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# Bone marrow chimeras—a vital tool in basic and translational research

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## Abstract

Bone marrow chimeras are used routinely in immunology research as well as in other fields of biology. Here, we provide a concise state-of-the-art review about the types of chimerisms that can be achieved and the type of information that each model generates. We include separate sections for caveats and future developments. We provide examples from the literature in which different types of chimerism were employed to answer specific questions. While simple bone marrow chimeras allow to dissect the role of genes in distinct cell populations such as the hematopoietic cells versus non-hematopoietic cells, mixed bone marrow chimeras can provide detailed information about hematopoietic cell types and the intrinsic and extrinsic roles of individual genes. The advantages and caveats of bone marrow chimerism for the study of microglia are addressed, as well as alternatives to irradiation that minimize blood-brain-barrier disruption. Elementary principles are introduced and their potential is exemplified through summarizing recent studies.

**Keywords** Bone marrow chimeras · Tools · Stem cells · Hematopoiesis · Irradiation damage

## Abbreviations

| | |
|---|---|
| BM | Bone marrow |
| BMC | Bone marrow chimera(s) |
| CNS | Central nervous system |
| DC | Dendritic cells |
| EAE | Experimental autoimmune encephalomyelitis |
| GFP | Green fluorescent protein |
| HLA | Human leukocyte antigen |
| HSC | Hematopoietic stem cell(s) |
| ILC | Innate lymphoid cells |
| KO | Knockout |
| MHC | Major-histocompatibility complex |
| TBI | Total body irradiation |
| WT | Wildtype |

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## Introduction

All immune cell types, as part of the hematopoietic system, undergo constant renewal from hematopoietic stem cells (HSC). This feature is exploited in research by generation and use of bone marrow chimeras (BMC). In this review, we want to introduce the reader into the various applications of BMC in biomedical research and discuss their advantages but also their pitfalls. In contrast to the situation in humans or in most other animal species, the frequent use of inbred mouse strains makes application of BMC for research purposes comparatively easy. Our discussion will thus focus on the mouse, but the concepts can be applied to other species where inbred lines, clones, or identical siblings are available.

As a basic principle, BMC are obtained by transplanting bone marrow (BM) or purified HSC between different subjects. The development of BMC dates back to the middle of the twentieth century. In the aftermath of the second World War and fearing further nuclear conflicts and accidents, there was an increase in medical research towards understanding and treating the consequences of exposure to whole-body irradiation. One of the most affected organs in this context is the

BM. Thus, and contrary to most recent discoveries, BM transplantation has been developed almost in parallel, in animal models as well as human subjects (for a detailed historical perspective of the first 25 years, see the review by Seller in 1970 [1]). Moreover, initial research in animal models was preferentially performed in dogs and other outbred species, since it was acknowledged at the time that inter-subject variability of undetermined factors could negatively affect the success of cell and organ transplantation [2]. As it proved very difficult to identify those factors, experimental animals were chosen in order to mimic the human situation. Initial studies in mice date back to 1949 when Jacobson and colleagues showed that shielding the spleen of mice with lead allowed them to survive otherwise lethal whole-body irradiation [3]. Yet, at that time, the nature of this protection remained unclear: was it dependent on humoral factors or was it cellular reconstitution? In 1956, Ford and colleagues used basic chromosome size and morphology to show that protection against irradiation was mediated by repopulation through donor cells [4]. These initial reports paved the way for the BM transplantation field, a field intimately linked to leukemia treatment and graft versus host disease. Accordingly, in 1956, Barnes and Loutit showed that mice can be treated for leukemia by irradiation followed by transplantation of normal mouse BM [5]. These studies were translated to humans by Thomas and colleagues who soon realized that only transplants of syngeneic BM, such as those from identical twins, were successful [6, 7]. From then on, transplantation in the human developed into a routinely used treatment for various diseases such as malignancies, autoimmune disorders, and germline mutations. A parallel interest in using BMC as a tool for basic and translational research developed. One prominent example of such studies is the work of Spangrude et al., published in 1988, describing the procedure of isolating mouse HSC and establishing phenotypic markers [8]. Currently, the use of BMC in research is widespread and different approaches have been developed that allow for a higher resolution in the analysis of different cell types and of gene functions, as discussed below and summarized in Fig. 1.

## Generating BMC

The first step in the generation of BMC is to remove the host hematopoietic system. Usually, this is achieved by exposing the animals to full-body gamma irradiation. DNA double strand breaks will lead to induction of cell death especially in rapidly dividing compartments such as HSC and other immune cell precursors. In mice, this cell ablation can be achieved by irradiating with a total of 9–12 Gy in 1 continuous cycle [9] or 2 cycles of 5.5–7 Gy with an interval of 3–4 h [10] for attenuation of side effects. An alternative to irradiation is chemical conditioning by use of busulfan [11, 12]. Ablation of

immune cells by irradiation is usually not complete, with host immune cells surviving for instance at mucosal sites and within the central nervous system. It is therefore important that host and donor-derived cells can be distinguished. One possibility for differentiating between host and graft cells is congenic markers that can be conveniently and reliably detected by flow cytometry. Commonly used surface molecules for that purpose are CD45.1 and CD45.2 or CD90.1 and CD90.2. Also, transgenic expression of fluorescent proteins such as green fluorescent protein (GFP) enables the tracking of donor-derived versus host cells. Researchers interested in studying adaptive immunity frequently use mice deficient in one of the recombination activating genes (*Rag1* or *Rag2*) or severe combined immunodeficiency (SCID) mice as hosts. In such a design, no residual host B or T lymphocytes can impact the analysis. As a consequence, the use of genetic markers is not necessary when the analysis is restricted to these two lineages.

The second step is to transplant the HSC or complete BM cells into the host by intravenous injection, or alternatively by intraperitoneal injection, albeit at lower efficacy [13]. Successful generation of BMC depends on several factors such as the histocompatibility between the host and donor, the secondary effects of the irradiation protocol, the presence of pathogens, and the age of both donor and host animals. Histocompatibility issues were the reason why so many studies have failed in the early days of BM transplantation, when the existence of polymorphisms in major-histocompatibility complex (MHC) genes, the human leukocyte antigen (HLA) and the mouse H-2 antigens, were not known. Irradiation itself can cause gastrointestinal dysfunction as the gut is the site of a large population of highly proliferative epithelial cells directly affected by the induction of double strand DNA breaks [14]. Moreover, the lack of a functional immune system can give rise to opportunistic infections, such as by *Pseudomonas aeruginosa*, which is a major cause of death in irradiated mice. Hence, irradiated mice must be given antibiotics for at least 2 weeks post-irradiation to avoid opportunistic infections [15]. Even though many scientists prefer to obtain BMC by use of individuals of identical sex or even only among females [10], sex mismatch was for some used as marker for determining chimerism (for example, [16]). There are, however, indications of problems associated with a sex mismatch [17]. Good protocols for optimized procedures to obtain BMC are given by Spangrude [10] as well as by Duran-Struuk and Dysko [18].

## Timeline of immune reconstitution

After transplantation of the BM, immune system reconstitution is self-propagating, with different immune lineages repopulating with individual time courses. In mice, full
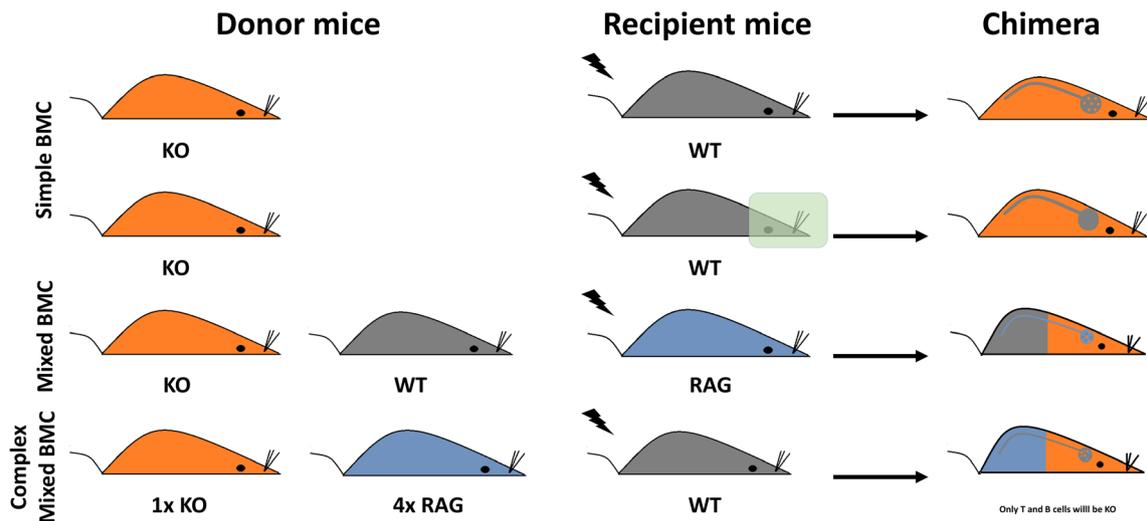
**Fig. 1** Summary of the BMC tools used in research. First row, simple BMC. Second row, simple BMC with head-shield (green)—immune resident cells are not ablated from the central nervous system. Third row, mixed BMC (1:1 ratio)—half of the immune cells of the chimeric mice are knockout (KO). Fourth row, complex mixed BMC (1:4 ratio)—wherein all T and B cells from the chimeric mice are KO, only 20% of the myeloid compartment is KO. Legend: KO knockout, WT wildtype, RAG Rag1 or Rag2 deficient mice that lack mature T and B cells

reconstitution takes around 8 weeks. The first lineages to be quantitatively fully reconstituted are the myeloid cell types, then macrophages, polymorphonuclear cells, and dendritic cells (DC), which are back after just 1 week after the transfer. They are followed by natural killer cells (week 2), B cells (week 3), and T cells (week 8) [19, 20]. Two points are of note: A) upon repopulation, all immune cell subsets overshoot their normal absolute cell counts and stabilization takes considerably longer than repopulation (Fig. 2, [19, 20]) and B) quantitative reconstitution is not always followed immediately by re-establishment of all cellular functions. Whereas DC appear to be fully functional after regaining their population size, NK cells only become fully functional 1 week after achieving full quantitative reconstitution as was found by correlation of cytokine production with reconstitution [20]. Taken together, for a fully functional immune reconstitution after BMC, host animals should be given at least 8 weeks of recovery time.

## BMC for the study of gene function in hematopoietic versus non-hematopoietic cells

In its most simple application, BMC allow to identify the functional consequences of a gene modification to either an irradiation-sensitive cell type, usually hematopoietic, or an irradiation-resistant cell type, usually non-hematopoietic. A plethora of studies have made use of this technique. Here we chose to highlight five recent exemplary studies conveying the experimental design behind the different BMC experiments. As the first example, we want to highlight the merits of a simple BMC experiment using a gene deficiency by summarizing the study of Kreymborg and colleagues [21]. In this

study, the role of B7.H3 in the development of spontaneous prostate cancer in mice was assessed. BMC allowed the researchers to determine that tumor growth was controlled by the presence of B7.H3 on non-hematopoietic cells (Table 1). The authors determined chimerism in their experiments by use of CD45.1/CD45.2 congenic system. This study also presents all the appropriate groups and controls that should be run in such a BMC experiment. Transplantation of gene-modified BM into wildtype and of wildtype BM into gene-modified animals allows to identify the effect distinguishing hematopoietic versus non-hematopoietic cell types. The two other types of chimeras, wildtype into wildtype and mutant into mutant, serve as controls for effects of the BMC procedure per se on cells and organism on wildtype or gene-modified genetic background.

## Mixed BMC for the study of cell type intrinsic vs extrinsic effects of a gene modification

When cells from different genotypes develop under identical conditions within the same individual, any difference observed between them regarding proliferation and differentiation is most likely cell-intrinsic. This can be achieved and exploited in mixed BMC. They allow to determine whether effects of a gene modification are extrinsic or intrinsic within the investigated hematopoietic cell type. Such mixed BMC are generated by, for instance, transplanting the BM of wildtype and that of genetically modified donors in equal proportions into irradiated recipients. This concept was used in the second example study, in which the intrinsic capability of T cells as IL-23-mediated driver of GM-CSF production was identified [22]. Here, the *Rag1*-deficient host precluded
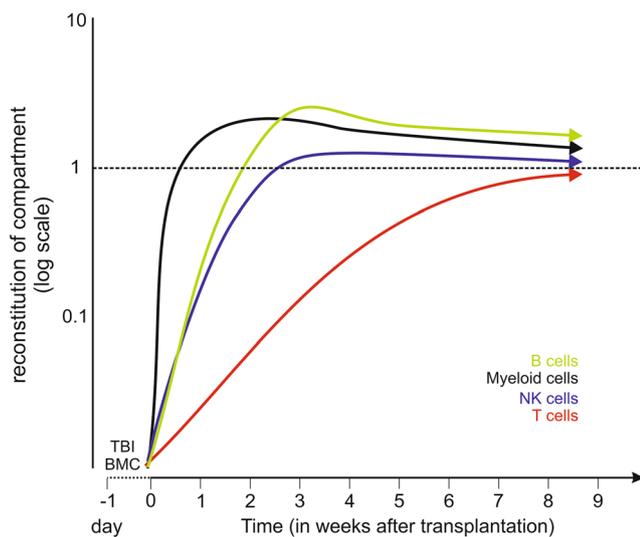
**Fig. 2** Timeline of immune reconstitution after BM transplantation in the mouse. This figure is based on [19, 20]). Legend: TBI total body irradiation

interference from surviving host T and B cells. Donor 1 was wildtype while the cells from donor 2 were deficient for one or the other IL-12 receptor subunit. As these receptors are solely expressed on T and not on B cells, the donor cells were a mix of fully functional B cells (from donor 1 and donor 2) and a mix of T cells in which half of them (from donor 2) were selectively incapable to respond to IL-12 + IL-23, or IL-12 alone (Table 2).

Mixed BMC can also be used to assess the effect of extrinsic factors on the differentiation of two cell populations from different donors in parallel in the same organism. In the third exemplary study, mixed BMC was used to assess the factors underlying impaired NK cell function during aging. Shehata and colleagues addressed the consequences of having NK cells derived from BM of young (CD45.1) and aged (CD45.2) mice transferred into both young and aged host mice [23]. The results suggest that aged NK cells are fully functional provided that the non-hematopoietic environment in which they reside is juvenile. Conversely, NK cells from young donors are not able to fully exert their functions when transferred into an old host. Hence, this study highlights the importance of the microenvironment in cellular function and may impact therapy approaches to NK cell impairment disorders.

## Complex mixed BMC to study the role of an individual gene in a particular cell type

An even more complex BMC approach allows the restriction of gene deficiencies to specific hematopoietic subpopulations. For this experimental design, BM from two donors are needed, one of them lacking the genetic ability to generate a certain

cell lineage. By combining a larger fraction of the lineage-deficient BM with a small fraction of BM carrying the gene modification of interest, cells carrying the gene modification of interest fully reconstitute the lineage-deficient compartment, while all other compartments are comprised mostly of cells proficient for the gene of interest. This experimental design was successfully applied by Gutcher and colleagues, as our fourth example [24] (Table 3), in which donor 1 was deficient for the gene of interest (*Il18r1*) and donor 2 lacked the cell type of interest (T and B cells). As is usual in such experiments, the two different BM were mixed in a 1:5 ratio for reconstitution. This resulted in the selective absence of the gene of interest in T and B cells developing from RAG-proficient but *Il18r1* deficient BM; the majority of cell types not requiring RAG for development expressed *Il18r1*. In this example, RAG-deficiency was used to facilitate filling of these compartments with *Il18r1*-deficient or proficient cells. By reducing the influence of myeloid compartment, the authors were able to observe that deficiency of IL-18 in T and B cells increased EAE severity.

For more analytical specificity, BM from JhT [25, 26] or μMT [27] animals can be used for analysis of the B cell compartment, and BM from Tcrb-deficient animals [28] for analysis of the T cell compartment. Whenever a factor is known whose absence leads to a block in the development of the cell type in question, the respective BM can be used for experiments with unequal ratios. Two examples of this usage are studies using different B cell deficient mice to provide insight into the function of IL-10 in the B cell lineage: 1) using JhT mice in a mixed chimerism setting, Stein and colleagues were able to exclude immunomodulation caused by the production of IL-10 by B cells as the players behind the mechanism of action of transcutaneous immunization [29]; whereas 2) Fillatreau studied the role of IL-10 producing B cells in the recovery remission phase of experimental autoimmune encephalomyelitis (EAE). Using μMT mice to generate mixed chimeras that lack the production of IL-10 in B cells, the authors observed more severe and non-remitting EAE progression than in the control mice [30].

Such lineage-specific BMC may also be obtained by toxin-mediated ablation of a specific cell lineage through use of, for instance, diphtheria toxin A or its receptor. BM from such mice can be used as donor 2, in order to restrict the gene deficiency to the cell type of interest.

## BMC for the study of peripheral and CNS myeloid cells

Within the central nervous system (CNS), BMC studies are very useful in distinguishing microglia, the yolk-sac-derived phagocyte of the CNS, from infiltrating macrophages. Here, the crucial factor is that only the healthy, undisturbed CNS shows a very

**Table 1** Experimental design and major findings using simple BMC. This study aimed to assess whether the role of B7.H3 in tumor progression is mediated by its expression in the hematopoietic system or rather in non-hematopoietic tissue. Therefore, the authors used a simple BMC design with four groups: group 1 is deficient for B7.H3 only in tissue due to transplant of wildtype HSC into B7.H3 knockout mice. Group 2 is the control, in which wildtype cells are transferred to wildtype mice. Group 3 is fully deficient for B7.H3 as knockout HSC were introduced into knockout mice. Group 4 is deficient for B7.H3 in the immune compartment. As wildtype recipients have similar tumor sizes whereas recipients deficient for B7.H3 have larger tumor sizes, regardless of the type of cells transplanted, the authors conclude that stromal B7.H3 expression is regulating tumor development. [21]

| Donor | Recipient | B7.H3 deficiency | Tumor size |
|---|---|---|---|
| Wildtype CD45.1 | B7.H3 KO CD45.2 | Tissue | Larger |
| | B7.H3 WT CD45.2 | None, wildtype situation | Control |
| B7.H3 KO CD45.2 | B7.H3 KO CD45.1 | Tissue and hematopoietic cells, full knockout situation | Larger |
| | B7.H3 WT CD45.1 | Hematopoietic cells | As control |

low level of surveillance by immune cells other than microglia. This feature of the CNS should be preserved so that only brain-resident cells from the host are present at the time of transplantation and no contamination with blood-derived cells into the CNS has taken place. To achieve this, the recipient mice can be head-shielded with a lead cover during irradiation. In our fifth and last example, Mildner and colleagues assessed the role of monocytes in a transgenic mouse model for Alzheimer's disease, by directly comparing whole-body irradiation with head shielding [31]. For identification of resident versus infiltrating macrophages, GFP-labeled BM cells were used instead of a congenic marker. One of the main findings of this study is that exposing the brain to irradiation has consequences to the resident cell population that could bias study conclusions. It should either be avoided or controlled for. An alternative to head-shield is the use of busulfan instead of irradiation for conditioning [32, 33]. Another possibility for populating the brain with a

haematopoietically derived cell types of interest is intranasal administration of HSC [34]. This technique bypasses the need to ablate the BM, thus shortening the process and decreasing adverse side effects. Yet, engraftment appears to be somewhat less effective than after conventional BMC.

## Caveats of using BMC

Box 1 Factors affecting the success of bone marrow chimeras. To successfully generate BMC, the immune system of the recipient mice must be first depleted and then reconstituted in a fashion that does not trigger major adverse side effects. Hence, several factors should be taken into consideration

| | |
|---|---|
| Radiation doses | Irradiating mice two times with a lower dosage, over a time period around 7 h, allows for a better ablation of the recipient hematopoietic compartment than a one-time irradiation protocol. It also reduces gastrointestinal side effects. |
| Mouse strain and detection markers | GFP expression in mice can lead to an anti-GFP response that can affect the detection rates of this marker. The magnitude of this response differs from strain to strain. CD45.1 cells have a slower rate to full immune compartment reconstitution than CD45.2 cells. |
| Gut microbiota | The decrease of gut microbiota diversity due to antibiotics use can impact the success rate of BMC, as gut bacteria produce metabolites that are required for appropriate development of certain leukocyte lineages. |

Although a highly useful tool for a variety of questions, BMC are obviously not free of limitations. They should be used solely in carefully controlled experimental designs and the researcher should be aware of its caveats. One such caveat is the use of allotypes (e.g., CD90.1 vs CD90.2) and fluorescent proteins to track the donor cells in the recipient mice. Depending on exact experimental circumstances, these molecules may be a target of an immune response. This was highlighted in studies in which tumor cells expressing an allotype (CD90.1) or fluorescent protein (eGFP) were

**Table 2** Experimental design and major findings using mixed BMC to study the relationship between IL-23 signaling in T cells and GM-CSF production. The authors adopted a mixed BMC scheme by co-transplanting in a 1:1 ratio wildtype HSC and HSC in which T cells lack the β1 subunit of IL12R (first row), T cells lack the β2 subunit of

the same receptor (second row), or fully express both subunits (third row). These mixtures were transplanted into *Rag1* mice, which lack T and B cells. Therefore, the authors could assess production of GM-CSF when T cells are differentially able to respond to IL-12 and IL-23, concluding that GM-CSF production is mainly regulated by IL-23. [22]

| Donor 1 | Donor 2 | Recipient | IL-23 response | GM-CSF production |
|---|---|---|---|---|
| Wildtype CD45.2 | *Il12r2β1* KO (T cells do not respond to IL23 + IL12) | *Rag1* CD45.1 | Halved | Decreased |
| | *Il12r2β2* KO (T cells do not respond to IL12) | | As control | Increased |
| | Wildtype (fully responsive) | | Control | Control |

**Table 3** Experimental design and major findings using complex mixed BMC for studying the role of IL-18R within the lymphoid compartment in EAE development. Gutcher et al. transplanted a mix of BM from *Il18r*-deficient (20%) and *Rag1*-deficient (80%) mice into wildtype recipients. Hence, all lymphoid cells were derived from the Il18r-deficient BM (as *Rag1* do not have mature T and B cells), whereas the myeloid cells were mostly derived from the *Rag1*-deficient, *Il18r*-sufficient BM. By diluting the myeloid compartment to 20% of knockout cells, the authors reduced its influence in EAE progression and were able to observe that a deficient IL-18 response in T and B cells leads to reduced EAE severity. [24]

| Donor 1 | Donor 2 | Recipient | *Il18r1* deficiency | EAE |
|---|---|---|---|---|
| Wildtype | – | Wildtype | None | Control |
| *Il18r1* KO | – | Wildtype | Hematopoietic cells | As control |
| *Il18r1* KO 1 part | *Rag1* 4 parts | Wildtype | T/B cells | Reduced |

transplanted into donor mice expressing the other allotype (CD90.2) or wildtype background, respectively. These experiments showed that both factors are capable of triggering an immune response [35–37]. It should be kept in mind that mouse strains are differentially capable of mounting immune responses to certain tracking molecules, with BALB/c mice being more immunocompetent against eGFP than C57BL/6 [38]. Finally, whereas CD45.2 cells transferred into CD45.1 expressing mice result in full reconstitution of the immune system, transplanting CD45.1 expressing cells into CD45.2 mice results in a reduction in the numbers of cells contributing to the long-term reconstitution of the immune system, due to lower homing capabilities of CD45.1 expressing cells [39].

Another issue with BMC is the effect of whole-body irradiation. We discussed above the effects on mucosal tissue of the gut, but other organ systems are also affected. From humans, it is known that at doses close to $LD_{50}$, the principal symptoms of the prodromal syndrome are anorexia, nausea, vomiting, and easy fatigability. Immediate diarrhea, fever, or hypotension indicates a supralethal exposure. In mice, the effect of irradiation on the skin is usually easily visible when black-haired recipients are used: it leads to graying of the coat. In humans, a dose as low as 0.15 Gy leads to temporary sterility in men and may induce symptomatic pneumonitis (forced breathing, inadequate gas exchange) in 5% of people [40]. Researchers should check for these symptoms after the irradiation cycles of the BM transplantation procedure. Lethal cerebrovascular syndrome is seen in humans following high-dose irradiation (> 100 Gy); small non-lethal effects on the CNS at lower doses should be therefore kept in mind. Another issue with BMC is the effect of irradiation in depleting the mucosal tissue from immune cells [41]. The reconstitution of the immune system occurs within each cell niche. Thus, while primary and secondary lymphoid organs may be completely repopulated by donor cells after irradiation, the same is not necessarily true for the mucosa. Complete ablation of immune cells by irradiation of the intestinal mucosa may not be achieved with the protocols

routinely used, generating a situation in which the host immune cells will compete and outnumber the donor cells in the mucosa. The presence of host immune cells can be a source of bias when studying conditions such as inflammatory bowel disease, and can be minimized by performing irradiation in neonatal mice or, when studying B and T lymphocytes, by use of RAG-deficient or SCID hosts. The resistance to radiation of immune cells residing in mucosal sites may be of special concern when studying innate lymphoid cells (ILC). ILC expansion after BMC has been reviewed by Vermijlen and Prinz [42], who highlight the drawback of using mice as models for the human situation, as the development of the innate immune cell subsets of mice has a natural lag phase compared with the human situation. While in humans, most ILC types develop during gestation, in mice, ILC mature and self-propagate after birth. A positive aspect of this difference is that, in contrast to the human situation, the reconstituted immune system after BMC in mice will better mimic the adult situation. As early as 1974, both the use of antibiotics and the composition of the microbiota have been shown to influence the success of BMC [43]. This was attributed to these factors providing resistance to infections, and also to metabolites such as those that can protect intestinal epithelial cells. Indeed, in a recent study, butyrate produced by the gut microbiota was shown to simultaneously stimulate Tregs function and regeneration of the intraepithelial cells damaged by irradiation [44]. Moreover, diversity of the gut microbiota at the time of BM transplant is a good indicator of success in patients, with lower mortality rate associated with higher microbiota diversity [45] [46]. Thus, antibiotics must be used with care so that dysbiosis is minimized. Alternatively, selective regeneration of the gut microbiota could be performed to enhance growth and function of those species that are beneficial. Here, innovative fecal microbiota transplantation (FMT) may prove useful [47]. Another alternative would be to, instead of irradiating the whole body, directly target the irradiation to the BM to decrease by standing organ damage [48].

## Future developments

Now well into their sixties, BMC have come of age, and clearly will remain a major and highly valuable technological tool. Their applications are being and will be further extended by optimal integration of established and novel technologies. For instance, repertoire sequencing of T and B cells is highly informative and CRISPR-Cas techniques are finding their way into BMC. Highly sophisticated imaging technology of individual cells in vivo will be useful to assess issues like competitive advantage of individual cells upon transfer in relation to tissue niches [49]. Finally, emerging technologies in DNA writers and molecular recorders will provide new opportunities for cell tracing and in vivo functionality [50].

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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