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Marrow-Derived Stromal Cell Delivery on Fibrin Microbeads Can Correct Radiation-Induced Wound Healing Deficits

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Abstract

Skin that is exposed to radiation has an impaired ability to heal wounds. This is especially true for whole body irradiation, where even moderate non-lethal doses can result in wound healing deficits. Our previous attempts to administer dermal cells locally to wounds to correct radiation-induced deficits were hampered by poor cell retention. Here we improve the outcome by using biodegradable fibrin microbeads (FMB) to isolate a population of mesenchymal marrow-derived stromal cells (MSC) from murine bone marrow by their specific binding to the fibrin matrix, culture them to high density in vitro and deliver them as MSC on FMB at the wound site. MSC are retained and proliferate locally and assist wounds gain tensile strength in whole body irradiated mice with or without additional skin only exposure. MSC-FMB were effective in 2 different mouse strains but were ineffective across a major histocompatability barrier. Remarkably, irradiated mice whose wounds were treated with MSC-FMB showed enhanced hair regrowth suggesting indirect effect on the correction of radiation-induced follicular damage. Further studies showed that additional wound healing benefit could be gained by administration of G-CSF and AMD3100. Collagen strips coated with haptides and MSCs were also highly effective in correcting radiation-induced wound healing deficits.

Introduction

There is growing concern about the dearth of medical countermeasures for the treatment of ionizing radiation injuries in the event of either a radiological or nuclear incident (Williams and McBride, 2011). This has led to many studies directed at correcting the acute radiation syndrome (ARS). Much is known about how different tissues individually respond to radiation but less about how damage to one tissue affects healing of another and how effects

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Conflict of interest

The authors state no conflict of interest.

are compounded by non-radiation injuries. The skin is particularly susceptible to compounded injuries.

In an ARS situation, rapid wound closure, re-epithelialization, and re-establishment of the skin tissue integrity are top clinical treatment priorities. These processes are compromised by irradiation, which seriously impedes the healing of physical or thermal skin wounds. Indeed radiation-impaired wound healing is a specific form of the general clinical challenge posed by non-healing wounds for which few effective treatment options are available (Olascoaga *et al.*, 2008).

Healing of skin wounds involves complex, well-orchestrated interactions between different cell types and extracellular matrix materials (Wu *et al.*, 2007b). After exposure to ionizing radiation, the time to expression of skin damage is determined by the slow turnover of dermal cells (Withers, 1967). However, trauma speed the proliferation rate of the affected cells and thereby greatly accelerates expression of any radiation-induced latent damage and wounded irradiated skin displays healing defects acutely (Gorodetsky *et al.*, 1988). The dose and the body volume are critical variables. The dermis is relatively radiation resistant but local stem/progenitor skin cells can be damaged so that they fail to replace those lost through normal turnover or through physical or thermal injury. On the other hand, damage to the more sensitive hematopoietic system through whole or partial body radiation exposure can compromise the pool of bone marrow-derived cells that contribute to the healing process. This includes stem cells, immune cells, endothelial progenitor cells, and fibrocytes all of which can contribute to healing either structurally or as regulatory influences (Wu *et al.*, 2007b). Sublethal whole body irradiation (WBI) therefore requires far less dose than skin only irradiation (SI) to delay healing of full thickness incisional wounds in mouse skin (Vegesna *et al.*, 1993). In a radiological incident the whole body dose received is therefore critical to the assessment of potential deficits in wound healing. The dose received by the hematopoietic system may however be very different from that received by the skin due to proximity to the radiation source.

In recent years, multiple animal injury models, as well human studies (Garcia-Gomez *et al.*, 2010), have shown that MSC are excellent candidates for enhancing tissue repair, including damage caused by radiation (Leclerc *et al.*, 2011). This is enhanced by reports that they may be effective across histocompatibility barriers (Shi *et al.*, 2010), although their true potential in this regard is still controversial.

Our approach to correcting radiation-induced wound healing problems using MSC was tempered by our previous experience. Based on an earlier study in pigs (Kruegler, 1978.), we reported that implanted neonate skin fibroblasts could partly correct radiation-induced wound healing deficits in mice (Gorodetsky *et al.*, 1991), findings that Dantzer (Dantzer *et al.*, 2003) later extended in a rat model using bone marrow-derived stromal cells. The primary factor responsible for our limited success at the time was that cells implanted directly into a wound site rapidly disappeared, with <1% remaining for more than a few days. To circumvent this problem, we developed a novel fibrin microbead (FMB) cell carrier (Gorodetsky, 2008; Gorodetsky *et al.*, 1999; Gorodetsky *et al.*, 2004). Matrix-dependent cells including MSC attach to the FMB in 3 dimensional suspension culture, allowing easy

removal of the non-attaching hematopoietic and epidermal cells. Attachment is mediated by newly described cell binding homologous C-terminal short peptides on β- and γ-chains of fibrin, termed haptides (Gorodetsky *et al.*, 1998; Gorodetsky *et al.*, 2003; Levy-Beladev *et al.*, 2010). MSC isolated on FMB proliferate to high density (up to 10^8/ml packed beads) in vitro, yielding up to a log more cells than conventional plastic adhesion-based culture methods.

When MSC-FMB are implanted in skin wounds in vivo, the FMB degrade slowly and the MSC are retained in high numbers to proliferate and differentiate normally within the target tissue. Also, FMB can support the viability of MSC for up to 10 days at room temperature, making cell transportation easy in emergencies (Gorodetsky *et al.*, 2011).

Here, we use the incisional wound healing model to examine the ability of MSC-FMB to correct radiation-induced damage. The ability of MSC-FMB to act across an allogeneic barrier was examined, as was the effects of addition of granulocyte colony-stimulating factor (G-CSF) and Plerixafor (AMD3100). Finally, the ability of MSC on haptized collagen strips to reduce radiation wound healing deficits was assessed.

Results

Dose/time effects of WBI and SI on gain in skin WTS

A dermal wound healing model was established to examine the effect of irradiating the skin only with or without total body exposure of the hematopoietic system with the aim of examining how different doses to these different organs, as might easily happen in a radiological situation, would interact.

Sublethal WBI of C3H mice using gamma rays compromised the ability of wounds in their skin to gain WTS by 2 weeks (Fig 1a). After 4 Gy WTS was approximately 10% lower; after 6 Gy 20%; and after 8 Gy 30%. These data agree closely with what we found previously (Vegesna *et al.*, 1993). In contrast, doses of around 15 Gy 150kVp X-rays to the skin only (SI) were required to reduce the gain in WTS by 50% at 2 weeks (Fig 1b), rather more than the 13 Gy that we first reported 20 years ago, but considering that the irradiator and tensiometer were different, the reproducibility of radiation effects on WTS measurements is remarkable.

The gain in WTS with time after radiation was also re-examined. We confirmed our earlier finding that WTS increased in unirradiated C3H skin in two phases with nearly half normal strength being recovered within 2 weeks (Fig 1c) and 90% by 4–5 weeks (Fig 1c) (Gorodetsky *et al.*, 1988). C57Bl/6 skin responded in a very similar manner though with slightly higher WTS values (not shown), which could have been due to sex or strain differences. The combination of 4 Gy WBI and 21 Gy SI, a scenario that mimics the expected scenario of a radiological incident where the skin might be compromised by a high dose while the whole body might receive a more moderate dose, resulted in a fairly consistent delay of around 10 days to gain the unirradiated level of WTS in C3H mice at 3 or 4 weeks (Fig 1c). The first “phase” of wound healing is most affected by radiation exposure (Gorodetsky *et al.*, 1988), so much so that the 2 week values are too low to be

reliable after these doses. We therefore chose to measure the effects of 6Gy WBI and 15Gy SI on WTS measured at 4 weeks, the combined WBI and SI deficit in WTS being more than additive (Fig 1d).

MSC-FMB compared to plastic adherent MSC populations

FMB were used as a substrate for culture of MSC to allow their rapid purification from hematopoietic cells and high-density expansion (Gorodetsky, 2008). Because culture on FMB may change the MSC phenotype, we have previously compared the flow cytometric profiles of MSC downloaded from FMB to those cultured on plastic (Rivkin *et al.*, 2007) using a wide range of putative MSC markers which were more characteristic to a pure population of MSC than the plastic isolated cells. For these experiments, the major differences were that MSC cultured on FMB (Fig 2a) showed bimodal distributions for CD44, CD49e and CD105 with increases in expression of CD49e and CD105. The bimodal distribution was largely contributed to by Sca1+, $CD44^{lo}$, $CD49e^{hi}$, $CD105^{hi}$ cells of smaller size (not shown). CD45+ and CD19+ cells were absent indicating purification from hematopoietic cells, which was not the case for cells grown on plastic, while the proportions of CD29 and CD31 positive cells were identical in the two populations (Rivkin *et al.*, 2007).

WBI, but not SI, impairs normal cell infiltration into wounds (Vegesna *et al.*, 1993), suggesting dependency of optimal wound healing on bone marrow-derived cells and that MSC may be a useful therapeutic intervention in combined radiation/wound healing scenarios. We considered that FMB-MSC implantation into the wound may additionally overcome the rapid loss of cells from the wound site by allowing MSC to download slowly from the FMB into the damaged tissue where they could proliferate and differentiate under local influences within the wound microenvironment. Importantly, if GFP-MSC from transgenic mouse bone marrow that were highly positive for GFP by flow cytometry were cultured and implanted on FMB into wounds in WBI+SI treated mice they could be detected after 4 days at higher numbers than if they came from plastic adherent cultures (Fig 2c,d). Additionally, they integrated in the repaired dermis and persisted for at least 4 weeks post-implantation in the healing wounds (Fig 2e) when the plastic isolated cells that were injected could not be detected.

Syngeneic MSC-FMB correct radiation-induced deficits in WTS

Repeated experiments consistently showed that implantation of FMB devoid of cells did not affect the gain in WTS in unirradiated (not shown) or irradiated (Fig 3a) skin. MSC-FMB implanted into wounds in SI mice also failed to affect gain in WTS (Fig 3a), as would be expected since the infiltrate is still effective. However, MSC-FMB loaded with approximately 10^6 syngeneic cells corrected a 19% WTS total deficit caused by 6 Gy WBI by around 80% at 4 weeks (Fig 3a). Pilot experiments showed that although improvement was evident at 2 weeks after wounding (not shown), it was markedly less dramatic than at 4 weeks, suggesting the implanted MSC delivered with FMB proliferated or differentiated in the site, as was also suggested by the data shown in Fig 2e.

The efficacy of syngeneic MSC-FMB at reducing the WTS deficit depended upon the number of MSC-FMB implanted but not on the strain of mice (Fig 3b,c). If mice were given

6 Gy WBI followed immediately by 15 Gy SI, 10^6 syngeneic MSC-FMB corrected a 39% total deficit in C57Bl/6 mice by 49%, and a 35% total deficit in C3H mice by 34% after 4 weeks of healing. If the dose of MSC-FMB was doubled, the degree of correction increased to 71% in both strains. Because of limitations in the volume that can be implanted into an incisional wound, it was not possible to test higher numbers of MSC-FMB.

The experimental schedule for the preparation of MSC-FMB was chosen as 2 days of rotational culture to allow MSC bone marrow cells to load onto FMB and a further 5 days to allow them to proliferate to a high density. While this was a reasonable experimental schedule, in an emergency setting a shorter preparation time might be needed. We therefore examined the efficacy of MSC-FMB implanted immediately after 2 days loading with similar cells loaded and cultured for the additional 5 days in vitro prior to implantation. Figure 4a shows that MSC-FMB implanted 2 days after loading were almost as effective in reducing radiation-induced wound healing deficits at 4 weeks as MSC that had been expanded on FMB in culture for 5 more days, suggesting that MSC proliferate in the wound at least as well as they can on FMB in vitro.

Consistent findings in all experiments were that irradiation with WBI and SI prevented hair from regrowing in the shaved skin and that, remarkably, MSC-FMB implantation into the wound promoted hair regrowth as early as 3 weeks after exposure, although hair color was not restored (Fig 4b).

Attempts to improve MSC-FMB efficacy

Our studies clearly showed that syngeneic MSC-FMB implants were effective at correcting radiation-induced deficits in wound healing. Since a limitation of an incisional wound system is the volume that can be added, we explored in parallel other possible strategies to enhance wound healing efficacy in pre-irradiated skin.

Because G-CSF is likely to be given to patients exposed to potentially lethal WBI doses in a high dose radiological incident, we examined whether G-CSF treatment of mice with and without Plerixafor (AMD3100), which enhances G-CSF-induced mobilization of hematopoietic progenitor cells, would assist or detract from MSC-FMB activity in correcting radiation-induced WTS deficiencies. MSC-FMB were 20% effective in reducing the WTS deficit and this was increased to 29% by in vivo G-CSF treatment and to 46% by an additional single injection of Plerixafor given after G-CSF (Fig 5a), indicating that hematopoietic mobilization can act in conjunction with MSC-FMB implantation to better enhance wound healing.

Additionally, collagen sponge sheets that had been coated haptides, 20mer peptides homologous to the cell binding domains on the c-termini of fibrin (“haptized”) (Marx *et al.*, 2008) were examined as an alternative support structure. These were used to purify MSC and were implanted in the bed under the wound, and were highly successful in correcting radiation-induced WTS deficits (Fig 5b).

MSC-FMB implants across an allogeneic barrier

Literature reports indicate that MSC can be immunosuppressive, even to the extent that they may be effective across histocompatibility barriers. But contradictory data were also presented (English and Mahon, 2011). This could have important clinical consequences in radiation settings as autologous MSC might be difficult to obtain from a patient exposed to potentially lethal WBI. MSC-FMB that could be administered as a commercial product would be ideal for treatment of to a large number of individuals, especially as FMB maintain MSC viable for long periods of time without incubation (Gorodetsky *et al.*, 2011). We therefore examined the ability of MSC-FMB to reverse radiation-induced wound healing deficits across an allogeneic barrier, but only syngeneic MSC were shown to be effective in this system (Fig 5c).

Discussion

Exposure to ionizing radiation sets in motion a train of events that can culminate in failure of one or multiple organs depending upon many factors, amongst which dose and volume are perhaps the most important. As shown in this study and elsewhere, the skin itself is moderately resistant to radiation effects (Vegesna *et al.*, 1993; Withers, 1966), but wound healing is heavily impacted by modest doses to the whole body that damage the hematopoietic system but that are themselves non-lethal (Ran *et al.*, 2004; Vegesna *et al.*, 1993). WBI decreases the cellular infiltrate into wounds far more than local skin irradiation (Vegesna *et al.*, 1993). It is not known which of the many bone marrow-derived cells that migrate into the wound site are most affected or the extent to which this effect extends to tissue repair in other non-hematopoietic organs (Wu *et al.*, 2007b).

MSC have been a major focus of many recent approaches aimed at improving tissue repair and regeneration (Garcia-Gomez *et al.*, 2010) and have been shown to aid wound healing in different preclinical models as well as in humans (Chen *et al.*, 2009; Conget *et al.*, 2010; Hu *et al.*, 2011; McFarlin *et al.*, 2006; Rogers *et al.*, 2008; Wu *et al.*, 2007a). In bone marrow, MSC represent a small fraction of all nucleated cells: less than 0.01% (Bartmann *et al.*, 2007)

FMB based technology is a convenient, simple and rapid technique to isolate and enrich matrix dependent mesenchymal cells that can be applied to tissue regeneration models (Ben-Ari et al., 2009). One ml of packed FMB can typically bind ~30–70×10^6 mesenchymal cells. Their doubling time at subconfluence in slowly rotating conditions is typically ~48hrs (Gorodetsky, 2008, Gorodetsky *et al.*, 2011). Cell binding to FMB seems based on interaction of small fibrin domains termed "haptides" with the cell membrane (Gorodetsky *et al.*, 2003; Levy-Beladev *et al.*, 2010). Haptides interact with the membranes of different cell types but mesenchymal cells once anchored secrete their own extracellular matrix that allows them to adhere strongly while other cells of hematopoietic origin are shed during rotation. As a result, the populations isolated on FMB are phenotypically purer than those isolated on plastic, even if their proportion in the population is originally extremely low, (Kassis et al.,;Rivkin *et al.*, 2007). It is notable that in the current study a subpopulation of small marker positive MSCs were found that were not seen in populations generated by plastic adherence and require further investigation. Multipotency and plasticity are well-

established features of MSC as is the impact of culture methods, which makes it imperative that each system for generating MSC is assessed on its own merits.

For wound healing, FMB provide a protein-based biodegradable support lasting about 2 weeks in vivo after implantation that carries non-trypsinized intact MSC, retains them in the wound site and allows their integration into the ongoing healing processes. MSC-FMB were effective in correcting deficits in healing of incisional wounds in mice whose hematopoietic system had been compromised by WBI exposure but did little to assist if only the skin was irradiated, suggesting that the MSC may have replaced some of the effects of a natural bone marrow-derived population that was required for optimal wound healing. This contrasts with the recent study by Focheron et al. (2012) who showed that adipose-derived stromal cells could heal the skin of minipigs irradiated to 50 Gy which normally would develop necrosis. This difference may be ascribed to differences in the dose and model but more likely to the fact that our studies on MSC-FMB were somewhat limited by the wound incision model, where the area in which therapeutics can be delivered is small. We were however able to show that treatment of mice with G-CSF with or without Plerixafor could augment the efficacy of MSC-FMB and that haptized collagen sponges are also a promising new delivery system for MSC that is not based on whole fibrin protein that may be a more flexible system for future investigations. It has been shown in many systems that MSC may function indirectly to improve tissue regeneration, and that the effect could be related to modification of inflammatory responses (Garcia-Gomez *et al*., 2010; Chamberlain *et al.* 2007; Dominici *et al*., 2006; Keyzer *et al*., 2007). Allogeneic MSC can therefore be effective across histocompatibility barriers as they have low immunogenicity and they do not need to survive long (Griffin *et al.*, 2010), as they most likely often act by secreting immunosuppressive factors (Tolar *et al.*, 2010). In our MSC-FMB system we were unable to demonstrate production of immunosuppressive cytokines (not shown) and further experimentation is needed to determine if this is an effect of the wound healing/radiation model or of the growth promoting FMB culture conditions (Gorodetsky *et al.*, 1998).

An unexpected impressive observation of this investigation was the recovery of hair follicles in the high dose irradiated skin as a result of subdermal MSC-FMB implantation into irradiated wounds. Hair follicles consist of many different epithelial and mesenchymal cell types geared towards the production of hair and may provide an additional source of stem cells, which appears in continuity with the basal stem cell layer and that appear to reside in the “bulge” region of the follicle shaft (Cotsarelis, 2006; Sun *et al.*, 2007; Yang and Cotsarelis, 2010). Since the mesenchymal cells are not expected to trans-differentiate to form hair follicles, these findings hint for an indirect secretory effect on these cells on the regeneration of a wide range of cell types in the injected area.

Materials and Methods

Mice, Skin Wounds, and Irradiations

C3Hf/Kam (H2-k) female and C57Bl/6 (H2-b) male gnotobiotic mice were used for wound healing studies at 8–10 weeks of age. They were bred at UCLA. MSC-GFP were cultured from bone marrow from C57Bl/6-Tg(ACTB-EGFP)1Osb/J mice obtained from the Jackson

Labs (Maine) and maintained at Hebrew University. All experiments were approved by the UCLA IACUC.

WBI was delivered to 8 mice at a time in a well-ventilated Lucite chamber without anesthesia using a γ -ray irradiator (137 Cs; AEC) at a dose rate of 67cGy/min. For SI, 150 kVp/20mA X-rays (Gulmay) and lead shielding were used to reduce the whole body dose. Mice were anesthetized with ketamine and xylazine (Bedford, MA) and placed in a lead box (1 mm thick) with a flap of dorsal skin pulled through a slit and held loosely using tape. The dose rate was 3.623 Gy/min, as determined using thermoluminescent dosimeters on the skin surface.

Mice were irradiated one day before wounding (Gorodetsky *et al.*, 1988). WBI plus SI were given sequentially with less than 15 minutes in between. Full thickness wounds approximately 2.5 cm long were made in the shaved dorsal skin of anesthetized mice (Gorodetsky *et al.*, 1988), with ketamine/xylazine as anesthetic. Cells, FMBs and solutions were implanted into the wound site in 50 μl volumes. Wounds were closed with 3~4 clips which were removed after 2 days. In some experiments 5 μg (micrograms) G-CSF (GenScript, NJ) or sterile phosphate-buffered saline (PBS) was given s.c. to C3H mice, once a day for 5 days followed on day 6 by 5 mg/kg AMD3100 (Plerixafor; Sigma-Aldrich, MO) or PBS.

FMB and MSC

FMB were made from fibrinogen and thrombin as described previously (Gorodetsky *et al.*, 2004). They were sterilized in 70% ethanol for 8 hours and dried. Meshing was used to select FMB with diameters ranging from 108-180μm. Before use they were hydrated in PBS, which expands their size by ~50%.

Bone marrow (BM) cells were collected by flushing the femurs and tibias of euthanized mice with Minimum Essential Medium (Invitrogen/GIBCO, NY) containing 1% antibiotics (penicillin/streptomycin/Amphotericin B; Mediatech, VA). Red cells were lysed using ACK buffer (Lonza, Basel, Switzerland) and cells were resuspended in MEM + 20%FBS (Invitrogen, Ca.) with antibiotics. For isolation of MSC on plastic, 10^8 BM cells were plated onto 100mm × 20mm plates. After 2 days in culture with 7% CO_2 at 37°C, the non-adherent cells were rinsed away and the remaining cells expanded for 5 more days with medium changes. The enriched population of MSC was harvested using 0.25% trypsin-EDTA (Invitrogen, Ca).

For MSC-FMB preparation, 100 μl of sterile hydrated FMB (equivalent to ~50 mg of dry FMB) were washed with PBS and mixed with a total of 10^8 BM cells in culture medium. These were put in a 50ml CultiFlask disposable bioreactor tube with vented cap that allowed gas exchange and reduced water loss by evaporation (Sartorius Stedim Biotech, Aubagne, France). The tube was rotated slowly in ~10–20 rpm at an angle of 20 degree (Gorodetsky *et al.*, 2004) for 2 days in an incubator. The non-adherent cells were removed by washing, and the MSC-FMB were cultured for another 5 days (unless otherwise indicated) by which time the beads were totally covered by MSC. This was confirmed by nuclear count using propidium iodide staining performed as described previously (Rivkin *et al.*, 2007). Details of

the cell yield, purity, marker expression and other technical issues have been described extensively in previous publications (Gorodetsky et al., 1999; Gorodetsky et al., 2004; Gorodetsky et al., 2008).

Unless otherwise stated, 15 μl volumes of MSC-FMB in a total of 50 μl PBS containing approximately 10^6 MSC were implanted into each wound prior to its closure.

Flow Cytometry

MSC markers were assessed by flow cytometry as before (Rivkin *et al.*, 2007) (FACSCalibur; BD Biosciences, CA). The markers used were CD105, Sca-1, CD44, CD49e (Clone 5H10-27, MFR5; BD),CD29, CD45 and CD31.

Collagen Sponges

Highly crosslinked strip sheets of collagen sponge with covalently bound cell binding 20mer Haptide sequences (Hapto Biotech, Israel) were sterilized with ethanol 70% for 8 hours, rinsed with PBS, and put under a vacuum for 30 minutes. 10^7 bone marrow cells were loaded onto 20mm long collagen sponge strips in 5 ml medium in 50 ml tubes and treated as for FMB. After 2 days, the non-adhered cells were rinsed off and after 5 more days of incubation, the strips were implanted into the sub dermal area of the wounds. P.I. staining confirmed a confluent layer of MSC attached to the collagen substrate.

GFP labeled MSC

For the follow-up of survival and integration of MSC implants in the wounds, MSC were isolated from bone marrow of GFP+ C57BL mice by incubation with FMB and implanted with FMB to WT C57BL mice.

Tensile Strength Measurement

At stated times after wounding, a square of skin containing the wound was removed from euthanized mice and cut into seven 2mm strips of 20mm in length with a multi-blade device so that each 2mm wide strip contained a horizontal wound sample (Gorodetsky *et al.*, 1988). The strips were spread on filter papers soaked in ice-cold PBS in covered petri dishes till WTS measurement as previously described (Gorodetsky 1988) using an Instron tensiometer (Model 3342, Instron, Norwood, MA). The skin strips were stretched at a rate of 1 cm/min to breaking point to obtain the peak WTS in gram force/2mm.

Since different radiation doses and times are used, for simplicity and comparison between experiments, the efficacy of the treatment can be presented as Deficit Reduction (DR), which is the reduction in the radiation-induced deficit due to the treatment,

$$\mathrm{DR{:}}1 - \frac{\text{Unirradiated WTS} - \text{Irradiated WTS (+treatment)}}{\text{Unirradiated WTS} - \text{Irradiated WTS (Ctrl)}}$$

and as Total Deficit (TD):

$$\text{TD:1} - \frac{\text{Unirradiated WTS} - \text{Irradiated WTS}}{\text{Unirradiated WTS}}$$

Student's t test was used to assess statistical significance.

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Abbreviations

FMB	Fibrin MicroBeads
MSC	Marrow-Derived Stromal Cells
WTS	Wound Tensile Strength
WBI	Whole Body Irradiation
SI	Skin Only Irradiation
BM	Bone Marrow
G-CSF	Granulocyte Colony-Stimulating Factor
AMD3100	Plerixafor

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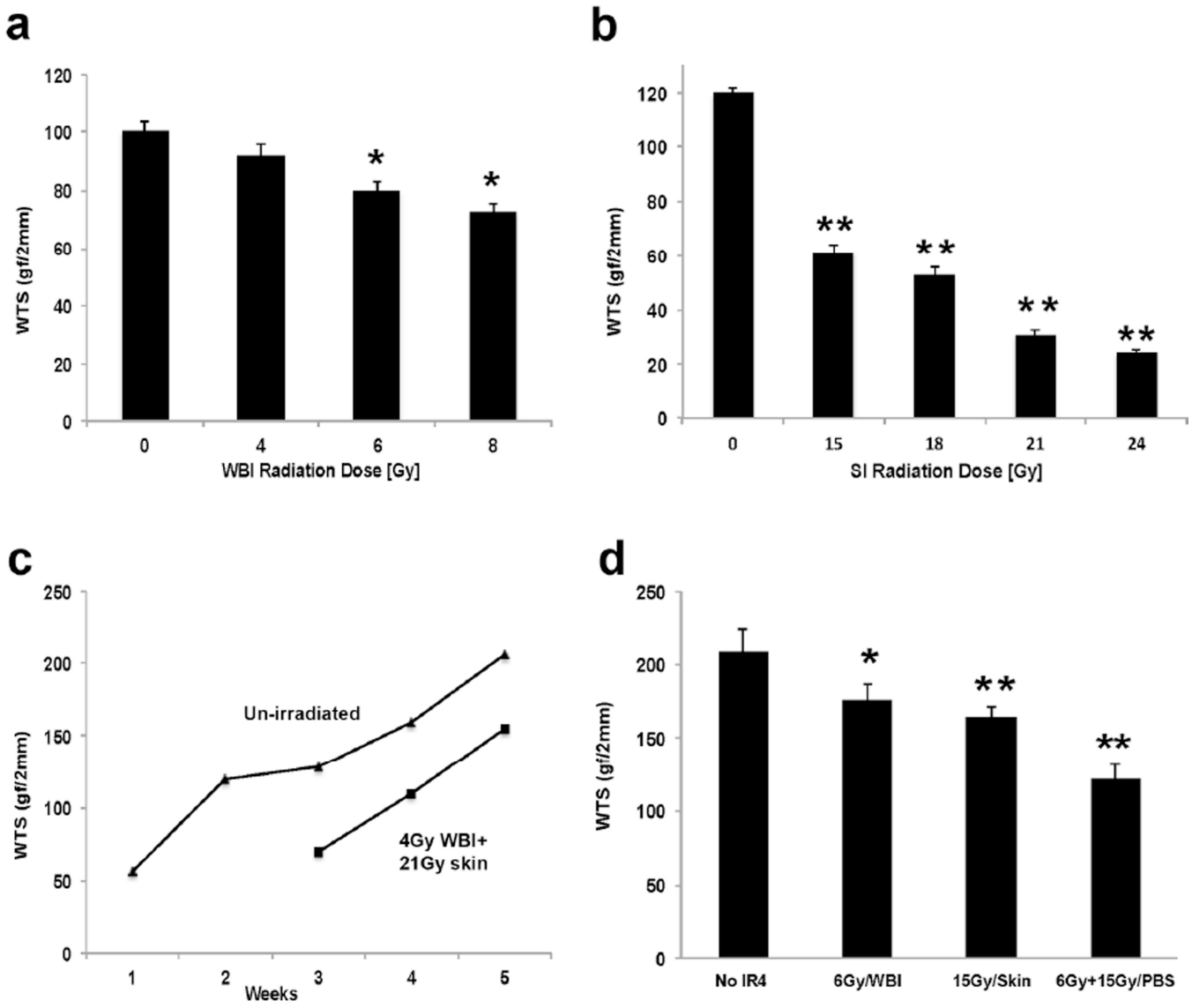

Figure 1. Effects of WBI and SI on wound healing in C3H mice

a) WTS was measured 2 weeks after varying doses of WBI exposure **b)** WTS measured at 2 weeks after varying doses of SI exposure. **c)** The effect of time on gain in WTS was assessed in un-irradiated skin (triangles) and skin exposed to 4Gy/WBI + 21Gy/SI (squares). WTS measurements at 2 weeks are unreliable within these high SI doses as the first "phase" of wound healing is most affected by radiation **d)** WTS was measured 4 weeks after 6Gy WBI or 15Gy SI, or both combined. Error bars indicate mean+/-SEM, n=4. Significance: **P*<0.05, ***P*<0.01 by Student's t-test. (WTS: wound tensile strength; WBI: whole body irradiation; SI: Skin only irradiation)

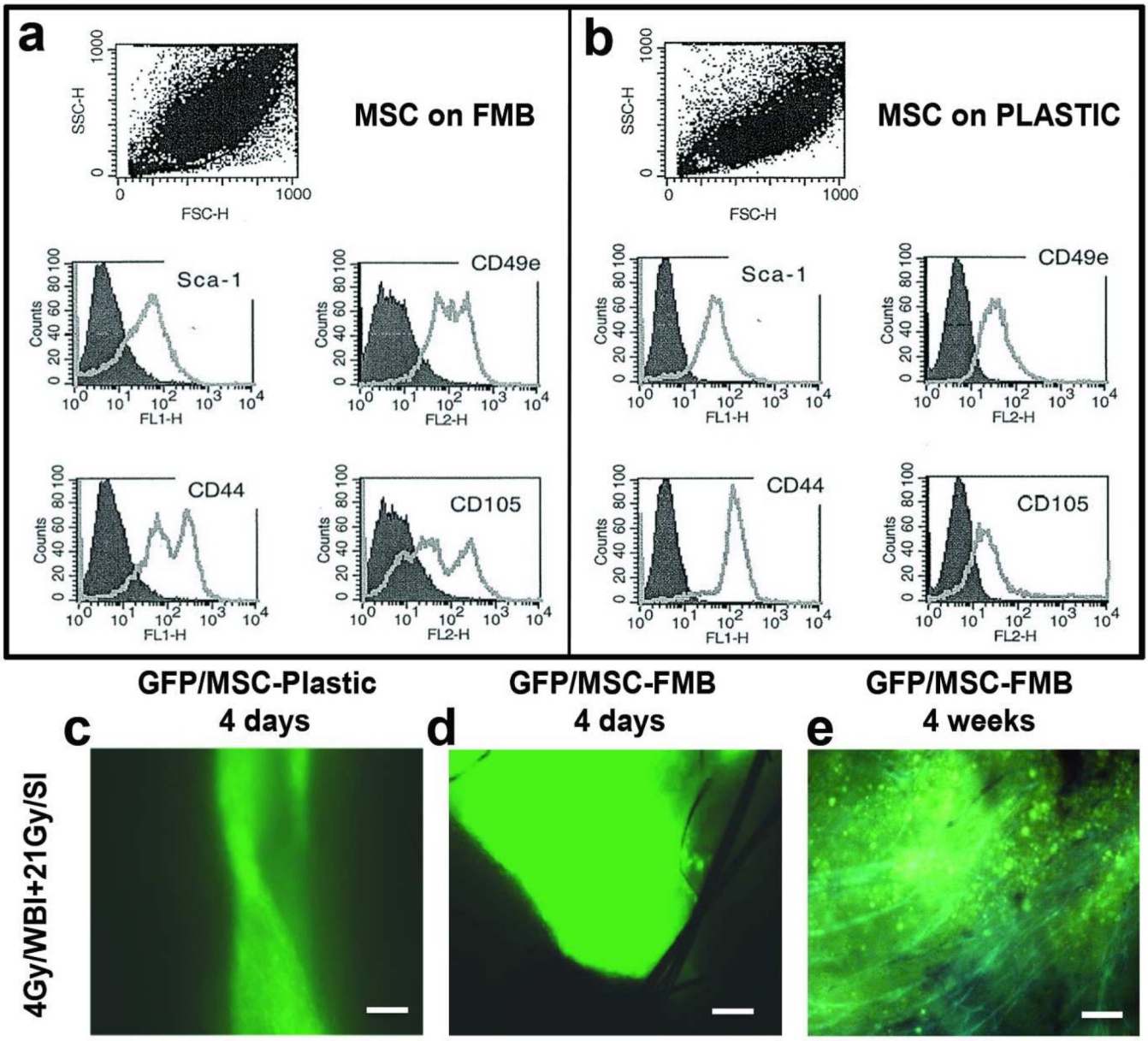

Figure 2. Phenotypic profile of C57Bl/6 marrow stromal cells –FMB

(a,b) Flow cytometric analysis of MSC markers Sca-1, CD49e, CD44, and CD105 of MSC grown on FMB or on plastic **c,d)** MSC/GFP grown on plastic or loaded on FMB were implanted in pre-irradiated wounds in C57Bl/6 mice. Scale bar: 200μm **e)** Four weeks after implantation, MSC/GFP-FMB were still clearly detectable in healing wounds but not in skin wounds implanted with MSC isolated and grown on plastic (not shown). Combination of fluorescence with dim light to demonstrate the fluorescence with the outline structures of the intact skin. Scale bar: 200mm (MSC: Marrow Stromal Cells; FMB: Fibrin Microbeads; WBI: whole body irradiation; SI: Skin only irradiation)

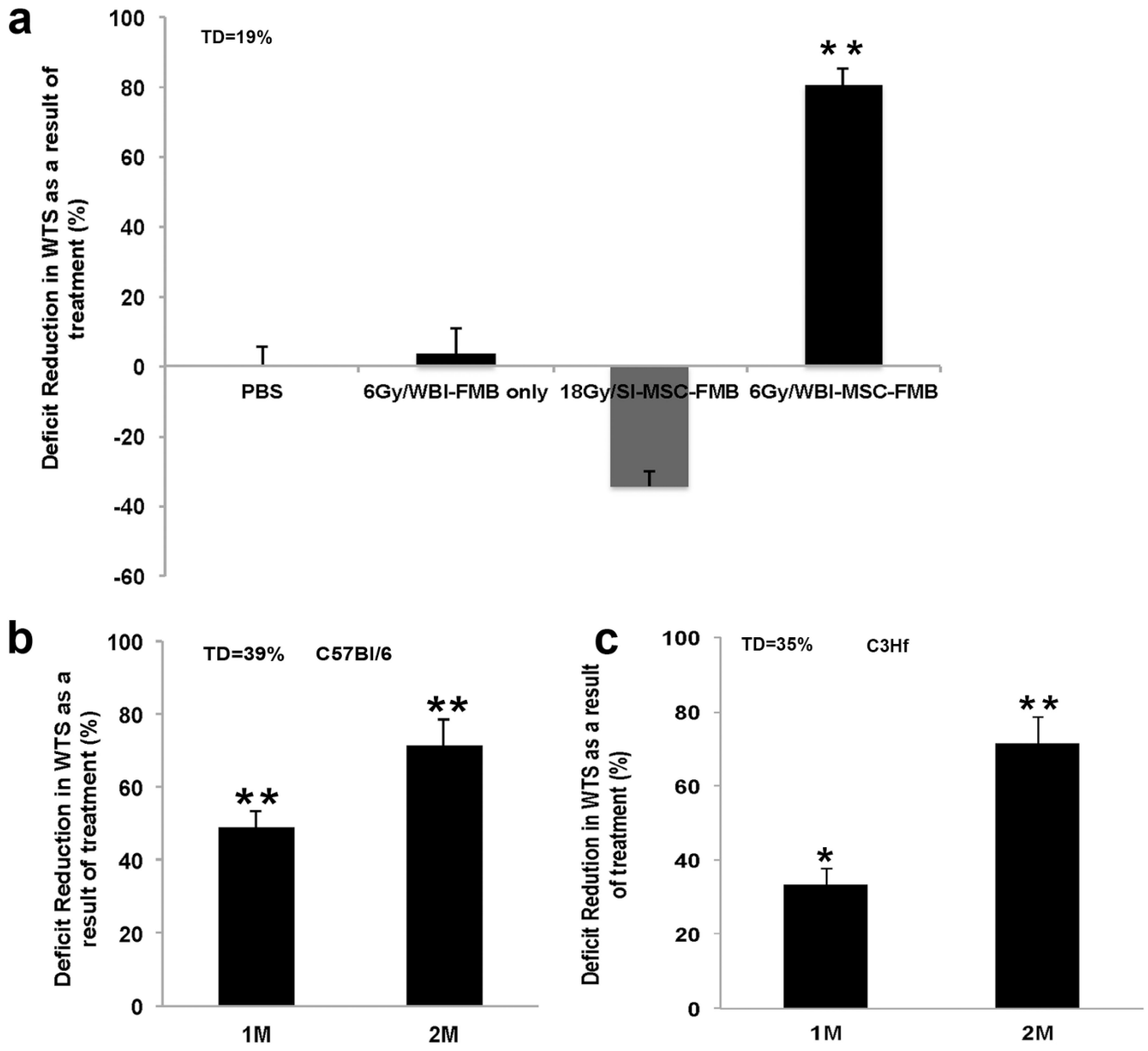

Figure 3. MSC-FMB correct deficits in gain in WTS caused by WBI

a) Syngeneic MSC-FMB were implanted in pre-irradiated C57Bl/6 mice and deficit reduction in WTS was assessed after 4 weeks of healing. Mice were exposed to either 18Gy SI or to 6Gy WBI. FMB alone were implanted as a control. **b)** 1×10^6 MSC-FMB and 2×10^6 MSC-FMB were implanted in pre-irradiated wounds of C57Bl/6 and C3H mice. Deficit reduction in WTS was assessed after 4 weeks of exposure to 6 Gy/WBI+ 15Gy SI. Error bars indicate mean+/−SEM, n=4. Significance: *P<0.05, **P<0.01 by Student's t-test. (WTS: wound tensile strength; WBI: whole body irradiation; SI: Skin only irradiation; MSC: Marrow Stromal Cells; FMB: Fibrin Microbeads; TD: Total deficit)

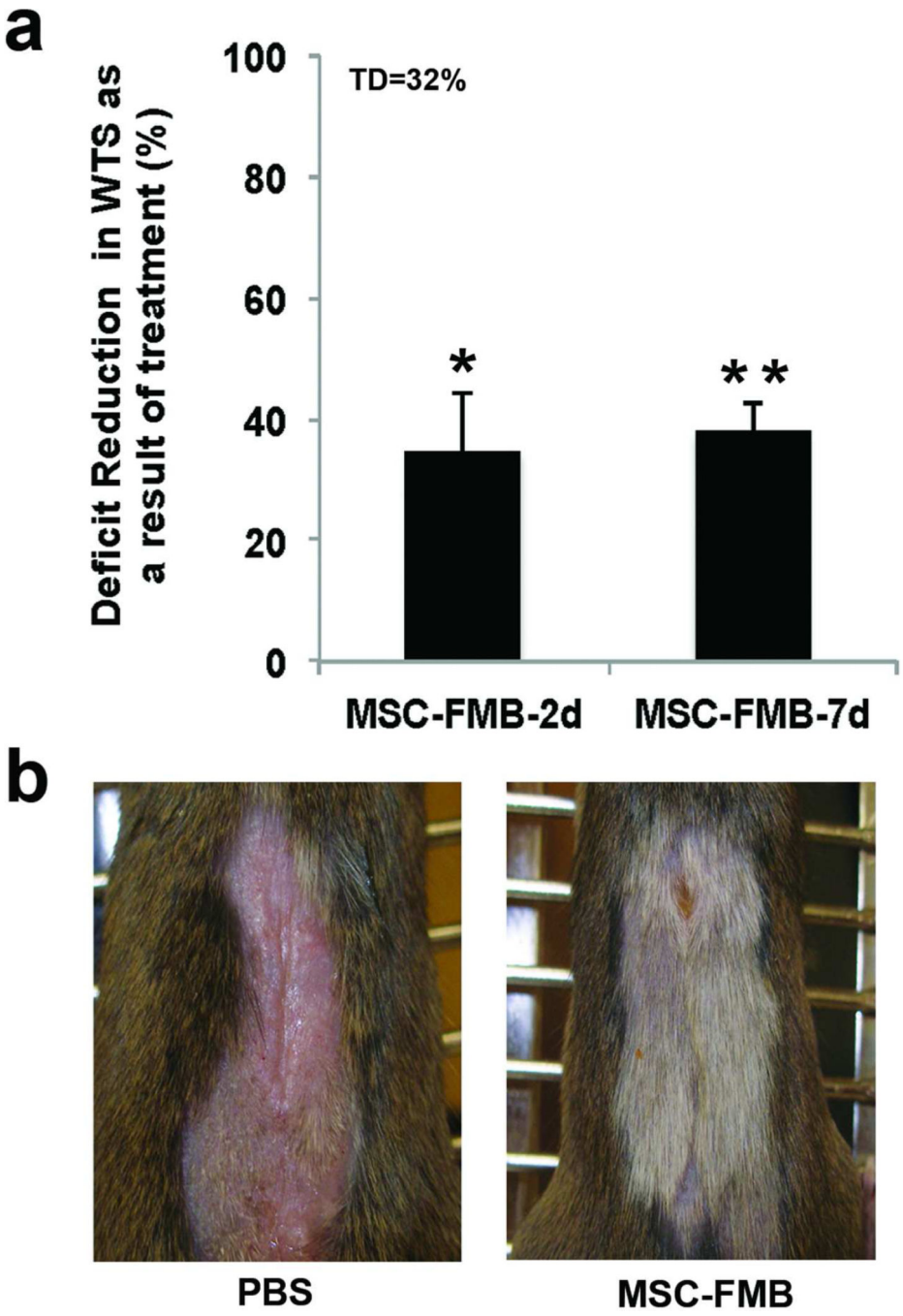

Figure 4. MSC-FMB implantation times and hair regrowth

a) 1.5×10^6 MSC-FMB were implanted into C3H mice wounds immediately after 2 days of loading onto FMB or after being expanded for a further 5 days in vitro. C3H mice were pre-irradiated with 4Gy/WBI + 21Gy/SI and WTS was assessed after 4 weeks of healing. Error bars indicate mean+/−SEM, n=4. Significance: *P<0.05, **P<0.01 by Student's t-test. **b)** Skin in C3H mice irradiated with 4Gy/WBI + 21Gy/SI and implanted with MSC-FMB regrew hair by 3 weeks after treatment, although color was not restored. (WTS: wound tensile strength; MSC: Marrow Stromal Cells; FMB: Fibrin Microbeads; TD: Total deficit)

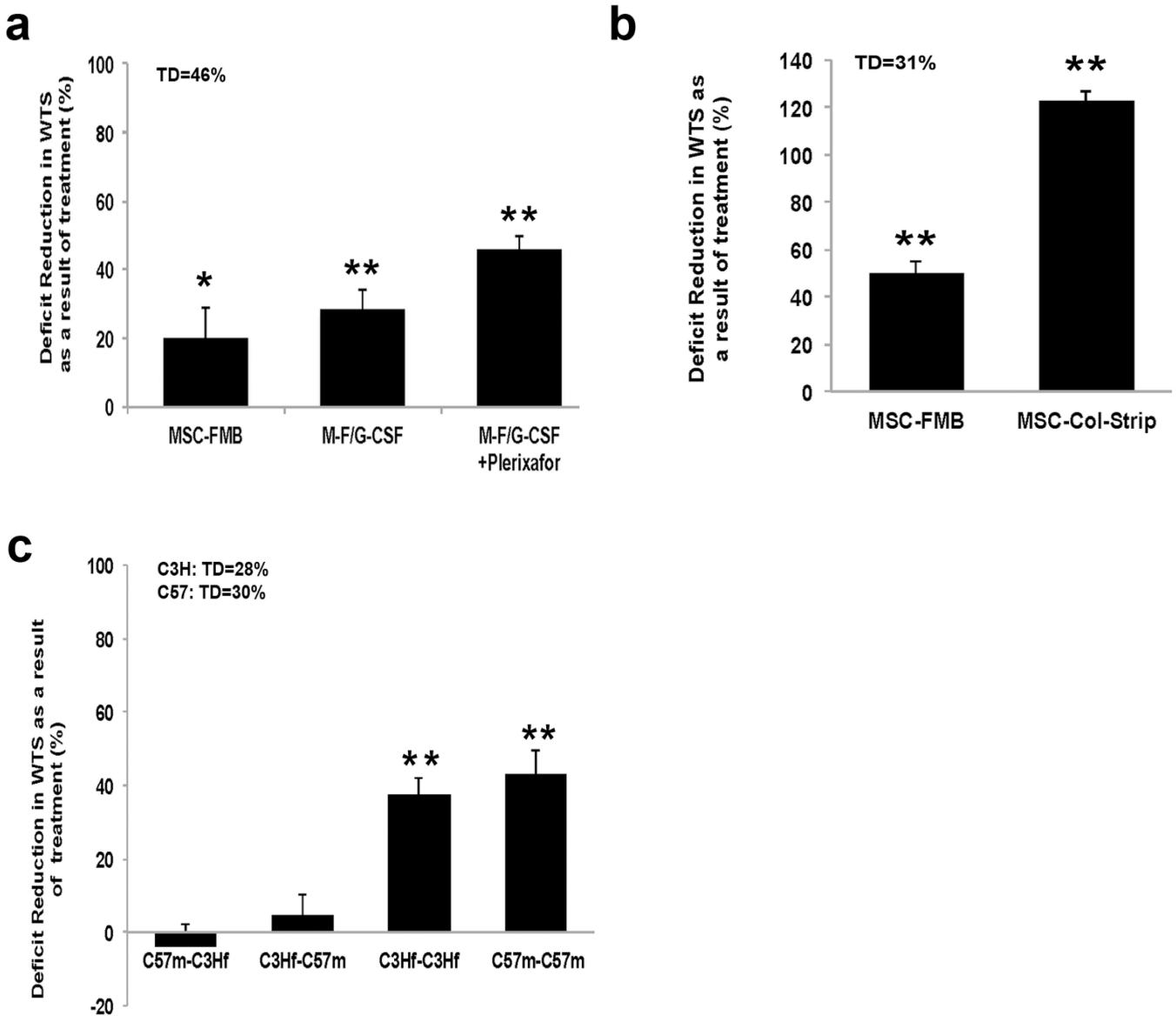

Figure 5. Hematopoietic mobilization, collagen sheets and allogeneic and syngeneic MSC

a) Five daily injections of G-CSF were given s.c. to previously wounded C3H mice pre-irradiated with 4 Gy/WBI+21 Gy/SI. An additional single injection of Plerixafor was administrated on day 6 **b)** MSC on haptized collagen sheets were implanted in the wound of C3Hf mice previously exposed to 4Gy/WBI + 21Gy/SI. Deficit reduction in WTS was measured 4 weeks after treatment **c)** C3H and C57Bl/6 mice were exposed to 4Gy/WBI +21Gy/SI, allogeneic or syngeneic MSC+FMB were implanted in skin wounds, and WTS was assessed at 5 weeks. Error bars indicate mean+/−SEM, n=4. Significance: **P*<0.05, ***P*<0.01 by Student's t-test. (WTS: wound tensile strength; MSC: Marrow Stromal Cells; FMB: Fibrin Microbeads; G-CSF: granulocyte colony-stimulating factor; TD: Total deficit)