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Inducible disruption of the *c-myb* gene allows allogeneic bone marrow transplantation without irradiation

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Abstract

Allogeneic bone marrow (BM) transplantation enables the *in vivo* functional assessment of hematopoietic cells. As pre-conditioning, ionizing radiation is commonly applied to induce BM depletion, however, it exerts adverse effects on the animal and can limit experimental outcome. Here, we provide an alternative method that harnesses conditional gene deletion to ablate *c-myb* and thereby deplete BM cells, hence allowing BM substitution without other pre-conditioning. The protocol results in a high level of blood chimerism after allogeneic BM transplantation, whereas immune cells in peripheral tissues such as resident macrophages are not replaced. Further, mice featuring a low chimerism after initial transplantation can undergo a second induction cycle for efficient deletion of residual BM cells without the necessity to re-apply donor cells. In summary, we present an effective *c-myb*-dependent genetic technique to generate BM chimeras in the absence of irradiation or other methods for pre-conditioning.

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Declaration of interests

The authors declare no competing interests.

Keywords

bone marrow; transplantation; hematopoietic stem cells; chimera; c-myb; conditional deletion

1 INTRODUCTION

1.1 Conventional BM depletion strategies and applications

The replacement of BM by donor hematopoietic stem cells (HSC) has become a cornerstone of today's cancer therapies in human patients (Thomas et al., 1957; Bensinger et al., 2001). In a similar vein, animal BM chimera models have significantly enriched life science research, allowing the study of biological pathways that affect hematopoietic cells (Hellman and Grate, 1967). However, irradiation causes undesirable side effects in the host organism. Dependent on the dose of ionizing irradiation, it induces local or systemic inflammation and may lead to organ failure (Paris et al., 2001). Through alterations of the hematopoietic niche, irradiation elicits leukemic transformation of non-irradiated donor HSC (Chamberlin et al., 1974; Duhrsen and Metcalf, 1990). In the brain, irradiation at doses necessary for BM myeloablation activates microglia and disrupts the blood brain barrier (Diserbo et al., 2002; Lumniczky et al., 2017). This causes BM-derived progenitors to enter the central nervous system and participate in microglia expansion (Priller et al., 2001; Hess et al., 2004). However, in mice with an intact blood brain barrier, e.g. in parabiosis assays or in models shielding the head region during irradiation, BM progenitors do not replenish microglia (Ajami et al., 2007; Mildner et al., 2007). Likewise, epidermal Langerhans cells (LC) are replaced by blood monocytes in response to radiation-induced inflammation (Ginhoux et al., 2006), whereas they are not replaced and self-renew by local proliferation in other BM chimera models (Chorro et al., 2009; Greter et al., 2012; Schulz et al., 2012). Thus, irradiation is a significant confounding factor in experimental research. Alternative chimera models that allow BM replacement following a less invasive conditioning regime are therefore desirable.

Busulfan chemotherapy is currently employed as an alternative pre-conditioning regime for BM transplantation in mice in the absence of irradiation (Wiebe et al., 1992; Westerhof et al., 2000). However, the method seems to be less efficient than irradiation-dependent regimes in achieving a suitable level of chimerism for myeloid cells in peripheral blood (Kierdorf et al., 2013). Further, the alkylating agent is cytotoxic to HSC and non-hematological tissues causing hepatotoxicity, veno-occlusive disease, sterility, carcinogenicity, and alteration of immune functions (van Pel et al., 2003; Nath and Shaw, 2007). Thus, as with irradiation, Busulfan conditioning is troubled by severe toxicities that limit its applications. We therefore developed an alternative approach that allows BM transplantation without conditioning by irradiation or chemotherapy. This chimera model is based on the inducible deletion of the *c-myb* gene, and we here provide a detailed and optimized protocol.

1.2 Role of c-Myb in hematopoiesis

c-Myb is an essential, evolutionary conserved regulator of hematopoiesis (Soza-Ried et al., 2010). It controls the proliferation and differentiation of HSC necessary for the formation of

a functional BM compartment. Absence of c-Myb results in failure of definitive hematopoiesis across species (Gewirtz and Calabretta, 1988; Mucenski et al., 1991; Soza-Ried et al., 2010). We and others have previously shown that induction of *c-myb* gene deletion in $Mx1^{Cre}$:$c\text{-}myb^{fl/fl}$ mice disrupts BM hematopoiesis and leads to profound reduction of all hematopoietic lineages (Lieu and Reddy, 2009; Schulz et al., 2012). The rapid depletion of the HSC pool allows the transplantation of donor BM without other pre-conditioning (Schulz et al., 2012; Hess et al., 2013). Monocytes and granulocytes are completely replaced by donor BM-derived cells, and consequently also myeloid cells within tissues that derive from BM progenitors (Schulz et al., 2012). In contrast, macrophage populations resident in tissues, including microglia and epidermal LC, remain of host origin months after transplantation. Whereas HSC-derived myeloid cells are dependent on c-Myb for their maintenance and renewal in adult tissues, resident macrophages persist in tissues in the presence of donor HSC and in the absence of c-Myb (Gomez Perdiguero et al., 2013).

1.3 Development of the protoctol

The BM transplantation model presented here allows a detailed analysis of BM-mediated functions in multiple models. In addition to the replacement of host BM by donor HSCs, the here presented transplantation model might also be useful for competitive assays. For example, researchers could potentially address functions of HSCs from different donors by injecting them simultaneously. Previously, distribution and engraftment of HSCs were mostly studied in parabiotic mice if adverse effects of irradiation were to be avoided (Bunster and Meyer, 1933; Herbert et al., 2008; Kamran et al., 2013).

2 MATERIAL AND METHODS

2.1 Animals

Recipient $Mx1^{Cre}$:$c\text{-}myb^{fl/fl}$:$CD45^{2/.2}$ mice were of C57Bl/6J genetic background. $CD45^{1/.1}$ mice of the same sex and genetic background were used as BM donors to allow easy quantification of the extent of chimerism by flow cytometry. Donor strains with fluorescence expression in HSC-derived cells are also suitable for an easy quantification of chimerism. Donor and recipient mice were between 12 and 20 weeks of age.

The mice are available from both public repositories as well as from commercial dealers. For example, $Mx1^{Cre}$ can be ordered from EMMA archive (strain ID: EM 01143) or The Jackson Laboratory (JAX, strain ID: 003556); $Myb^{fl/fl}$ from JAX (strain ID: 028881) or the KOMP Repository(Skarnes et al., 2011); CD45.1 mice from the RIKEN BRC facility (strain ID: RBRC00144), Charles River (strain ID: 564) or JAX (strain ID: 002014). This list is intended for information purposes and is not exclusive.

All experimental animals must be handled according to governmental and institutional regulations regarding the use of animals for research purposes. All animal experiments in this article were approved by the Government of Bavaria (Regierung von Oberbayern), Germany.

2.2 Reagent Setup: poly(I:C)

To prepare poly(I:C) high molecular weight 50 mg (Cat. # tlrl-pic-5, InvivoGen) aliquots we recommend to work under sterile conditions (laminar flow cabinet). Add 50 ml of endotoxin-free physiological water (supplied) to the 50 mg poly(I:C) vial and heat solution for 10 minutes to 68°C (Thermomixer, Eppendorf). Mix by pipetting gently up and down until the poly(I:C) is completely dissolved, then let the solution cool down for 1 hour at room temperature (20°C) to allow proper annealing. This preparation step is critical since rapid cooling strategies have a negative influence on poly(I:C) quality and decrease its effectiveness due to improper annealing. Additionally, the potency of poly(I:C) might vary between different providers.

2.3 Antibiotic treatment

Prophylactic antibiotic treatment was performed by adding 400 mg Sulfamethoxazol + 80 mg Trimethoprim (Cotrim, Cat. # PZN-03928197, Ratiopharm GmbH) to acidified drinking water (pH 2.7–3.0) (Duran-Struuck and Dysko, 2009).

2.4 Poly(I:C)-mediated bone marrow depletion

Transfer the poly(I:C) solution (1 μl = 1 μg) at a dose of 10 μg poly(I:C) per g BW into 1 ml syringes. Inject with a 26G needle into the peritoneum. Injections are carried out every other day for n= 3–5 times.

2.5 Isolation of donor bone marrow

Sacrifice the donor mouse by cervical dislocation in deep isoflurane anesthesia (Cat. # AP/DRUGS/220/96, CP Pharma). Isolate the bones from all surrounding tissue using a scalpel and transfer them into sterile PBS (Dulbecco's Phosphate Buffered Saline, Cat. # P5493-1L, Sigma-Aldrich) in a Petri dish on ice (4°C). To ensure isolation of BM cells in sufficient numbers, we recommend the use of at least 4 bones per planned transplantation (preferably humerus and femur, however, radius and tibia can be used as well). Proceed with bone marrow isolation at a sterile bench.

Cut the bone on both ends with sterile scissors to remove the epiphysis and open the BM cavity. Flush the bone marrow with 10–15 ml ice-cold (4°C) PBS supplemented with 2% FCS (vol/vol) (Cat. # S 0615, Biochrom GmbH) using a 26G needle. Prepare a single cell suspension by repetitive pipetting with a large (20G) needle attached to a 10 ml syringe. Pass the single cell suspension through a 70 μm cell strainer into a 15 ml Falcon tube. Centrifuge at 300 g (4°C) for 5 minutes and discard supernatant. For efficient erythrocyte lysis, resuspend the cell pellet in 3 ml erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA) and wait for 5 minutes at room temperature. Thereafter add 30 ml of PBS containing 2 mM EDTA (Cat. # 03690-100ML, Sigma-Aldrich) to stop erythrocyte lysis reaction.

Centrifuge at 300 g (4°C) for 5 minutes and discard the supernatant. Resuspend the cell pellet in 50 –100 μl PBS and keep on ice. Take 1 μl of this suspension for cell counting after staining with Trypan Blue (mix one volume of cell suspension with one volume of Trypan blue, dead cells will appear blue under a bright field microscope; Cat. # T8154, Sigma-

Aldrich). Adjust to the desired cell concentration in a volume of 200 μl (per mouse) for injection. Throughout this part of the protocol, keep cells at 4°C and avoid unnecessary resting periods.

2.6 Bone marrow transplantation

On the day after the last poly(I:C) application, inject the desired number of donor cells into recipient mice. We recommend to inject 10×10^6 cells in a volume of 200 μl PBS intravenously in the tail vein using a 26G needle.

2.7 Analysis of chimerism

4–6 weeks after transplantation, collect 25 μl of blood from chimeric mice into EDTA-coated tubes (Microvette® 100 μl, Cat. # 20.1278.100, Sarstedt AG & Co). Add 3 ml of room temperature 1x erythrocyte lysis buffer and incubate for 5 minutes. Erythrocyte lysis is important to allow easy identification of leukocyte subpopulations by flow cytometry. After 5 minutes of cell lysis, add 4 ml of PBS containing EDTA (2 mM) to stop erythrocyte lysis reaction and centrifuge at 300 g (4°C) for 5 minutes. Thereafter, stain your cell samples with desired antibodies and prepare the samples for analysis according to standard flow cytometry protocols.

3 RESULTS

3.1 Experimental design

We here describe a *c-myb*-dependent genetic technique to generate BM chimeras in a three-step protocol over six weeks. The protocol involves a BM depletion period of 4–8 days by poly(I:C)-mediated disruption of the *c-myb* gene, the isolation of donor cells (1 hour) and subsequent BM transplantation (1 hour) to achieve a chimerism over 95% after about six weeks (Fig. 1).

We recommend a concomitant antibiotic treatment to prevent opportunistic infections in the period of BM depletion. Therefore, mice are treated with Cotrim (400 mg Sulfamethoxazol + 80 mg Trimethoprim) in acidified drinking water (pH <3 by addition of hydrochloric acid to reduce bacterial contaminations) available ad libitum (Duran-Struuck and Dysko, 2009) (Fig. 1).

3.2 Poly(I:C)-mediated BM depletion

Polyinosinic:polycytidylic acid (poly(I:C)) is a toll-like receptor-3 (TLR3) agonist and a potent inducer of the Mx1 promoter, thus enabling gene targeting in mice (Kuhn et al., 1995). We have previously shown that a repetitive 7-fold injection of poly(I:C) every other day leads to deletion of BM cells and a high level of chimerism for myeloid cells in peripheral blood (Schulz et al., 2012). However, earlier studies suggested that deletion can be achieved with three injections of poly(I:C) (Kuhn et al., 1995). In this protocol, we show determined that repetitive (n=3–5) injections of poly(I:C) induce efficient depletion of BM hematopoiesis in $Mx1^{Cre}$:*c-myb*$^{fl/fl}$ mice, as demonstrated by myeloablation in peripheral blood (Fig. 2).

3.3 Isolation and transplantation of donor HSCs

After effective BM depletion, we isolated *CD45*$^{.1/.1}$ donor HSCs and transferred them to *CD45*$^{.2/.2}$ depleted recipients on the day after the last poly(I:C) injection. Consequently, a switch from CD45$^{.2/.2}$ to CD45$^{.1/.1}$ expressing leukocyte populations is observed in peripheral blood (Fig. 3a). The chimerism of individual cell populations gradually increases after BM transplantation. While short-lived cells such as granulocytes are rapidly replaced, other cells like lymphocytes persist significantly longer (Fig. 3b). Chimerism in steady state is typically analyzed 6 weeks after BM transplantation (Flomerfelt and Gress, 2016).

3.4 Poly(I:C)-mediated BM transplantation prevents tissue injury

In contrast, tissue-resident macrophages, like brain microglia and epidermal LCs, are not affected by this protocol and remain of recipient origin (Fig. 3c). This is of particular interest since conventional irradiation-based models have been shown to cause tissue injury, which leads to loss of resident macrophages and their replacement by BM-derived cells (Ginhoux et al., 2006). It is noteworthy, that similar strategies applied in humans have led to analogue results: Lung alveolar macrophages are replaced by BM-derived cells after irradiation (Thomas et al., 1976), whereas alveolar macrophages persist in transplanted lung tissue for many years after grafting (Eguiluz-Gracia et al., 2016). Likewise, human epidermal LC persist in donor skin after forearm transplantation (Petruzzo et al., 2003; Kanitakis et al., 2004; Kanitakis et al., 2011). Together, we here describe a protocol that allows efficient BM transplantation in the absence of irradiation, thereby maintaining host tissue macrophages.

3.5 Optimizing poly(I:C) treatment cycles

Survival rates depend on the extent and duration of myeloablation, and is therefore associated with the amount of poly(I:C) treatment cycles. In our hands, we achieved a chimerism >90% with a 100% survival rate by applying 3 injections every other day within a 6-day treatment period (Fig. 4a, b). In comparison, in models of allogeneic BM transplantation using irradiation survival rates of 80–90% have been described (Cui et al., 2002; Guest et al., 2015). The relative percentage of chimerism critically depends on the extend of BM depletion before transplantation. Short poly(I:C) injection strategies increase survival rates and are associated with a lower percentage of chimerism. Vice versa, extensive poly(I:C) injections provide better rates of chimerism with increasing lethality. Therefore, we analyzed these two parameters in relation to poly(I:C) treatment cycles and identified three injections to provide optimum results in our setting (Fig. 4a, b).

3.6 Alternative protocol in case of low chimerism

In the case of insufficient chimerism after a first (usually short) round of poly(I:C)-mediated BM depletion, a second round of poly(I:C) injections is possible and recommended. During this period, mice are protected from opportunistic infections by previously transplanted donor BM cells and can therefore be easily treated with additional poly(I:C) injection cycles. This strategy increases the rate of chimerism of initially about 60% after two injections to almost 100% after four additional injections of poly(I:C) (Fig. 4c).

4 DISCUSSION

We provided a detailed protocol that allows efficient generation of BM chimeras harnessing a conditional, *c-myb*-dependent HSC depletion model. While we found three poly(I:C) injections to give optimal results, chimerism and survival rates might vary in dependence on various factors, such as the experience of the scientist in handling mice (e.g. tail vein and i.p. injections), environmental conditions (e.g. animal husbandry) and other factors (e.g. mouse genetic background). In the following, we therefore discuss potential pitfalls in BM transplantation procedures as well as specific aspects related to the conditional model described here (Tab. 1).

4.1 Level of expertise needed to implement the protocol

The protocol requires experience in animal handling and careful daily observations for signs of sepsis. To ensure a sufficient standard of hygiene to obtain BM chimeras, harvesting of marrow cells should be performed under sterile conditions. This protocol has been successfully performed in mice between 12 and 20 weeks of age with a body weight between 20 and 25g. It is suited for female and male mice. Possible modifications with younger mice need to be evaluated.

4.2 Limitations

(i) Potential side effects of poly(I:C)—As discussed above, poly(I:C) induces type I interferons, thus mimicking inflammatory responses to systemic viral infection (Alexopoulou et al., 2001). Therefore, an increase in body temperature of 0.5–1.0°C during a period of 3–6 hours can occur (Hopwood et al., 2009; Zhu et al., 2016). In rats, a temperature rise of up to 1.6°C above baseline 3 hours after poly(I:C) injection has been described (Fortier et al., 2004). This can be accompanied by a reduced cage activity during that time. Thereafter, mice should be back to normal behavior. The dose described here of 10 μg/g body weight (BW) poly(I:C) injected at 2-day intervals is overall well tolerated. A reduction in the number of thymocytes, known to be sensitive to stress (Haeryfar and Berczi, 2001), has been described (Kuhn et al., 1995). Function of other organs, as analyzed by histology or flow cytometry, was not altered (Kuhn et al., 1995; Schulz et al., 2012). Rather, at this lower dose poly(I:C) was reported to induce tolerance to LPS and reduce liver injury(Jiang et al., 2005). At 20 μg/g BW, poly(I:C) induces focal necrosis in the liver (Dong et al., 2004) and at 30 μg/g BW injury of the small intestine has been reported (Zhou et al., 2007). Thus, the dose of the inducing agent poly(I:C) applied in our protocol is efficient and causes little side effects. Further, we agree with previous reports that its transient effects do not impair the utility of this inducible gene targeting system (Kuhn et al., 1995).

(ii) Reduced housing hygiene may induce background recombination—Suboptimal hygiene standards as found in conventional mouse housing promotes endogenous interferon production, which in *Mx1*^{Cre} mice can induce background recombination. This might alter the response to administered poly(I:C). Recombination of up to 20% in splenic tissue of untreated *Mx1*^{Cre} mice has been reported (Kuhn et al., 1995), whereas background recombination in the BM is unknown. We therefore suggest that all mice are maintained under specific-pathogen-free conditions. Nevertheless, we have

successfully applied the *Mx1*^{Cre} model under conventional housing conditions in previous work (Schulz et al., 2012). Alternatively, researchers could switch to an inducible system that is more tightly controlled, however, this may come at the expense of excision rate (Feil et al., 1996). Further, background recombination is a feature of various mouse strains and has also been reported for estrogen (ER^{T}) or tetracycline (tTA/tetO) dependent systems (Kemp et al., 2004; Bersten et al., 2015).

(iii) Genetic model requires cross-breeding—Whereas BM transplantation based on irradiation or chemotherapy can be carried out with most strains available, our model depends on the deletion of the *c-myb* gene and therefore requires homozygosity of the floxed alleles (i.e. *c-myb*$^{fl/fl}$) in mice as well as the presence of an inducible deleter Cre (e.g. *Mx1*^{Cre}). Therefore, cross-breeding is required to establish this strain. However, all mice discussed in this protocol are available both from public repositories as well as from commercial dealers (see method section).

4.3 Scope and applicability

The *c-myb*-dependent transplantation model described here is efficient in generating BM chimeric mice. Further, macrophages are not replaced in tissues following BM transplantation, which allows to adequately address their biological functions. The latter represents an important issue, since macrophages have been shown to populate most organs during early fetal development and are maintained by limited self-renewal (Schulz et al., 2012; Hashimoto et al., 2013; Yona et al., 2013; Mass et al., 2016; Mass et al., 2017). Both their ontogeny as well as the local organ environment contribute to shaping the cellular identity of tissue-resident macrophages (Gosselin et al., 2014; Lavin et al., 2014). The here presented BM transplantation model takes the biology of these important immune cells into account, since BM progenitor cells but not mature macrophages in tissues are affected by loss of *c-myb* (White and Weston, 2000; Schulz et al., 2012; Jin et al., 2016). Therefore, the chimera model presented here has a broad range of applications.

Further, we expect that the protocol can be modified by employing the conditional deletion of other transcription factors regulating hematopoiesis downstream of c-Myb. For example, Cited2 is critical for HSC maintenance in humans and mice, and is activated by c-Myb (Zhao et al., 2011). Conditional deletion of the *Cited2* gene in mice results in loss of HSC and BM failure, phenocopying the disruption of *c-myb* (Kranc et al., 2009). Further, *Cited2*$^{fl/fl}$ mice are publically available to scientists through public resources, e.g. EMMA archive (stock ID: EM 03111). This further extends the applicability of the model.

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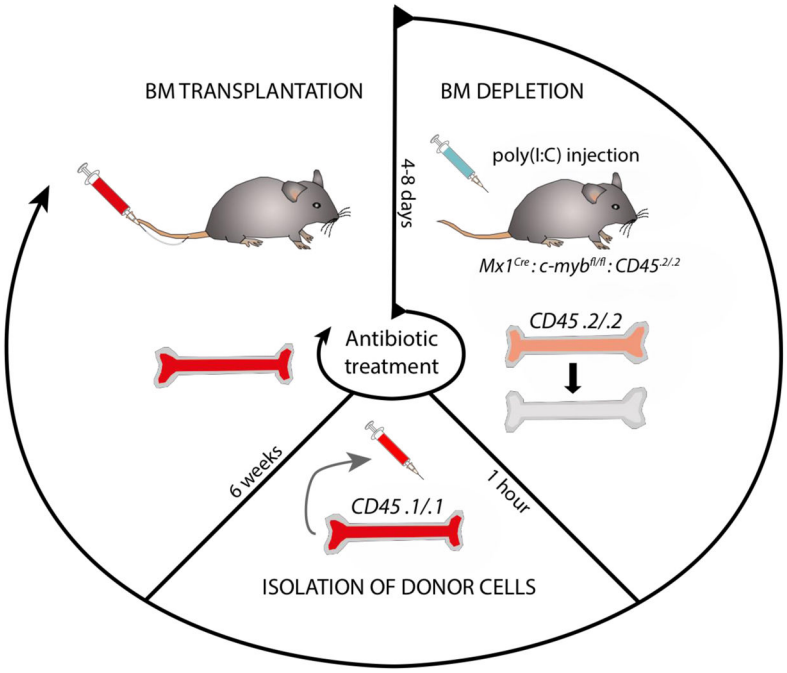

Figure 1. Experimental design

BM depletion is achieved by repetitive injections of poly(I:C) in *Mx1*^{Cre}:*c-myb*$^{fl/fl}$:*CD45*$^{.2/.2}$ mice (n=3–5). *CD45*$^{.1/.1}$ donor BM cells are isolated and injected in BM-depleted recipients to establish BM chimeras. 3 days prior until 30 days after the first poly(I:C) injection, transplanted mice receive a prophylactic antibiotic treatment. Approximate timing is indicated in the graph.

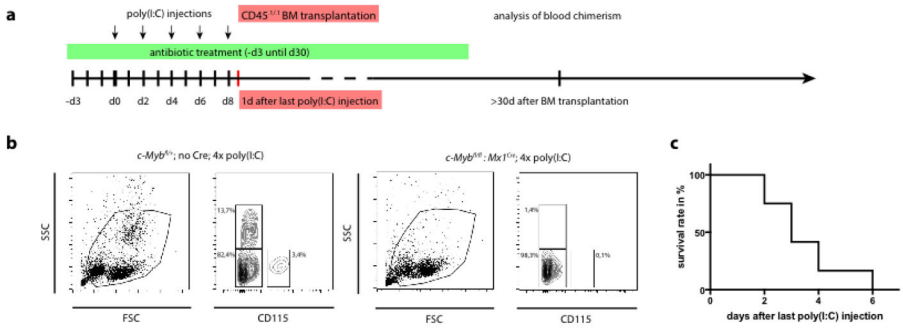

Figure 2. Flow cytometry analysis and survival after BM depletion

(a) Depicted is a time line of the experimental procedure. (b) Blood flow cytometry profile with and without genetic c-Myb-based BM depletion using 4 doses of poly(I:C); analysis 2 days after last poly(I:C) injection. (c) Survival rates after 5 doses of poly(I:C) without BM reconstitution (n=12).

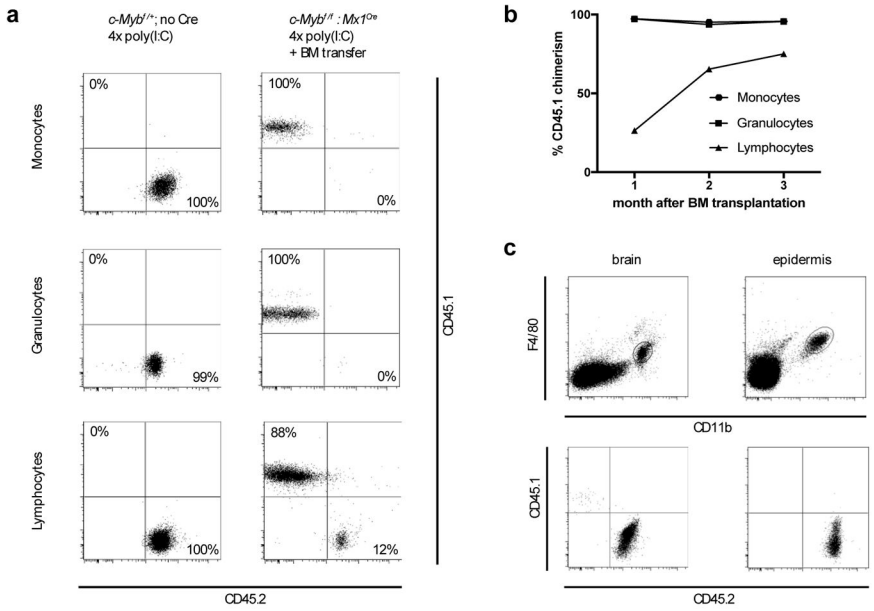

Figure 3. Chimerism after BM transplantation

(a) Blood chimerism in control and BM-depleted mice (4x poly (I:C)) with subsequent BM transfer. (b) Change of chimerism in peripheral blood over time after BM transplantation; graph shows one representative experiment. (c) Analysis of chimerism for tissue macrophages in the brain (microglia) and epidermis (LC).

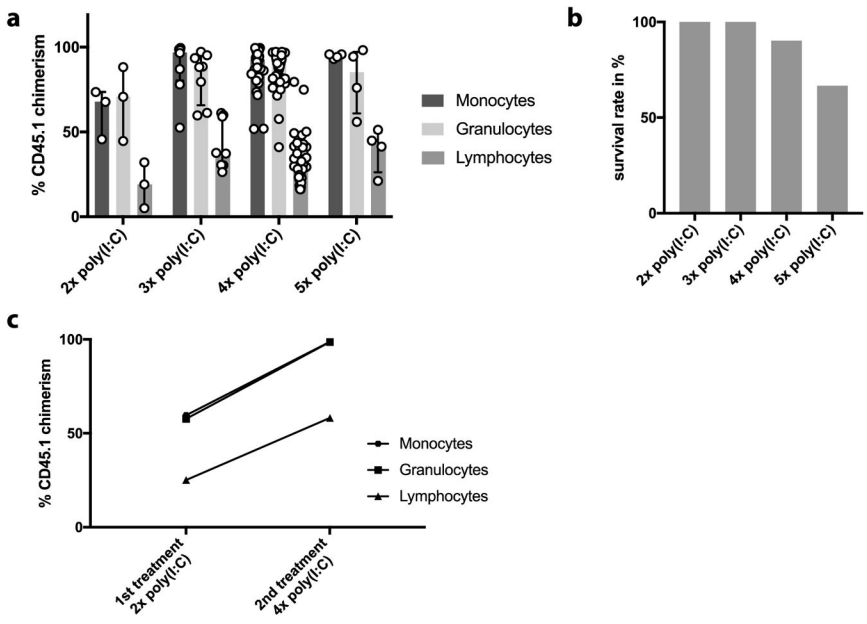

Figure 4. Rates of blood chimerism and survival

(a) Chimerism rate in monocyte, granulocyte and lymphocyte lineages is dependent on treatment duration and number of poly(I:C) injections; error bars indicate median ± interquartile ranges. (b) Bars indicate average survival rate after bone marrow transplantation in relation to the amount of poly(I:C) injections (treatment duration) (at least 4 animals were analyzed per group). (c) Mice receiving 2x poly(I:C) injections presented a low blood chimerism and were re-injected with poly(I:C) in a second round of treatment (4x poly(I:C)), which effectively increased the level of chimerism; graph shows one representative experiment.

Table 1

Troubleshooting.

Problem	Possible reason	Solution
Mice suffer from dehydration	Acidified drinking water might lead to dehydration	Check quality of drinking water; acidifying the water to a pH < 2.7 can result in dehydration (Duran-Struuck and Dysko, 2009)
Early lethality after poly(I:C) injection	Infection due to i.p. injection (contaminated needle, injury of abdominal organs)	Deep anesthesia and careful injection technique with mouse tilted head down
Low quality of donor HSCs	Donor HSCs might be contaminated or partially dead	Check quality of the BM cells isolated (live/dead stain)
		We routinely transplant whole BM in this protocol. In case of problems with this procedure, it could be possible to carry out cell sorting and injection of long- term HSC ($lin^{-}Sca^{-}1^{+}Kit^{+}150^{+}CD48^{-}$) in lower numbers (Gomez Perdiguero et al., 2015).
Low level of chimerism	Poor transplantation procedure (e.g. improper injection, infection)	Revisit transplantation procedures and optimize technique
	Low quality of donor HSCs	Optimize BM quality (see “Low quality of donor HSCs”)
	Insufficient initial BM deletion	Optimize BM depletion strategy (see “Insufficient BM deletion”)
		Independent of the reason for a low level of chimerism in blood, an additional cycle of poly(I:C) treatment can be performed to deplete recipient BM cells (see “Alternative protocol in case of low chimerism”, results section)
Insufficient BM depletion	Poly(I:C) ineffectiveness due to wrong reagent or insufficient annealing	Use reagents as presented in the protocol (check origin and batch), avoid rapid cooling of poly(I:C) solution after heating phase
	BM deletion efficiency depends on mouse strain	Adapt BM deletion protocol for desired mouse strain or switch to alternative genetic background if possible
Difference in levels of chimerism between mice	Poor i.v. injection technique	Optimize intravenous injection technique
	Heterogenous quality of injected BM	Check quality of the BM cells isolated (live/dead stain) (see “Low quality of donor HSCs”)
High lethality during experiment	Mostly due to Infections (health status of recipient mice, husbandry conditions, appropriate injection technique)	Check housing conditions and general husbandry; check techniques for mouse handling
	Dehydration of transplanted mice	Problems due to acidified water (see “Mice suffer from dehydration”)
	Low quality of donor HSCs	Optimize BM quality (see “Low quality of donor HSCs”)