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H2-mismatched transplantation with repetitive cell infusions and CD40 ligand antibody infusions without myeloablation

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Summary. Graft rejection and graft-versus-host disease are major problems in mismatched marrow transplants along with toxicity from standard myeloablative host treatments. We have established a tolerization model, using 1 Gy irradiation, which reduces stem cell capacity to < 10% of control while causing minimal myelosuppression, donor antigen pre-exposure (spleen cells), CD40-ligand antibody blockade and high levels of marrow (40×10^6 cells), which allows for stable long-term multilineage engraftment in H2-mismatched murine marrow transplants. We now show that the establishment of 'microchimaerism' (0·5–3·8%) sets the stage for macrochimaerism, with subsequent marrow infusions in H2-mismatched mice with CD40-ligand blockade only. Neither irradiation nor spleen cell exposure were necessary. When 40 x 10^6 bone marrow cells were infused on weeks 0, 12, 14 and 16, blood engraftment was about seven times the single 40×10^6 control. When marrow cells were given on weeks 0, 3, 4, 5 and 6, engraftment at 24 weeks post transplant was 17·9 ± 1·2%, compared with 2·7 ± 0·8% for the single 40×10^6 control ($P = 0{\cdot}009$). We have shown stable, long-term multilineage chimaerism and established that the schedule of marrow administration, not the total cell dose, is critical for tolerization. This approach indicates that microchimaerism can tolerize for subsequent marrow infusions and produce macrochimaerism. This strategy could be applied in clinical human transplants.

Keywords: bone marrow transplantation, allograft, immune tolerance, co-stimulation blockade, B7 antigen.

Recently we have described an approach for establishing long-term multilineage chimaerism in H2-mismatched mice (B6.SJL to BALB/c), using low-level recipient irradiation (1 Gy), donor spleen antigen pre-exposure, co-stimulator blockade with CD40-ligand monoclonal antibody (mAb) (anti-CD40L) and infusion of 40×10^6 donor marrow cells (Quesenberry *et al*, 2001). In that system, B6.SJL spleen cells (10^7) were injected 10 d prior to transplantation. A hamster anti-CD40L mAb was given i.p. on d −10, −7, −3, 0 and +3, and the recipient BALB/c mice were exposed to gamma irradiation immediately prior to infusion of 40×10^6 B6.SJL marrow cells on d 0. Using this approach, multilineage chimaerism, without rejection or graft-versus-host disease (GVHD) was established up to 47 weeks, close to half the expected murine lifespan. We noted both a cell and antibody dose response for chimaerism. These tolerant mice accepted donor skin grafts and rejected third party grafts. Using the above conditioning schema without irradiation, we also observed some low-level chimaerism in hosts given two doses of B6.SJL marrow (40×10^6 cells) on d 0 and week 14.

Attractive components of this approach include the absence of GVHD and the low-level host irradiation, in which only a relatively mild and transient myelosuppression occurs (Stewart *et al*, 1998). This avoids the toxicity of higher dose regimens and, presumptively, many of the deleterious effects of 'cytokine storm' (Ferrara, 1993). The success of this approach was clearly based on relatively high levels of infused marrow cells: a quasi 'mega stem cell' approach, such as described in previous reports (Aversa *et al*, 1994; Bachar-Lustig *et al*, 1995; Rachamim *et al*, 1998).

In these previous studies, we observed some low-level augmentation of engraftment in irradiated BALB/c hosts given B6.SJL marrow (40×10^6 cells) on d 0 and week 14. A number of studies in the past have indicated that marrow chimaerism may induce tolerance to various solid organ grafts. The administration of marrow with antilymphocyte serum (ALS) after skin grafting in substantial major

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histocompatibility complex (MHC) class I disparate combinations resulted in immunological tolerance to skin allografts (Gozzo *et al*, 1970). Subsequent work using chromosome marking showed persistence of donor bone marrow cells in the spleen at levels of less than 4%; this has been referred to as microchimaerism (Monaco *et al*, 1975).

Subsequent studies, both *in vitro* and *in vivo*, indicated an involvement of host-derived regulatory T cells and donor-derived bone-marrow-vetoed T cells in these tolerizing phenomena (Maki *et al*, 1981). Similar approaches, using polyclonal ALS and post-organ transplant donor marrow infusion, produced tolerance to kidney allografts in mongrel dogs (Hartner *et al*, 1986), non-human primates (Thomas *et al*, 1987) and possibly in man (Barber *et al*, 1991). In 1992, long-term survivors of liver and kidney allografts were found to have evidence of circulating donor cells (Starzl *et al*, 1992, 1993). These data indicated that persistent microchimaerism might be important for organ transplant tolerization. Other work using total lymphoid irradiation has shown that infusion of bone marrow cells can give tolerance to skin grafts using post-transplant total lymphoid irradiation (Slavin *et al*, 1977). In combined bone marrow and organ transplantation in rodents, long-term heart allograft survival was obtained (Ko *et al*, 1999). Studies also indicated that chronic rejection was seen with non-chimaeric tolerance, while there was a lack of chronic rejection with chimaeric tolerance (Slavin *et al*, 1978; Strober *et al*, 1979; Zeng *et al*, 1999; Strober *et al*, 2000). A number of subsequent studies have continued to show that marrow chimaerism secondary to previous marrow transplantation may be able to tolerize for acceptance of kidney (Sayegh *et al*, 1991; Jacobsen *et al*, 1994; Helg *et al*, 1995; Sorof *et al*, 1995) or lung (Svendsen *et al*, 1999) solid organ grafts. Recently, a double marrow and kidney transplantation was performed using a non-myeloablative approach (Spitzer *et al*, 1999).

MHC-incompatible transplants are associated with increased GVHD and immune incompetence (Zinkernagel *et al*, 1980a,b). The induction of mixed chimaerism, in which both host and donor mature T cells are present, leads to both immunocompetence and tolerance to self and donor (Ildstad *et al*, 1985). Initial studies, using lethal radiation and mixtures of both allogeneic donor and host marrow, have resulted in tolerance and immunocompetence (Ildstad & Sachs, 1984). Subsequently, investigators have worked to reduce the level of myeloablation and several non-myeloablative regimens have been utilized, producing mixed chimaerism with tolerance (Sharabi & Sachs, 1989; Sykes *et al*, 1997). Such approaches have been used in concordant rat to mouse transplants (Sharabi *et al*, 1990) and these studies have been extended to miniature swine (Fuchimoto *et al*, 2000) and cynomolgus monkeys (Kawai *et al*, 1995, 1999; Kimikawa *et al*, 1997).

Altogether, these studies have indicated that induction of marrow microchimaerism, whether achieved before or after solid organ grafting, is capable of inducing a long-term stable tolerance to the solid organ graft. Thus, marrow microchimaerism may provide a platform in general for donor organ graft acceptance (Wekerle & Sykes 2001). Based on this, and our initial results, we hypothesize that marrow microchimaerism can tolerize for subsequent marrow macrochimaerism. We have explored this possibility in the present studies, evaluating engraftment with no host irradiation, co-stimulator blockade, and varying schedules and numbers of donor marrow cells.

METHODS AND MATERIALS

Mice. Six to 8 week-old BALB/c (CD45·2,H2K^{d}) and congenic B6.SJL-Ptprc^{a} (CD45·1,H2K^{b}) mice were purchased from Taconic Farms (Georgetown, NY, USA). BALB/c severe combined immunodeficient (SCID) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed for at least 1 week before experimental use. Mice were maintained in micro-isolator cages in a conventional clean facility and given ad libitum access to autoclaved mouse chow and acidified water. The animals were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and Roger Williams Medical Center, and the recommendations of the National Research Council (1996).

Murine anti-CD40L mAb production and quantification. The MR-1 hybridoma line (gift from R. J. Noelle, Dartmouth Medical School, Dartmouth, NH, USA) produces a hamster anti-mouse anti-CD40L mAb. The isotype of the antibody is Armenian Hamster IgG, Group 3, k. BALB/c SCID mice were primed with pristane R (2, 6, 10·14-Tetramethylpentadecane, Sigma, St Louis, MO, USA) 0·5 ml i.p. Seven to 10 d later, 2×10^{6} MR-1 cells (viability > 75%) were injected i.p. Ascites were collected and incubated at 37°C for 1 h then 4°C overnight, centrifuged at 3000 r.p.m. for 30 min, and the supernatant was filtered through a 0·2 μm filter. We stored aliquots at −70°C and thawed them immediately prior to use. We used an enzyme-linked immunosorbent assay (ELISA) to quantify Hamster IgG in the ascites. The plates (Becton Dickinson, CA, USA) were coated with 100 μl capture antibody at 2 μg/ml affinity purified mouse anti-Armenian Hamster IgG (H + L) (BD PharMingen, San Diego, CA, USA) and incubated at 4°C overnight. Unknown concentrations of ascites and commercial anti-CD40L (BD PharMingen) were added to the appropriate wells and incubated at 4°C overnight. Biotinylated goat anti-hamster IgG (Roche Molecular Biochemicals, Indianapolis, IN, USA) was added to the wells, followed by avidin-horseradish peroxidase at the appropriate dilution. Tetramethylbenzidine (TMB) peroxidase enzyme immunoassay (EIA) substrate kit (Bio-Rad, Hercules, CA, USA) was used to perform the colour reaction and plates were read at 650 nm. A standard curve was made from the known concentration of a commercial anti-CD40L (BD PharMingen), and unknown ascites concentrations were calculated.

Donor specific and anti-CD40L exposure. Normal male B6.SJL spleen cells (10×10^{6}) were injected into BALB/c recipients by tail vein 10 d before bone marrow transplant. Anti-CD40L (1·6 mg) was injected i.p. prior to injection of B6.SJL spleen cells (d −10) and on d −7, −3, 0 and +3 (standard) or d −10, −3 and 0 (3 × CD40L).

Irradiation. We delivered 1 Gy whole-body gamma irradiation (WBI) 2 hours prior to transplant at a rate of 0·85–0·86 Gy/min in one fraction using a ^{137}Cesium source (Gammacell 40, Nordian, Kanata, Ontario, Canada).

Transplantation. We harvested B6.SJL bone marrow cells from tibiae and femurs using phosphate-buffered saline (PBS), filtered through a 40 μm strainer (Becton Dickinson, Franklin Lakes, NJ, USA), washed once, counted in crystal violet and resuspended for injection in PBS. We infused 40×10^6 or, alternatively, 200×10^6 (in two injections of 100×10^6 given 15 min apart) B6.SJL male bone marrow cells into male BALB/c i.v. in 0·5 ml of PBS on d 0. We used two schedules for repetitive transplants:

1. 40×10^6 on d 0 and week 12, 14 and 16 (total 160×10^6)
2. 40×10^6 on d 0 and week 3, 4, 5 and 6 (total 200×10^6)

These schedules were chosen to encompass a short and long time interval between the first and subsequent marrow infusions. In some experiments, we compared donor-specific spleen cells with no donor pre-exposure (see above). For all transplants, we used anti-CD40L as described above (Table I and Fig 1).

Evaluation of donor engraftment and lineage analysis. We incubated ~50 μl of blood for 20 min at room temperature (RT) with anti-mouse fluorescein isothiocyanate (FITC)-CD45·1 mAb (A-20 line, PharMingen), lysed the samples for 10 min at RT with 1·3 ml erythrocyte-lysing solution (150 mmol/l NH_4Cl, 10 mmol/l $NaHCO_3$, 1 mmol/l EDTA, pH 7·4), washed the samples with PBS and resuspended in PBS and 1% paraformaldehyde (Sigma). We measured donor cell percentage by dividing FITC-positive cells by total nucleated cells. For bone marrow and spleen evaluation, we incubated 2×10^6 cells in PBS – 5% heat-inactivated fetal calf serum (HIFCS, HyClone Laboratories, Logan, UT, USA) with FITC-CD45·2 mAb and biotin-CD45·1 mAb (PharMingen) for 15 min at 4°C, washed, and secondary labelled with conjugated streptavidin allophycocyanin (SAv-APC) (Molecular Probes, Eugene, OR, USA). Donor chimaerism was calculated as CD45·1/(CD45·1 + CD45·2). For lineage analysis, we labelled bone marrow with rat-anti-mouse Ly6G/GR-1 (inhouse 8C5 hybridoma), CD45R/B-220 (inhouse RA3 hybridoma) or CD3 (T cells) (PharMingen) mAbs. Donor specificity was detected using a mouse-anti-mouse biotin-CD 45·1 mAb (PharMingen). We secondary labelled with a FITC-polyclonal goat anti-rat mAb (Southern Biotechnology Associates, Birmingham, AL, USA) and SAv-APC (Molecular Probes).

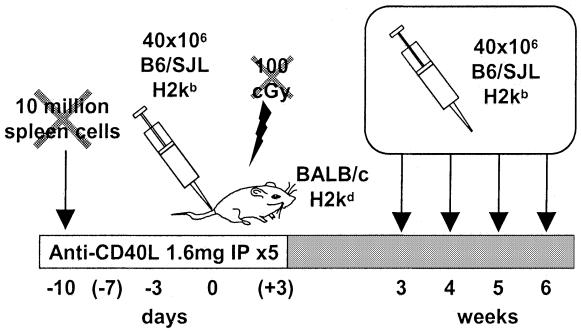

Fig 1. Schematic design of early repetitive transplants protocol. B6.SJL marrow cells were injected to non-irradiated BALB/c recipients on d 0 and weeks 3, 4, 5 and 6 post transplant. Anti-CD40L (3 or 5 × 1·6 mg i.p.) was given without donor spleen cell exposure.

Statistics. We used the non-parametric Wilcoxon rank-sum test for comparison and the trend test developed by Cuzick for testing trend (Cuzick, 1985). The level of significance was set at 0·05 (two-sided). Data are presented as mean ± one standard error of the mean (SEM).

RESULTS

Low-dose irradiation and spleen cell exposure with co-stimulator blockade

In four experiments, we confirmed that high levels of donor marrow chimaerism could be achieved up to 24 weeks post transplant when 40×10^6 B6.SJL cells were transplanted into 1 Gy treated BALB/c hosts with donor spleen cell pre-exposure and CD40L blockade. The mean blood donor chimaerism at 24 weeks for four experiments was 38·2 ± 5·4% ($n = 20$) when spleen cells were given 10 d prior to transplant. When spleen cells were not administered, mean

Table I. Transplant schemes used for allogeneic tolerization.

Model	Spleen cells (B6.SJL on d −10)	BALB/c host irradiation	Anti-CD40L (1·6 mg i.p. on day)	B6.SJL marrow (i.v. on d 0)	Repetitive marrow (40×10^6 on week)
Low-dose irradiation	10^7 or none	1 Gy	−7, −5, −3, 0, +3	40×10^6	none
Delayed repetitive transplant	10^7 or none	none	−7, −5, −3, 0, +3	40×10^6	12 or 12 & 14 or 12, 14 & 16
Early repetitive transplant	none	none	−7, −5, −3, 0, +3	40×10^6	3, 4, 5 & 6
Cell dose *vs* transplant schedule	none	none	−7, −5, −3, 0, +3 or −7, −3, 0	40×10^6 or 200×10^6*	3, 4, 5 & 6 or none

*In two injections of 100×10^6 given 15 min apart.

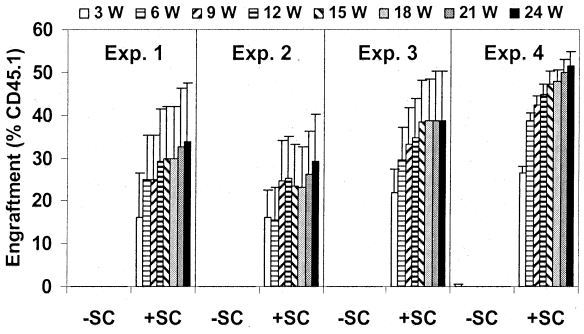

Fig 2. Single allogeneic transplant into 1 Gy irradiated, spleen pretreated and anti-CD40L treated hosts. BALB/c hosts were infused with 0 (–SC) or 10×10^6 B6.SJL spleen cells (+SC) on d −10 and administered 1·6 mg of anti-CD40L antibody on d −10, −7, −3, 0 and +3. On d 0, BALB/c mice were exposed to 1 Gy WBI and infused with 40×10^6 B6.SJL marrow cells. Engraftment of donor cells was determined as percentage (%) CD45·1 cells in peripheral blood at 3–24 weeks post transplantation ($n = 5$).

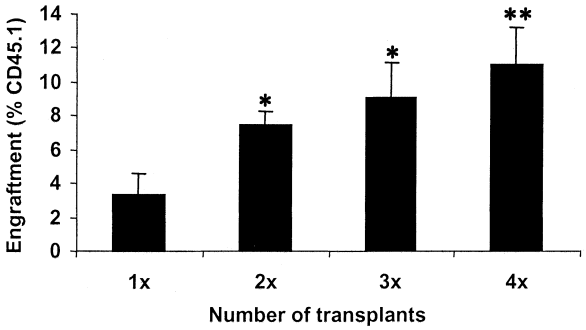

Fig 3. Delayed repetitive allogeneic transplants: combined engraftment at 21 weeks post injection. Marrow cells were injected 1–4 times (1 × – 4 ×) at week 0, week 0 and 12, week 0, 12 and 14 or week 0, 12, 14 and 16 into non-irradiated BALB/c hosts injected with anti-CD40L (d −10, −7, −3, 0 and +3) and spleen cells (d −10). Engraftment in peripheral blood was determined by measuring the percentage of CD45·1 nucleated cells. $^*P \leq 0{\cdot}05$ *vs* 1 × transplant. $^{**}P \leq 0{\cdot}001$ *vs* 1 × transplant ($n = 8$–10).

chimaerism was 0·0 ± 0·0% ($n = 20$) (Table I and Fig 2). In order to address the possible role of engrafting spleen cells in this regimen, BALB/c mice were injected with B6.SJL spleen cells 10 d prior to 1 Gy host irradiation with anti-CD40L administered on d −10, −7, −3, 0 and +3 without the administration of marrow cells. Chimaerism was not demonstrable at 3, 6, 9 or 12 weeks.

Delayed repetitive transplant with or without spleen cell exposure

Irradiation (1 Gy), while minimally myelosuppressive, still exposes the subject to significant irradiation and may be myelotoxic in patients previously exposed to cytotoxic therapies (Ballen *et al,* 2002). Thus, we explored different approaches for obtaining allochimaerism without irradiation. B6.SJL marrow cells (40×10^6) were injected at week 0, weeks 0 and 12, weeks 0, 12 and 14, and weeks 0, 12, 14 and 16. Blood engraftment was then measured at varying times up to 26 weeks post initial marrow infusion. Recipient BALB/c mice all received 10×10^6 spleen cells intravenously on d −10 and anti-CD40L (d −10, −7, −3, 0 and +3). Combined results from two experiments showed a mean blood B6.SJL allochimaerism for one, two, three and four transplants of 3·3 ± 1·3%, 7·5 ± 0·8%, 9·1 ± 2·1% and 11·0 ± 2·1%, respectively, at 21 weeks post cell infusion ($n = 8$–10) (Fig 3). Compared with a single transplant, a significantly higher engraftment was found for two and three transplants ($P \leq 0{\cdot}05$) and four transplants ($P < 0{\cdot}001$). A test for trend was significant ($P < 0{\cdot}01$).

In one of the experiments presented in Fig 3, we evaluated whether donor spleen cell exposure could be eliminated. Recipient BALB/c mice either received 10^7 spleen cells i.v. on d −10 or did not. Anti-CD40L was given. At 26 weeks, for mice given spleen cells, chimaerism was 4·8 ± 1·6% for mice given 40×10^6 B6.SJL marrow cells on d 0. When a 1–3 × additional 40×10^6 marrow cells were infused on weeks 12, 14 and 16, chimaerism was 9·4 ± 0·8%, 10·0 ± 2·5% and 15·7 ± 3·0% respectively ($n = 5$). When spleen cells were eliminated, chimaerism was 1·7 ± 0·8%, 3·6 ± 2·5%, 1·9 ± 1·4% and 12·1 ± 2·2% for one, two, three and four transplants respectively ($n =$ 4–5) (Fig 4). When the results of weeks 21 and 26 were combined, the difference between spleen cell exposure *vs* none was reaching significance ($P = 0{\cdot}054$) for one transplant and was not significant for four transplants ($P =$ 0·17). Thus, the requirement for spleen cells to facilitate marrow engraftment was no longer apparent with a total of four marrow cell injections.

Early repetitive transplant without spleen cell exposure

We next evaluated the allochimaerism that would result when B6.SJL marrow was given to BALB/c hosts earlier

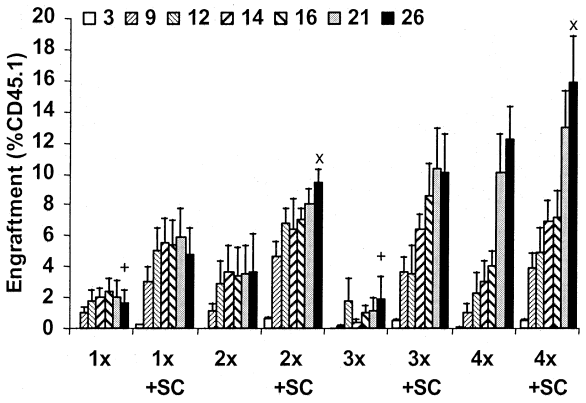

Fig 4. Delayed repetitive allogeneic transplants: evaluation of spleen pre-exposure. Transplant was performed in the same way as described in Fig 3 (1–4 times) with (+SC) or without spleen cell pre-exposure (d −10). Engraftment in peripheral blood is shown. At week 26, significant differences were found between the 1 × +SC and the 2 × +SC or 4 × +SC groups (×), and between the 4 × and the 1 × or 3 × groups (+) (all $P < 0{\cdot}05$, $n = 4$–5).

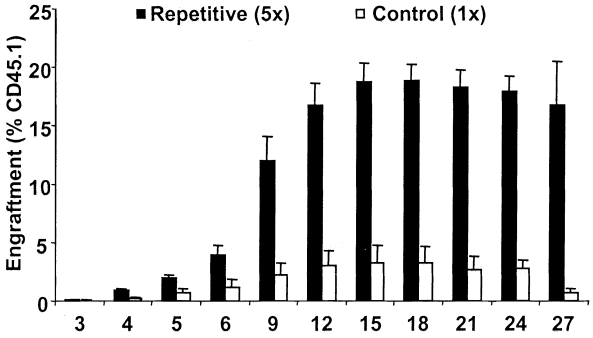

Fig 5. Early repetitive allogeneic transplants without irradiation or spleen cell exposure. B6.SJL marrow cells (40×10^6) were injected on d 0 (controls) or d 0, weeks 3, 4, 5 and 6 (repetitive) into non-irradiated BALB/c hosts injected with anti-CD40L (1·6 mg i.p. d −10, −7, −3, 0, +3), without spleen cell pre-exposure. Engraftment was measured in blood as percentage of CD45·1 nucleated cells from week 3–27 post transplant. All differences were significant from week 4 to week 27 ($P \leq 0{\cdot}021$).

after the initial infusion of marrow cells. We also added an additional 40×10^6 cells infusion. Thus, BALB/c mice received a total of five infusions of 40×10^6 B6.SJL marrow cells given at time 0, and at 3, 4, 5 and 6 weeks after initial infusion. Anti-CD40L was given on d −10, −7, −3, 0 and +3 (Fig 1). Engraftment was measured in blood from 3 to 27 weeks post transplant. Maximum engraftment reached a plateau at 15 weeks and persisted for at least 27 weeks. The average engraftment from week 15–27 was 18·1 ± 0·8% *vs* 2·6 ± 0·4% in the $1 \times 40 \times 10^6$ control group (Fig 5). The mice did not present any clinical signs of acute or chronic GVHD and appeared healthy. At week 24, one mouse died in each group secondary to anaesthesia for blood collection. The difference between the five times transplant and the control ($1 \times 40 \times 10^6$ cells) groups was significant from week 4 to week 24 (all $P = 0{\cdot}009$, $n = 5$) and at week 27 ($P = 0{\cdot}021$, $n = 4$).

Comparison of cell dose with transplant schedule

We tested whether the total cell dose or the schedule of marrow infusion was important for B6.SJL allogeneic engraftment, in non-irradiated, anti-CD40L treated BALB/c mice. Results of engraftment with 200×10^6 B6.SJL bone marrow cells given at time 0 were compared with results with 40×10^6 marrow cells given five times at weeks 0, 3, 4, 5 and 6 post transplant (total dose of 200×10^6). We also tested the effect of reducing the anti-CD40L dose from $5 \times 1{\cdot}6$ mg i.p. to $3 \times 1{\cdot}6$ mg i.p. No spleen cell pre-exposure was employed and no host irradiation was administered (Fig 1). Engraftment was measured in blood at 8, 21 and 57 weeks post transplant (Fig 6). When 200×10^6 cells were given as a single injection (with five injections of CD40L), engraftment approached 0, whereas when the same total dose was given as $5 \times 40 \times 10^6$ on weeks 0, 3, 4, 5 and 6 post transplant (with five or three injections of anti-CD40L), the combined blood engraftment was 3·6 ± 0·7%, 11·7 ± 2·9% and 6·2 ± 1·2% ($n = 8$–9) at 8, 21 and 57 weeks post transplant respectively. Significant differences were found between the $1 \times 200 \times 10^6$ and the $5 \times 40 \times 10^6$ groups for all time points ($5 \times$ or $3 \times$ anti-CD40L) ($P < 0{\cdot}05$). The $1 \times 40 \times 10^6$ group appeared to give more, albeit minimal, engraftment than the 200×10^6 group. The two anti-CD40L doses ($5 \times$ *vs* $3 \times$) were not significantly different, but at 57 weeks, chimaerism was present in 4/4 and 3/4 mice in the $5 \times$ CD40L and

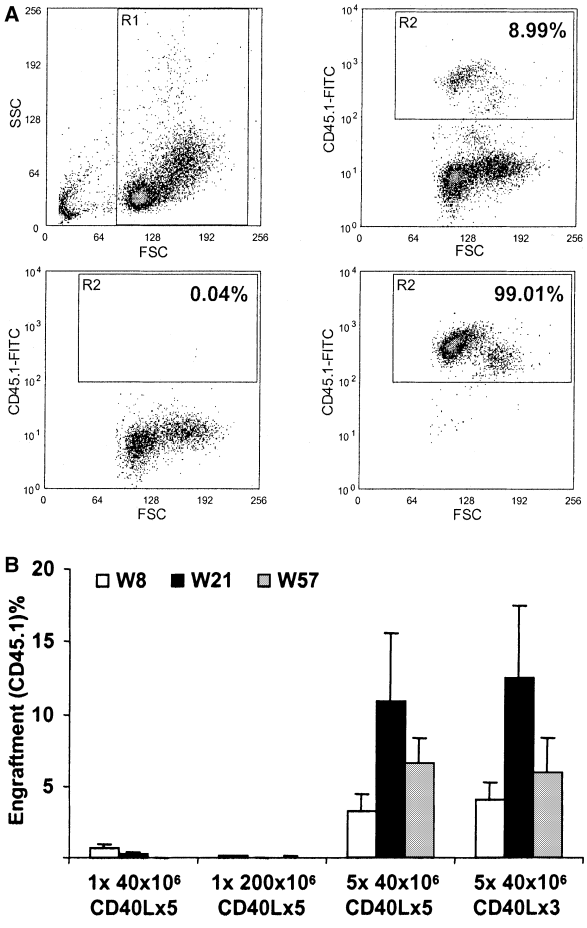

Fig 6. Comparison of total cell dose *vs* fractionated schedule. Forty (Group 1: $1 \times 40 \times 10^6$ control) or 200×10^6 (Group 2: $1 \times 200 \times 10^6$) B6.SJL marrow cells were injected on d 0 into non-irradiated BALB/c hosts injected with anti-CD40L (1·6 mg i.p. d −10, −7, −3, 0, +3). Marrow cells (40×10^6) were given on d 0, week 3, 4, 5 and 6 with anti-CD40L (1·6 mg i.p. d −10, −7, −3, 0, +3) (Group 3: $5 \times 40 \times 10^6$, CD40L × 5) or reduced dose of anti-CD40L (1·6 mg i.p. d −10, −3, 0) (Group 4: $5 \times 40 \times 10^6$, CD40L × 3). (A) Example of peripheral blood FACS analysis on mouse 2, Group 3 at week 57. Nucleated blood cells were gated on the forward and side scatter plot (R1, upper left). The percentage of FITC-positive cells (CD45·1) was then measured (R2, upper right). Negative BALB/c (bottom left) and positive B6.SJL (bottom right) blood controls are shown. (B) Donor engraftment was measured in blood at week 8, 21, and 57 post transplant. Significant differences were found between the two $5 \times 40 \times 10^6$ groups *vs* $1 \times 200 \times 10^6$ group at 8 and 21 weeks (all $P \leq 0{\cdot}05$). No difference was found between the two anti-CD40L doses.

3 × CD40L groups respectively. One mouse died of anaesthesia for blood collection in each high-dose group (200×10^6 and $5 \times 40 \times 10^6$ with two anti-CD40L doses). No mouse showed evidence of GVHD.

At 57 weeks, the animals were sacrificed, and bone marrow (BM) and spleen were analysed for engraftment. Similarly to blood, when 200×10^6 cells were injected on d 0, engraftment was 0·0 ± 0·0% and 0·0 ± 0·0% in BM and spleen respectively (data not shown). The $5 \times 40 \times 10^6$ groups (5 × and 3 × anti-CD40L) showed 3·0 ± 0·7% and 2·7 ± 1·8% for BM, and 7·6 ± 1·6% and 6·8 ± 3·4 for spleen (Fig 7A). As for blood engraftment, significant difference was also observed between the $1 \times 200 \times 10^6$ dose and the two $5 \times 40 \times 10^6$ groups ($P < 0.05$), while these latter did not significantly differ from each other.

Lineage analysis was performed on bone marrow of the $5 \times 40 \times 10^6$ cell groups (5 × or 3 × anti-CD40L), using CD45·1 and lineage-specific markers B220 (B cells), CD3 (T cells) and GR1 (granulocytes). All chimaeric mice showed trilineage engraftment (Fig 7B and C).

DISCUSSION

We have confirmed that, using a high dose of marrow cells, CD40L blockade, low-dose irradiation and spleen cell pre-exposure, high levels of chimaerism can be obtained in a fully mismatched transplant model. Spleen cell pre-exposure and irradiation could both be eliminated using higher marrow cell doses given as repeated infusions at delayed times after initial transplant. When 40×10^6 marrow cells were given at d 0, week 12, 14 and 16 with anti-CD40L and no irradiation or spleen cell exposure, chimaerism averaged 12·1% and was not significantly different from the spleen-cell-exposed mice. Based on these observations, we designed a new transplant schedule where recipient BALB/c mice received 40×10^6 B6.SJL marrow cells on d 0, week 3, 4, 5 and 6 with anti-CD40L alone. We showed stable, long-term multilineage engraftment, averaging 18·1%. These observations demonstrated that irradiation is not

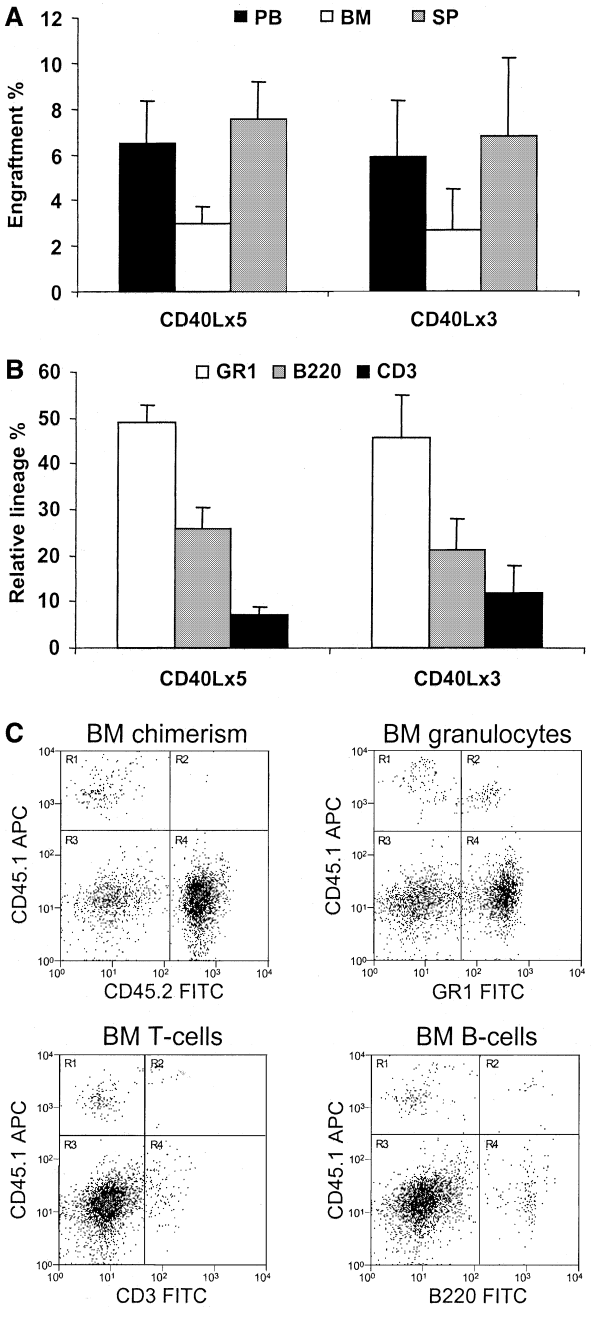

Fig 7. Cell dose *vs* fractionated schedule: 1 year chimaerism and lineage. Bone marrow was transplanted as indicated in Fig 6. At 57 weeks post initial transplant, mice were sacrificed. The two groups having received early repetitive marrow infusions ($5 \times 40 \times 10^6$ marrow cells with five or three doses of CD40L), and showing persistent peripheral blood chimaerism, were analysed for engraftment and chimaerism. (A) We analysed donor engraftment in bone marrow (BM) and homogenized spleen (SP) cells by fluorescence-activated cell sorting (FACS®). A double labelling with CD45·1 (donor) and CD45·2 (host) was used. The chimaerism was calculated as the ratio of $CD45{\cdot}1^+$ over the sum of $CD45{\cdot}1^+$ and $CD45{\cdot}2^+$. Peripheral blood (PB) chimaerism already depicted in Fig 6 is shown for comparison. (B) Relative lineage percentage was analysed in bone marrow of mice with more than 1% chimaerism at week 57. Three different markers were used: GR1 (granulocytes), B220 (B cells), and CD3 (T cells). Specific donor phenotype was identified using CD45·1. There were four and two mice analysed in groups CD40L × 5 and CD40L × 3 respectively. Percentage represents the percentage of CD45·1 cells labelled with a specific lineage antibody. (C) Example of bone marrow FACS analysis on mouse 3, Group 4 at week 57. Nucleated blood cells were gated on the forward and side scatter. A biotinylated CD45·1 antibody secondary stained with Streptavidin-APC was used to identify donor cells. To measure chimaerism in the bone marrow, a FITC-conjugated CD45·2 antibody was used and the ratio of $CD45{\cdot}1^+$ (R1, upper left) over the sum of $CD45{\cdot}1^+$ and $CD45{\cdot}2^+$ (R2, upper right) gave the percentage of chimaerism. Lineage analysis was performed using CD45·1-APC and a GR1 mAb (upper right), CD3 mAb (lower left) or a B220 mAb (lower right). In each histogram, the ratio R2/(R1 + R2) gave the percentage of lineage-positive cells among cells of donor origin.

needed to obtain engraftment and, thus, any form of myeloablation or host stem cell depletion is not necessary to obtain stable long-term engraftment. In a syngeneic model (male into female BALB/c), when 40×10^6 marrow cells were injected, in the absence of irradiation, the average engraftment was 7·7% (Colvin *et al,* 2000). We hypothesize that the final chimaerism obtained results from a direct competition between host and donor stem cells. A theoretical application of this hypothesis would predict that 40×10^6 donor cells divided by 525×10^6 host cells, which is the measured BALB/c total mouse marrow cellularity (Lambert *et al,* 2000), equals 7·6%. This theoretical result corresponds to the observed result, thereby supporting our hypothesis. When the same calculation is applied to the allogeneic system, 200×10^6 over 525×10^6 should give 38% in the absence of an allogeneic effect. The measured result (17·9%) corresponds to approximately 50% of the engraftment observed in a syngeneic transplant. Whether this decreased efficiency is a pure allogeneic effect or is due to strain differences remains an open question.

Interestingly, the role of irradiation may be deleterious to engraftment as the four experiments done with low-dose irradiation (1 Gy) and absent spleen cell pre-exposure (Fig 2) gave no chimaerism while when irradiation was omitted; the $1 \times 40 \times 10^6$ groups without spleen cells gave consistently low but significant engraftment. This is consistent with the observations of Hendrikx *et al* (1996) that irradiation decreases homing of marrow cells to bone marrow and, thus, decreases engraftment potential. Furthermore, irradiation may inhibit important host regulatory cells involved in tolerance.

The question remained whether the cell dose alone determined engraftment or whether the cell administration schedule was important. When comparing the infusion of 200×10^6 marrow cells on d 0 with the administration of the same total dose in five fractionated doses of 40×10^6 (d 0, week 3, 4, 5 and 6), the chimaerism was obtained only in the fractionated schedule. Previous work in a syngeneic male BALB/c to female BALB/c transplant model has indicated that 200×10^6 in one injection gave the same level of engraftment as when 40×10^6 cells were given daily for 5 d, and engraftment was measured at 7–14 weeks post cell infusion (Ramshaw *et al*, 1995). These observations indicate that a single injection should provide the recipient with the same number of transplantable cells as multiple smaller injections. Thus, the marked difference in engraftment observed in the allogeneic setting (H2-mismatch) between 200×10^6 marrow cells given in one or five times over several weeks can be attributed to a time-dependent specific allogeneic effect. Schedule-related tolerance could be dependent on marrow microchimaerism achieved from time 0 injection, tolerizing for later marrow engraftment in a similar fashion as occurs with solid organ engraftment. Whether this is due to antigen level, or critical ratios of donor or host regulatory cells, remains an open question.

These cellular kinetic approaches have allowed for the elimination of irradiation or antigen pre-exposure. We have also shown that a 40% reduction in anti-CD40L did not adversely influence engraftment. This was expected given the long serum half-life of antibody (> 15 weeks) after the five injection schedule (Quesenberry *et al,* 2001). Further reduction in antibody dose should eventually be feasible.

In summary, we have shown that reaching multilineage chimaerism in a fully mismatched transplant model is feasible by establishing an initial microchimaerism using a relatively high dose of marrow cells and co-stimulatory blockade alone. The secondary enhancement of chimaerism is obtained by repetitive administration of marrow cells either early (3 weeks) or late (12 weeks) after initial infusion, and persists for more than 1 year. The achievement of non-toxic allochimaerism in the absence of irradiation is a testable approach in the clinical setting, with appropriate co-stimulation blockade, for correction of haematopoietic genetic diseases or immune therapy of malignancies.

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