTS169 anti-CD8 mAb (Bioceros BV, Utrecht, The Netherlands) was administered via *IP* injection (200μg/mouse) to deplete $CD8^+$ T cells on days 28 and 29 after BMT and further continued twice weekly. RLI donor mice were given 200 μg of anti-CD8 mAb on day -2 and day-1 before sacrifice.

Flowcytometry

Flowcytometry studies were performed on peripheral blood and spleen cells collected at indicated time points using a FACS Canto (BD Biosciences, Belgium) and mAb against mouse Thy1.1, Thy1.2, CD3, CD4, DX5, IFN-γ (intracellular staining, according to the manufacturer's instruction) or the appropriate isotype control Ig (Serotec, BD Biosciences).

Mixed lymphocyte reaction

3×10^5 MACS-isolated $CD4^+$T cells isolated from RLI chimeras and control chimeras were stimulated with 1×10^4 MACS-isolated $CD11c^+$DC (Miltenyi Biotec, The Netherlands) isolated from naive AKR and C3H mice, in a final volume of 200μL/well in a flat-bottomed 96-well plate for five days at 37°C and 5%CO_2. Cultures were harvested after a 16 h pulse with 1μCi [3H]TdR. Results are expressed as stimulation index (mean counts per minute of stimulated cells/means counts per minute of non-stimulated cells).

Statistical analysis

The Mann-Whitney U test was used to estimate the level of statistical significance of differences between groups of data. The log rank test was used to estimate the level of significance of differences in survival ($P<0.05$ was considered as evidence for statistical significance; Bonferroni's correction was applied when multiple comparisons were performed).

Results

Recipient lymphocyte infusion induces a late-onset and partial decrease in donor T-cell chimerism

First, we determined the kinetics of donor chimerism in peripheral blood taken at regular time intervals from chimeras given RLI on day 21 (RLI-chimeras) and chimeras not given RLI (control chimeras). In these experiments, all chimeras remained clinically healthy and survived long-term (*data not shown*) indicating that RLI is a safe proce-

dure. Control chimeras showed progressively increasing donor T-cell chimerism reaching high-level donor T-cell chimerism at week 10 after BMT (mean 72.6±1.3 SE, n=11) and remaining stable until the end of follow up (day 220). In chimeras given RLI at week 3, donor T-cell chimerism also followed a progressive increase, similar to that of control chimeras, until week 6; at this time point chimerism first stabilized and from week 8 onwards, it progressively decreased to a lower level of mixed chimerism at week 16 (mean % 36.9±2.0 SE, n=12), which was maintained long-term (Figure 1A). We conclude that RLI elicits a slow and limited host-*versus*-graft T-cell response, as evident from the late-onset and partial decrease in donor T-cell chimerism.

We further documented that RLI elicited *in vivo* T-cell alloreactivity prior to the stabilization and decrease of donor chimerism: in *in vitro* MLR assays, $CD4^+$ T cells obtained from RLI-chimeras on day 35 after BMT mounted a limited but clear proliferative response against donor and host antigens (Figure 1B).

The limited lymphohematopoietic host-versus-graft reactivity provoked by RLI is associated with a significant antileukemic effect

Next, we showed that the limited lymphohematopoietic host-*versus*-graft alloreactive T-cell response elicited by RLI is sufficient to elicit an antileukemic effect. RLI chimeras and control chimeras were challenged with BW5147.3 leukemia cells on day 28 after BMT. Animals were inspected daily and follow up was terminated on day 130.

Whereas control chimeras showed 100% mortality from leukemic disease between day 37 and 63 after BMT, RLI chimeras showed a significant survival benefit with 64% mortality (occurring between day 53 and 130), and 36% long-term survival (P=0.01, log rank test). In a selected experiment, we included a group of mice treated with donor lymphocyte infusion and leukemia challenge, and confirm previous studies,[16,18] the DLI-challenged group showed 66% long-term survival after leukemia challenge (Figure 2).[16]

The antileukemic effect of RLI requires a bone marrow graft of allogeneic origin and a sufficient level of allogeneic donor chimerism

Having demonstrated that *in vivo* alloreactivity accompanies the antileukemic effect, we further documented the prerequisites for RLI to generate an antileukemic effect.

First, we challenged syngeneically transplanted AKR→AKR chimeras with RLI on day 21 and with leukemia cells on day 28. In these animals an antileukemic effect could not be observed (mortality 100% by day 98 after BMT in both groups) (Figure 3B). Next, we administered RLI at an early time point, i.e. day 7 after BMT, and challenged these mice with BW5147.3 leukemia cells on day 14. In contrast to the day-21 RLI effect on leukemia-free survival, chimeras given RLI on day 7 did not exhibit a survival benefit over controls (93.3% mortality by day 130 after BMT in both groups) (Figure 3C). Studies of the kinetics of chimerism revealed that RLI in the early post-transplant period prevented progressive engraftment, but on the other hand did not lead to complete graft rejection

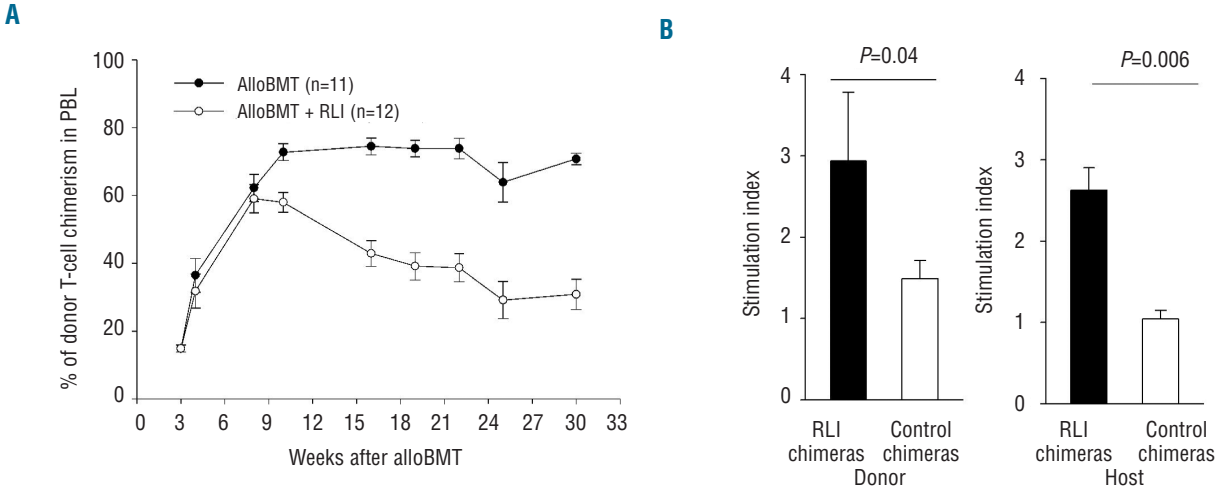

Figure 1. Evolution of donor T-cell chimerism and *in vitro* T-cell response of chimeric $CD4^+$ T cells two weeks after recipient lymphocyte infusion (RLI). (A) Evolution of peripheral blood donor T-cell chimerism in animals receiving an allogeneic bone marrow transplantation (BMT) only (AlloBMT), compared to animals receiving an allogeneic BMT and RLI on day 21 (AlloBMT+ RLI). Results are shown from a total of 11 'alloBMT' mice and 12 'alloBMT+RLI' mice, from 2 identically designed experiments. Results are presented as mean % ± SE. (B) At day 35 after BMT, $CD4^+$ T cells were isolated from chimeras given RLI at day 21 (n=6) and control chimeras (n=5) and stimulated *in vitro* with $CD11c^+$ DCs isolated from C3H (donor) or AKR (host) mice. The proliferative response from individual mice is shown. P=0.04 between the RLI chimeras and control chimeras for the proliferative response against donor antigens and P=0.006 between the RLI chimeras and control chimeras for the proliferative response against host antigens as tested by Mann-Whitney U. Data from 2 identical independent experiments are shown.

(mean donor T-cell chimerism on day 21 in RLI-day 7 chimeras was 9.8%±0.6 SE, remaining stable until end of follow up, whereas in control chimeras this was 26.8´%±4.2 SE (n=9), with a further progressive increase to 85.5%±2.3 SE (n=9) at day 70 after BMT) (Figure 3D). Finally, RLI administered to naive AKR mice or to AKR mice given total body irradiation only (without BMT) failed to generate an antileukemic effect (Figure 3A).

Taken together, these experiments show that a bone marrow graft of allogeneic origin and a sufficiently high level of donor chimerism are critical prerequisites for RLI to induce an antileukemic effect.

The antileukemic effect of recipient lymphocyte infusion involves CD8⁺ T cells and NK cells

The observation that RLI elicits a limited lymphohematopoietic host-*versus*-graft T-cell response while producing a significant antileukemic effect suggested that in addition to T cells, also non-T cells (in particular NK cells) may take part in the antileukemic effector mechanism. To investigate the role of CD8⁺ T cells and NK cells in the

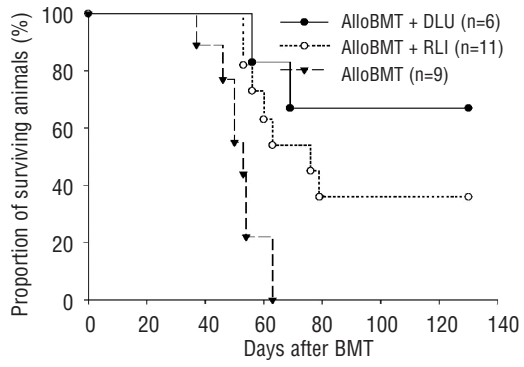

Figure 2. Antileukemic effects of recipient lymphocyte infusion (RLI) in C3H→AKR chimeras. The Kaplan-Meier leukemia-free survival is shown of C3H→AKR chimeras following challenge with BW5147.3 on day 28 after allogeneic bone marrow transplantation (BMT) in mice given DLI on day 21 after allogeneic BMT (AlloBMT+DLI, n=6), in chimeras given RLI 21 days after allogeneic BMT (AlloBMT+RLI, n=11) and in control chimeras not given cell therapy (AlloBMT, n=9). Results are shown from 2 identically designed experiments. *P*=0.01 between "AlloBMT+RLI" and "AlloBMT" as tested by the log rank test.

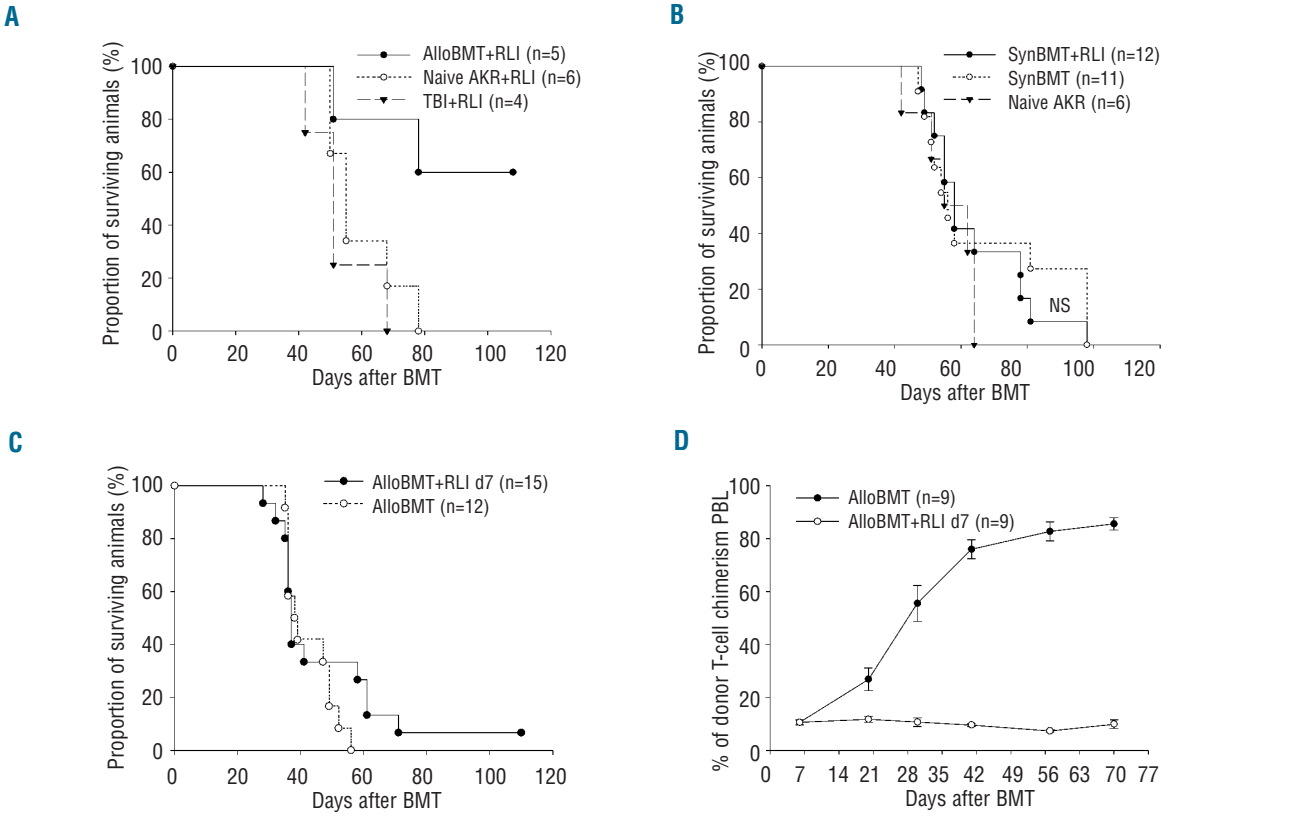

Figure 3. Prerequisites for the antileukemic effect after recipient lymphocyte infusion (RLI). (A) Kaplan-Meier leukemia-free survival after leukemia challenge on day 28 in allogeneic BM chimeras given RLI on day 21 after bone marrow transplantation (BMT) (AlloBMT + RLI, n=5), in naive AKR mice given RLI (Naive AKR + RLI, n=6) and in mice given RLI after TBI only, without an allogeneic BM graft (TBI + RLI, n=4). Results are shown from one experiment. *P*=0.03 between "AlloBMT+RLI" and "Naive AKR+RLI", *P*=0.04 between "AlloBMT+RLI" and "TBI+RLI" as tested by the log rank test. (B) The Kaplan-Meier leukemia-free survival is shown after BW5147.3-challenge of syngeneic AKR BM-chimeras given RLI on day 21 after BMT (SynBMT+RLI, n=12), of syngeneic AKR BM-chimeras not given RLI (SynBMT, n=11) and of naive AKR mice (Naive AKR, n=6). Results are shown from 2 identically designed experiments. NS, not significant: *P*=0.9 between "SynBMT + RLI" and "SynBMT" as tested by the log rank test. (C) Kaplan-Meier leukemia-free survival after leukemia challenge on day 14 after allogeneic BMT in allogeneic BM-chimeras given RLI on day 7 after BMT (AlloBMT + RLI d7, n=15) and in allogeneic chimeras not given RLI (AlloBMT, n=12). Results are shown from 3 identically designed experiments. NS, not significant: *P*= 0.35 between "AlloBMT+ RLI d7" and "AlloBMT" as tested by the log rank test. (D) Evolution of donor T-cell chimerism in allogeneic C3H→AKR chimeras (AlloBMT, n=9) and in allogeneic chimeras challenged with RLI on day 7 (AlloBMT+RLI d7, n=9). Results are shown from 2 identically designed experiments. Results are expressed as % donor T-cell chimerism and presented as mean % ± SE.

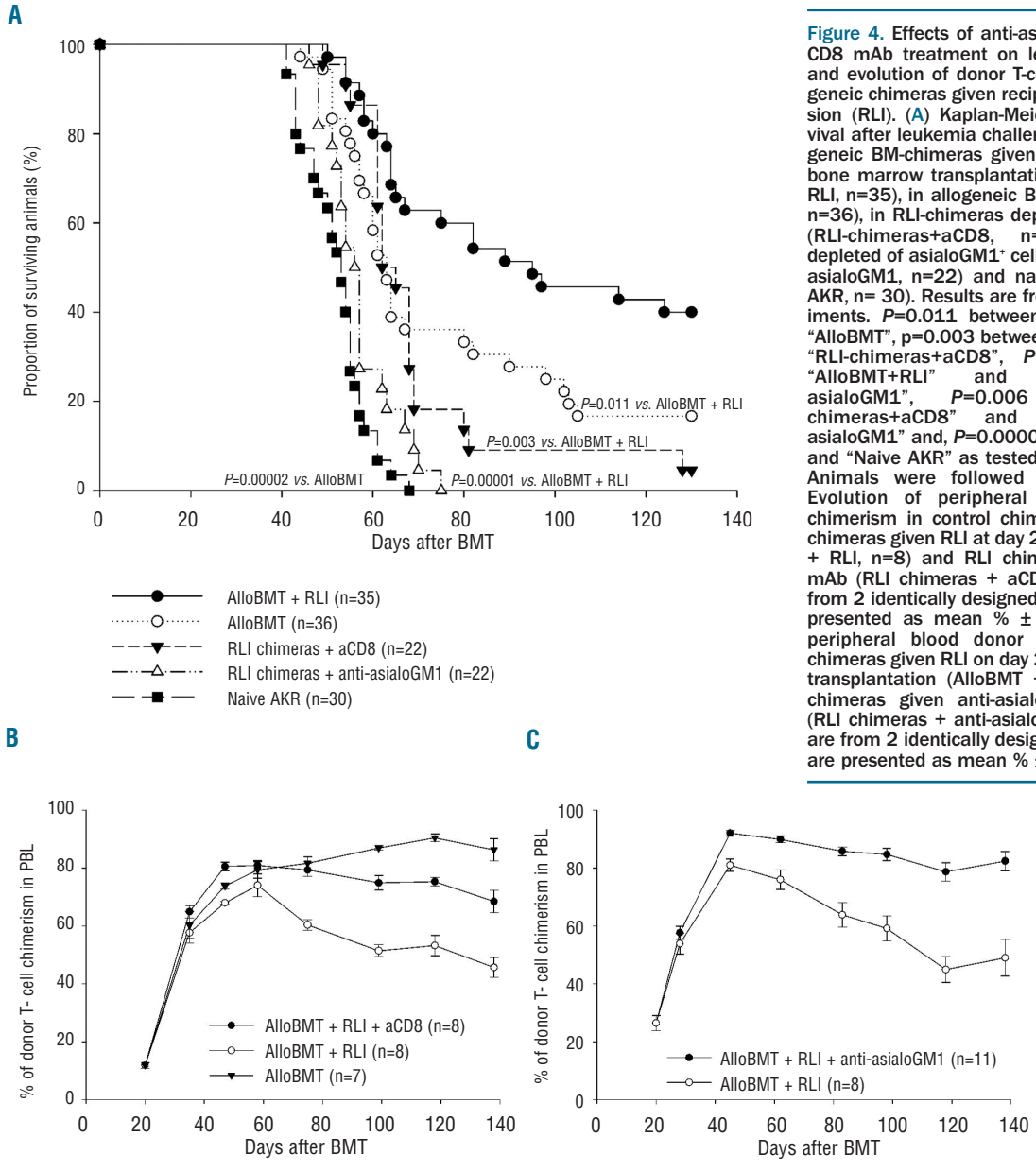

Figure 4. Effects of anti-asialo-GM1Ab and anti-CD8 mAb treatment on leukemia-free survival and evolution of donor T-cell chimerism in allogeneic chimeras given recipient lymphocyte infusion (RLI). (A) Kaplan-Meier leukemia-free survival after leukemia challenge on day 28 in allogeneic BM-chimeras given RLI on day 21 after bone marrow transplantation (BMT) (AlloBMT + RLI, n=35), in allogeneic BM-chimeras (AlloBMT, n=36), in RLI-chimeras depleted of $CD8^+$ T cells (RLI-chimeras+aCD8, n=13), RLI-chimeras depleted of asialoGM1$^+$ cells (RLI-chimeras+anti-asialoGM1, n=22) and naive AKR mice (naive AKR, n= 30). Results are from a total of 7 experiments. *P*=0.011 between "AlloBMT+RLI" and "AlloBMT", p=0.003 between "AlloBMT+RLI" and "RLI-chimeras+aCD8", *P*=0.00001 between "AlloBMT+RLI" and "RLI-chimeras+anti-asialoGM1", *P*=0.006 between "RLI-chimeras+aCD8" and "RLI-chimeras+anti-asialoGM1" and, *P*=0.00002 between "AlloBMT" and "Naive AKR" as tested by the log rank test. Animals were followed until day 130. (B) Evolution of peripheral blood donor T-cell chimerism in control chimeras (AlloBMT, n=7), chimeras given RLI at day 21 after BMT (AlloBMT + RLI, n=8) and RLI chimeras given anti-CD8 mAb (RLI chimeras + aCD8, n=8). Results are from 2 identically designed experiments and are presented as mean % ± SE. (C) Evolution of peripheral blood donor T-cell chimerism in chimeras given RLI on day 21 after bone marrow transplantation (AlloBMT + RLI, n=8) and RLI chimeras given anti-asialoGM1 Ab treatment (RLI chimeras + anti-asialoGM1, n=11). Results are from 2 identically designed experiments and are presented as mean % ± SE.

antileukemic effect of RLI we administered anti-asialoGM1 and anti-CD8 antibodies *in vivo*: in order to obtain complete depletion of these $CD8^+$ T cells and NK cells, both the RLI donor mice and bone marrow recipients were given depleting antibody treatment.

In these experiments, RLI chimeras showed a significantly better survival rate after leukemia challenge than did control chimeras (*P*=0.011, log rank test) (Figure 4A). The removal of $CD8^+$ T cells led to a significant reduction of the survival benefit relative to RLI chimeras (*P*=0.003, log rank test). When asialoGM1$^+$ cells were depleted, this resulted in an even more pronounced reduction of survival benefit (*P*=0.00001, log rank test) (Figure 4A).

In the current C3H→AKR strain combination, due to the lack of expression of the NK1.1-marker, we chose anti-asialoGM1-Ab to deplete NK cells. In addition to NK cells this antibody targets asialoGM1$^+$$CD8^+$ T cells, a minor subset of $CD8^+$ T cells, of which the significance is incompletely understood. AsialoGM1$^+$$CD8^+$ T cells have been reported to correspond with naive, antiviral or alloreactive $CD8^+$ T cells.[19-21] We documented the evolution of chimerism in anti-CD8-mAb and anti-asialoGM1-Ab treated mice, and found that the characteristic chimerism changes disappeared in both treatment groups (Figure 4B-C). We conclude that the abrogation of the lymphohematopoietic host-*versus*-graft response after anti-CD8-mAb treatment is due to the removal of alloreactive $CD8^+$T cells, and since similar effects were seen after anti-asialoGM1-Ab treatment, that asialoGM1$^+$$CD8^+$ T cells represent the alloreactive $CD8^+$ T-cell subset. These data imply that effects seen with anti-asialoGM1 antibody treatment may, at least in part, be attributed to depletion of this $CD8^+$ T-cell subpopulation.

However, the survival of asialoGM1-depleted chimeras was significantly worse than of CD8-depleted RLI chimeras (*P*=0.006, log rank test). These data indicate that

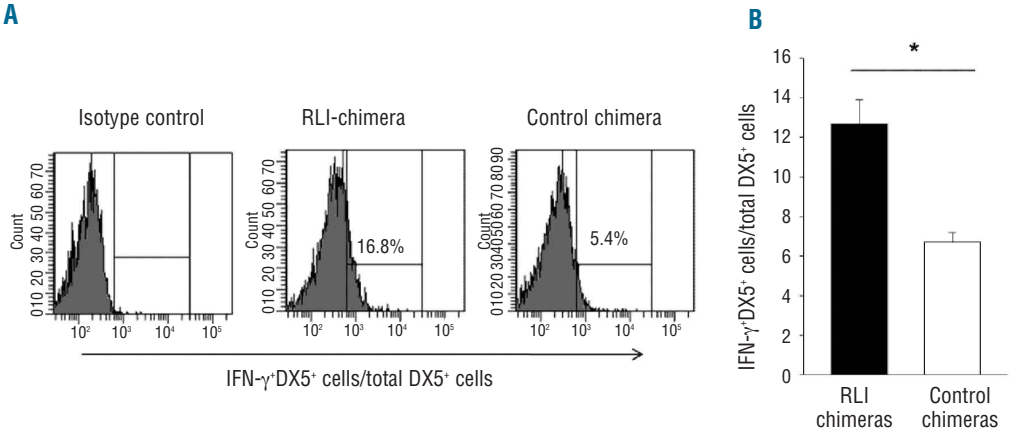

Figure 5. IFN-γ expression by NK cells in control and recipient lymphocyte infusion (RLI) chimeras. Intracellular IFN-γ expression was determined using flowcytometry on splenic DX5+ cells of control chimeras and RLI chimeras, on day 10 after RLI. (A) Representative histograms of IFN-γ expression in an RLI-chimera and a control chimera; the percentage IFN-γ+ cells is indicated as determined using isotype control antibody staining. (B) Mean IFN-γ expression in RLI chimeras (n=5) and control chimeras (n=4). *P=0.02 as tested by Mann-Whitney U.

the RLI-induced antileukemic effect in bone marrow chimeras is dependent not only on CD8+ T cells, but, importantly, it also involves asialoGM1+ cells that are not CD8+ T cells, in this case NK cells. Consistent with this, using intracellular flowcytometry, we documented that ten days after RLI, the proportion of IFN-γ producing NK cells in RLI chimeras was significantly higher than that in control chimeras (mean 12.7%±1.2 SE in RLI-chimeras (n=5) *vs.* 6.7%±0.5 SE in control chimeras (n=4), P=0.02, Mann-Whitney U test) (Figure 5).

Discussion

Strategies that exploit host-*versus*-graft lymphohematopoietic T-cell reactivity for the induction of antileukemic effects hold the theoretical advantage of avoiding GvHD. Conversely, such strategies carry the risk of complete graft rejection, as evident from clinical observations and from the findings in a recently published MHC-mismatched mouse model.[10-12] In the current study, we showed that in a model of MHC-matched allogeneic BMT, which is representative of 50-85% of all clinical transplants, RLI is not associated with a complete loss of donor chimerism. We observed a slow and limited lymphohematopoietic host-*versus*-graft response that was, however, associated with a significant antileukemic effect dependent on CD8+ T cells and NK cells. These data indicate that RLI in an MHC-matched, multiple miHC-mismatched setting holds the advantage of preserving mixed chimerism and transplant tolerance, and reveals a novel role for NK cells in the antileukemic effect of adoptive cell therapy.

Looking at the kinetics of donor chimerism as a reflection of lymphohematopoietic host-*versus*-graft reactivity, we found that in the MHC-matched setting, RLI elicits a late-onset and only partial decrease of chimerism. This chimerism evolution is in contrast with the rapid conversion from mixed to full donor chimerism following donor lymphocyte infusion in the same experimental model[18] and with strong lymphohematopoietic alloreactivity generally seen in patients after donor lymphocyte infusion therapy.[4-8] We postulate that following lethal irradiation, infused healthy donor bone marrow cells have a competitive advantage over residual host-type bone marrow cells and that adoptively transferred donor lymphocytes reinforce this effect, leading to a rapid conversion from mixed to full donor chimerism. Moreover, in the context of donor lymphocyte infusion, non-tolerant donor T cells are confronted with abundant host antigen presenting cells since donor chimerism at day 21 only amounts to around 15% which results in extensive donor T-cell activation and total elimination of recipient hematopoietic cells. In contrast, in the case of antigen presenting cells, non-tolerant recipient T cells, infused at a similar time point will encounter a small number of donor antigen presenting cells only. Our data are also in discrepancy with the rapid loss of donor chimerism following RLI in the MHC-mismatched setting, which is probably due to the strong mismatch in MHC-antigens. We postulate that the minor mismatch in transplantation antigens explains why in our model the chimerism changes are slow to appear and result in a partial rejection of donor cells.

In vitro, CD4+ T cells of RLI chimeras generated a distinct proliferative response against donor and host antigens. Whereas the anti-donor response can be attributed to the reactivity of the non-tolerant T cells from the RLI-inoculum against donor antigens, the anti-host response indicates that donor T cells in the chimera mount an alloresponse when they encounter additional host antigen presenting cells from the RLI-inoculum. This may explain the biphasic evolution of donor chimerism after RLI, with an initial further increase (donor-anti-host) and a subsequent decrease (host-anti-donor).

Despite the limited lymphohematopoietic host-*versus*-graft response, RLI in the MHC-matched setting elicits a significant antileukemic response. In concordance with the concept that lymphohematopoietic alloreactivity is critical for antileukemic responses, we determined firstly that this RLI-antileukemic effect could only be elicited in mice given irradiation and a bone marrow graft of allogeneic (and not syngeneic) origin. Secondly, RLI in the early post-transplant period, when donor lymphohematopoiesis and the amount of donor antigen presenting cells is very low, did not produce an antileukemic effect.

From the significant reduction in leukemia-free survival after anti-CD8-mAb treatment, we conclude that CD8+ T cells play a role in the protection of an RLI-chimera against

challenge with leukemia. This confirms findings in the MHC-mismatched model where the anti-tumor response involved RLI-derived CD8+ T cells.[13] The observation that the characteristic chimerism changes were abrogated in anti-CD8-mAb and anti-asialoGM1-Ab-treated chimeras indicates the critical role of alloreactive CD8+ T cells and identifies asialoGM1+CD8+ T cells as alloreactive T cells, consistent with a previous report by others.[20] Interestingly, a recent report showed asialoGM1+CD8+ T cells to have a central memory phenotype and to exhibit early IFN-γ production, leading the authors to propose a critical role in Th1-mediated immunity such as in tumor immunity.[19]

We found that also NK cells contributed to the RLI-induced antileukemic effect. This was evident from the finding that the survival of asialoGM1-depleted RLI-chimeras was significantly worse than that of CD8-depleted RLI-chimeras. In addition, we documented that RLI led to an increase in IFN-γ expressing NK cells relative to control chimeras. The role of NK cells in antileukemic responses after transplantation is being increasingly acknowledged,[22] particularly in the setting of KIR-ligand mismatched allogeneic hematopoietic stem cell transplantation, where they might contribute to anti-tumor effects through killing on the basis of 'missing-self'.[23,24] In contrast, in this study, we work with an MHC-matched transplantation model and NK cells from naive AKR and C3H mice are not able to lyse BW5147.3 or C3H and AKR blasts *in vitro* (*data not shown*). We, therefore, postulate that the lymphohematopoietic alloreactivity provoked by adoptively transferred non-tolerant T cells provides a cytokine environment that (as a bystander phenomenon) activates NK cells, thereby providing them with the capacity to recognize and lyse recipient-type tumor cells. This corresponds with the *in vitro* phenomenon known as "lymphokine-activated killer cells".[25-27] This contribution of NK cells to the RLI-induced antileukemic effect is supported by preliminary findings in the MHC-mismatched model showing increased expression of CD69 by NK cells after RLI.[14]

It should be noted that in the current experimental setup, *in vivo* antibody treatment may also interfere with a protective mechanism present in chimeras independently of RLI. In this respect, we noted that allogeneic bone marrow chimeras show a significantly better survival after leukemia challenge as compared to naive recipient mice, whereas such a protective effect was not seen for syngeneically transplanted chimeras. Studying the mechanisms responsible for this effect, we were able to demonstrate that MACS-purified CD8+ T cells, isolated from day 28-chimeras failed to lyse BW5147.3 cells *ex vivo* (^{51}Cr release assay, *data not shown*), arguing against a role for CD8+ T cells in the protection of allogeneic chimeras. In contrast, MACS-purified NK cells from allogeneic chimeras, but not those from syngeneic chimeras or naive donor- or host-type mice exhibited pronounced cytotoxic reactivity against recipient-type tumor cells *ex vivo* (12% specific lysis in allogeneic chimeras relative to 0% in syngeneic chimeras, 0% in naive AKR and 0.4% in naive C3H mice, one of 3 experiments, *data not shown*). When taking these data into account, we conclude that the reduction in leukemia-free survival of anti-asialoGM1-Ab-treated RLI-chimeras is, at least in part, due to interference with the NK-dependent protection present in allogeneic chimeras independently of RLI.

On the other hand, these observations further support our hypothesis that lymphohematopoietic T-cell reactivity gives rise to NK-cell activation: in particular, we postulate that alloreactive CD4+ T cells, when producing lymphohematopoietic graft-*versus*-host reactivity during engraftment, provide cytokines that activate NK cells, thereby providing these NK cells with anti-tumor activity.

Reportedly, the dose of anti-asialoGM1 Ab used in the current study depletes NK cells, but does not remove NKT cells *in vivo*, owing to the low expression levels of asialoGM1 in these cells[28,29] suggesting that in our model iNKT-cells do not play a central role in RLI-induced anti-tumor immunity, as opposed to their role in the MHC-mismatched model as recently reported by Saito *et al.*[14]

Interactions between NK cells and CD8+ T cells have been reported.[30-34] It has been shown that NK cells can induce proliferation and/or differentiation of CD8+ T cells into cytolytic effector T cells,[32-34] and that CD8+ T cells can become activated via IL-12, produced by dendritic cells in response to IFN-γ producing NK cells.[30,31] Whether or not such interactions contribute to the RLI-effect remains to be determined.

In conclusion, in the MHC-matched setting, RLI elicits lymphohematopoietic host-*versus*-graft reactivity that is limited but sufficient to provide an antileukemic effect. Long-term mixed chimerism is preserved and may offer a platform for additional graft-*versus*-leukemia inducing therapy with donor lymphocyte infusion. The antileukemic effect is dependent on CD8+ T cells and NK cells: the data indicate that NK cells are activated as a bystander phenomenon during lymhohematopietic T-cell alloreactivity and thus support a novel type of NK involvement in anti-tumor responses after post-transplant adoptive cell therapy.

Whereas the current study was performed in mice having mixed chimerism, in the clinical setting, patients often evolve to full donor chimerism; we postulate that RLI in such patients, due to the abundance of donor-type antigen presenting cells, would result in similar, if not stronger, lymphohematopoietic T-cell alloreactivity and bystander activation of NK cells. Finally, insight into the immune effects of RLI may open interesting perspectives for the treatment of therapy-resistant solid tumors. Clinical data is available which provide a scientific background for the use of allohematopoietic stem cell transplantation in the treatment of renal, colon and ovarium carcinoma and pancreatic tumors.[35-38] In this setting, where avoidance of GvHD is a particular objective, RLI may activate potent antitumor effects, specifically for tumors that are NK sensitive.

Authorship and Disclosures

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