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REVIEW

Regulation of mitochondrial function and endoplasmic reticulum stress by nitric oxide in pluripotent stem cells

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Abstract

Mitochondrial dysfunction and endoplasmic reticulum stress (ERS) are global processes that are interrelated and regulated by several stress factors. Nitric oxide (NO) is a multifunctional biomolecule with many varieties of physiological and pathological functions, such as the regulation of cytochrome c inhibition and activation of the immune response, ERS and DNA damage; these actions are dose-dependent. It has been reported that in embryonic stem cells, NO has a dual role, controlling differentiation, survival and pluripotency, but the molecular mechanisms by which it modulates these functions are not yet known. Low levels of NO maintain pluripotency and induce mitochondrial biogenesis. It is well established that NO disrupts the mitochondrial respiratory chain and causes changes in mitochondrial Ca^{2+} flux that induce ERS. Thus, at high concentrations, NO becomes a potential differentiation agent due to the relationship between ERS and the unfolded protein response in many differentiated cell lines. Nevertheless, many studies have demonstrated

the need for physiological levels of NO for a proper ERS response. In this review, we stress the importance of the relationships between NO levels, ERS and mitochondrial dysfunction that control stem cell fate as a new approach to possible cell therapy strategies.

Key words: Endoplasmic reticulum stress; Mitochondrial function; Nitric oxide; Pluripotency; Cell differentiation; Mitochondrial biogenesis

Core tip: Several studies have focused on the role of nitric oxide (NO) in regulating many physiological functions, such as metabolism and pluripotency. NO has been established to act as a potent agent for the control of stemness by promoting the expansion of pluripotent cells. NO regulates mitochondrial function and endoplasmic reticulum stress. In pluripotent stem cells, both of these factors are related to the control of cell fate and may contribute to the mechanism by which NO regulates the maintenance of pluripotency. This provides additional evidence supporting the use of NO as an alternative small molecule for the conservation and expansion of cultured pluripotent cell lines necessary for implementing a cell therapy programme.

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INTRODUCTION

Actions of nitric oxide in cells

Nitric oxide synthase (NOS) modulates the L-arginine-to-L-citrulline pathway, by which nitric oxide (NO) is synthesized[1,2]. NO, a short-lived free radical, reacts with oxygen, superoxide, cytochrome c oxidase (CcO) and other molecules when it acts independently of cyclic guanosine monophosphate (cGMP), and this interaction is dose-dependent. Therefore, at high levels of NO, reactive oxygen species and NO interact to contribute to protein posttranslational modifications through S-nitrosylation and S-nitration[3-5]. When it acts in concert with cGMP, NO activates soluble guanylate cyclase (sGC), catalysing the conversion of GTP into cGMP, which controls a variety of physiological effects in multiple tissues[6,7]. The role of NO/cGMP in embryonic development and cell differentiation is a subject of intensive investigation; however, it is not clear whether the action of NO in stem cell biology is mediated *via* the cGMP pathway. It has been reported that bone marrow stem cell potency and differentiation are independent of the sGC/cGMP pathway[8-12]. Moreover, many effects of NO on stem cell pluripotency and differentiation are independent of this pathway, and thus the mechanism by which NO modulates the differentiation of embryonic stem cells (ESCs) remains unclear[13,14].

Physiological functions of NO

NO has been described to have important roles as a regulator of multiple physiological functions: It is a principal mediator of the immune system in the inflammatory response and a neurotransmitter in the central nervous system; acting as a second messenger, it has multiple biological effects that have been implicated in numerous physiological functions in mammals, such as regulation of blood pressure *via* smooth muscle relaxation and inhibition of platelet aggregation[13,15-17]. NO has been reported to affect gene expression at the levels of transcription and translation and has been associated with the regulation of cell survival and proliferation in diverse cell types[14,18]. Moreover, important processes such as growth, survival, proliferation, differentiation, and the pathologies of various diseases such as cancer, diabetes, and neurodegenerative diseases are mediated by functions of NO[19,20]. NO has also been shown to be involved in the control of heart functions and cardiac differentiation/development[21,22].

NO is considered one of many molecules that act on specific cell signalling pathways involved in embryonic development, specifically playing a dual role in the control of ESC differentiation and morphogenesis[23].

This dual role is determined by the NO concentration: It has been demonstrated that a low NO concentration maintains pluripotency, whereas a high concentration induces differentiation[14,18,23]. A high NO concentration has been reported to cause oxidative and nitrosative stress and apoptosis, processes partly responsible for cell death during chronic and degenerative disease. Moreover, embryonic stem cell differentiation is promoted by pharmacological treatment with high NO concentrations[18,24,25]. Our group has reported[18] that exposure to high concentrations of a NO donor (DETA-NO) promotes the differentiation of mouse ESCs induced by down-regulation of the pluripotency genes *Nanog* and *Oct4*. However, low NO concentration has been shown to promote cell proliferation and survival. Specifically, our group has demonstrated that the exposure of ESCs to a low concentration of DETA-NO promotes the expression of self-renewal genes and prevents the differentiation of mouse and human ESCs[14]. Therefore, NO is also considered a regulator of cellular respiration (oxygen-sensitive pathways) and metabolism, with a major role in regulating the hypoxia response by modulating the activity of CcO, a component of complex Ⅳ, which is involved in the final processes of the mitochondrial electron transport chain[4,16,26-28].

High NO

NO is a small molecule that has dual roles in the control

of ESC differentiation and tissue morphogenesis. High concentrations of NO promote differentiation. It has been reported that in ESCs, the production of NO is necessary for cardiomyogenesis because the maturation of terminally differentiated cardiomyocytes is prevented by NOS inhibitors[22]. Mouse ESC (mESC) differentiation is promoted by high DETA-NO concentrations, which induce the down-regulation of *Nanog* and *Oct4* expression. NO represses Nanog *via* the activation of p53, which is associated with covalent modifications such as Ser315 phosphorylation and Lys379 acetylation. Moreover, the expression of the definitive endoderm markers FoxA2, Gata4, Hfn1-β and Sox17 is increased by exposure to high concentrations of DETA-NO[8,18]. It has been reported that the NO concentration regulates signalling pathways implicated in the survival and homeostasis of RINm5F cells. Thus, high NO can cause oxidative and nitrosative stress and apoptosis[18,24,25]. Several studies report that NO induces apoptosis in various cell types, such as pancreatic beta cells[29,30], thymocytes[31] and hepatocytes[32]. In ESCs, high levels of DETA-NO promote nitrosative stress, inducing apoptotic events in part of the ESC population. The remaining ESC population will be resistant to nitrosative stress and express the cytoprotective genes haeme oxygenase-1 and HSP70, representing the start of a differentiation programme[18].

Mitochondria and stemness

Mitochondrial modulation is emerging as a mediator of stem cell proliferation and differentiation. Mitochondrial function is known to be fundamental to cellular health. The two actions that maintain mitochondrial function are fission and fusion processes, collectively termed mitochondrial dynamics (MD). Altering the balance of MD results in changes to mitochondrial morphology and increases the incidence of age-related disorders, such as neuromuscular degeneration, and of metabolic disorders, such as obesity, impaired glucose tolerance, and diabetes[33,34]. Many of these disorders have been shown to originate due to alterations in the function, morphology and number of mitochondria. The volume and efficacy of the mitochondrial mass is considered a determining factor in the production of reactive oxygen species (ROS) and the response to the oxidative stress level[35]. It has been reported that ROS levels are lower in undifferentiated cells. ESCs have been reported to resist oxidative stress better than differentiated cells and to contain a large complement of active mitochondria[36]. In addition, it has been shown that the expression levels of pluripotency markers are downregulated in mESCs when the mitochondrial DNA copy number is increased[37]. In general, pluripotent stem cells (PSCs) have a low mitochondrial population with low energy potential; most of the energy comes from glycolysis, which is limited only by a low ATP reservoir that precludes glucose phosphorylation to glucose 6-phosphate, which is required for uptake into the cells. Several types of differentiated cells, such as those that have differentiated into the trophectoderm of mice and rats, have been described as having more elongated mitochondria, with higher membrane potential and more O_2 consumption[38]. When cells are differentiated, the number of mitochondria is observed to increase, and the mitochondrial morphology shows characteristics observed in mature cells[39-41]. This behaviour is similar to that described in tumour cells, which have a decreased respiratory rate associated with an enhancement of anaerobic glycolysis due to a uniform transcriptional reduction of mitochondrial components. Human ESCs (hESCs) have been reported to have only a few mitochondria with immature morphology, and it has been found that the mitochondrial mass, the intracellular ROS level and the expression of antioxidant enzymes increase with differentiation[41]. Thus, the ROS produced in the differentiated cells might play an important role in cell signalling and differentiation[41-43]. Dynamic changes in mitochondrial energy metabolism, ROS and antioxidant enzymes have been shown to affect differentiation propensity. In fact, mitochondrial biogenesis is controlled by the expression of oxidative metabolism genes, among which are mitochondrial transcription factor A (Tfam), nuclear respiratory factor (NRF-1) and peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α)[44,45], which has been described to regulate mtDNA transcription and replication.

NO has been described as a physiological regulator of the mitochondrial respiratory chain and has been reported to interact with oxygen bound to CcO located in the inner mitochondrial membrane. CcO has a higher affinity for NO than for oxygen, which suggests that this interaction and its biological consequences are dependent on the redox state and turnover of CcO[4,46]. It has been shown that NO maintains normal cellular ATP levels by inhibiting mitochondrial respiration and increasing glycolysis[28]. This activity of NO is an important mechanism by which NO can modulate cellular responses to hypoxia in mammalian cells.

NO AND MITOCHONDRIAL BIOGENESIS

Mitochondrial biogenesis and mitochondrial dynamics

Mitochondrial biogenesis (MB) can be defined as the growth and division of pre-existing mitochondria as a mechanism to adjust the cellular energy balance in response to an environmental change or a change in the general status[47,48]. This process is regulated by a wide range of substances, including benzodiazepine, Ca^{2+} fluxes, and thyroid hormones such as T3, which controls metabolic rates in vertebrates[49,50]. Growing evidence suggests that the delicate equilibrium between mitochondrial fission and fusion is vital for many mitochondrial functions, including metabolism, energy production, Ca^{2+} signalling, ROS production and apoptosis[49,51-53]. For example, in some neurodegenerative diseases, there is a reduction in the expression levels of fusion proteins such as optic atrophy type 1, mitofusin-1 and mitofusin-2 and an increase in the expression of fission proteins such as dynamin-related protein-1 and fission related protein-1[54]. It has been reported that mitochondrial

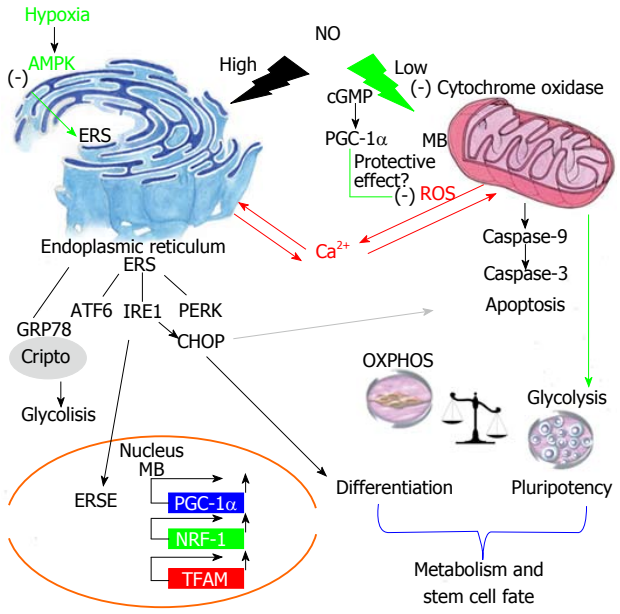

Figure 1 Nitric oxide dose effect on mitochondrial function and endoplasmic reticulum stress. High NO activates an ER stress response that induces apoptosis, at least in some types of cells, such as β-cells and macrophages. Depletion of ER Ca^{2+} and activation of the ERS pathway, including ATF6 activation and CHOP induction, was also detected in these cell types after treatment with NO. The soluble protein CRIPTO (also known as TDGF1) and its cell-surface receptor, 78 kDa glucose-regulated protein (GRP78), have crucial roles in promoting quiescence and in the maintenance of many cell types, including haematopoietic stem cells (HSCs). Under hypoxic conditions, HIF1α binds to hypoxia responsive elements (HREs) in the promoter region of CRIPTO and activates its expression. CRIPTO then binds to GRP78 and stimulates glycolytic metabolism-related proteins. This acts as a link between the ERS response and cell metabolism that finally controls stem cell fate. In addition, a low concentration of NO inhibits CcO and induces AMPK, which activates glycolysis. This can help to maintain pluripotency and cell proliferation. NO could have a protective effect under hypoxic conditions because the activation of AMPK inhibits CHOP expression and prevents apoptosis. This figure shows the relationship between NO, mitochondrial function and ERS and its impact on stem cell development. ERSE: Endoplasmic reticulum stress element; MB: Mitochondrial biogenesis; OXPHOS: Oxidative phosphorylation. AMPK: AMP-activated protein kinase; ATF 6: Transcription factor 6; CcO: Cytochrome c oxidase; CREB: cAMP response element binding protein; ERS: Endoplasmic reticulum stress; ESCs: Embryonic stem cells; Sgc: Soluble guanylate cyclase; cGMP: Cyclic guanosine monophosphate; HIF: Hypoxia inducible factor; HSP70: Heat shock protein 70; JNK: c-Jun-NH2-terminal kinase; MAPK: Mitogen-activated protein kinase; MB: Mitochondrial biogenesis; MD: Mitochondrial dynamics; NO: Nitric Oxide; NOS: Nitric oxide synthase; NRF-1: Nuclear respiratory factor 1; OXPHOS: Oxidative phosphorylation; PERK: Protein kinase-like endoplasmic reticulum kinase; PSCs: Pluripotent stem cells; PGC-1α: Peroxisome proliferator activated receptor γ coactivator 1α; ROS: Reactive oxygen species; Tfam: Mitochondrial transcription factor A; UPR: Unfolded protein response.

fusion permits inter-mitochondrial interaction and the exchange of membrane and matrix components; fusion can help to maintain the mitochondrial membrane potential and mitochondrial function[55]. Moreover, it has been demonstrated that mitochondria and mtDNA can be transferred between cells through cytoplasmic projections[56], which is a compensatory mechanism that allows mammalian cells with non-functional mitochondria to restore aerobic respiration. In addition, mitochondria also require numerous proteins from the cytosol that are transported through the matrix[57].

NO induces mitochondrial biogenesis

It is well known that treatment with low concentrations of NO induces MB in various mammalian cells types as well as in animal tissues. The MB induced by low concentrations of NO is mediated by activation of guanylate cyclase-dependent cGMP and is related to the increased expression of PGC-1α, NRF-1 and Tfam (Figure 1), and it also affects the mitochondrial function[58] (Figure 1). It has also been reported that NO/cGMP-dependent MB activates oxygen consumption and ATP production and subsequently increases mitochondrial function in U937, L6 and PC12 cells, among other cell types[59]. However, a previous study showed that during spontaneous differentiation of hESCs, the expression of MB markers was increased. In addition, that study found a discrepancy between regulatory factors of MB and mitochondrial activity: The mitochondrial activity in differentiated cells was higher than in undifferentiated cells, and differentiated cells showed no relationship between the expression of PGC-1α, NRF-1 and Tfam expression and mitochondrial function. Moreover, transmission electron microscopy showed that cellular organelles were immature in undifferentiated hESCs compared to cells undergoing differentiation. They concluded that hESCs possess immature machinery for the assembly of functional mitochondria[41]. Therefore, we consider in this review that low NO induces MB, but the expression of mitochondrial biogenesis markers alone may not be indicative of mitochondrial function.

Signalling for MB is activated by peroxisome PGC-1α and involves the expression of several transcription factors, resulting in the upregulation of proteins encoded by both nuclear and mitochondrial genomes[60].

PGC-1α is a transcription factor that regulates oxidative metabolism and adaptive thermogenesis and is the main inducer of MB in cells[61]. PGC-1α regulates the activities of cAMP response element binding protein and nuclear respiratory factors (NRFs). It provides a direct link between external physiological stimuli and the regulation of MB. PGC-1α has been described to have an important role in aging-related diseases, including neurodegenerative diseases such as Alzheimer's. PGC-1α activates a protective response through the induction of many antioxidant enzymes to decrease the ROS level, including superoxide dismutase and glutathione peroxidase 1. In addition, Ca^{2+} and ROS regulate MB by activating PGC-1α, leading to an increase in the mitochondrial mass[62]. It is very important to mention that PGC-1α was found to regulate the adaptive response at sub-lethal levels of different toxins that improve mitochondrial function in a process known as mitohormesis[60,63].

Interestingly, this hormetic response is controlled by the cell-tolerated increasing expression of PGC-1α, which in turn induces a balanced expression of fusion/fission genes. However, deregulation of PGC-1α expression through either stable down-regulation or overexpression renders cells more susceptible to toxic insult, leading

to mitochondrial fragmentation and cell death. This result has also been supported by the activation of Mtn2 expression[64]. In contrast, while PGC-1α permits the tolerance of a certain level of toxins in the cell, the prolonged expression to non-physiological levels of PGC-1α has a negative effect on mitochondrial function and the viability of the cells[65]. This suggests that the maintenance of a homeostatic PGC-1α expression level may offer a promising strategy for neuroprotective therapies against some toxicants[60].

NITRIC OXIDE REGULATES MITOCHONDRIAL FUNCTION

NO plays a very important role in regulating mitochondrial function and cell metabolic activity. It has been described that endothelial nitric oxide synthase (eNOS) is associated with the outer mitochondrial membrane in neurons and endothelial cells, which suggests that NOS regulates mitochondrial function[66] (Figure 1). The chemical structure of NO allows the interaction with haemoglobin and the release of O_2 for mitochondrial consumption[67]. As introduced earlier in this review, a mechanism for the regulation of mitochondrial function is the binding of NO to CcO, the terminal enzyme in the electron transport chain[68]. It competes with O_2 as the last electron receiver, inhibiting the activity of the enzyme and preventing water formation and ROS generation[69,70]. Furthermore, at a low concentration of O_2, both physiological and low concentrations of NO inhibit CcO and induce a switch to glycolysis that permits adaptation to hypoxic conditions[28,71]. However, a high concentration of NO also inhibits other mitochondrial complexes of the respiratory chain (complexes Ⅰ, complexes Ⅱ and complexes Ⅲ), increasing superoxide anion (O_2^-) production and inducing cell death[70,72] (Figure 1).

The hypoxia response is mediated by hypoxia inducible factor (HIF). HIF1α is the isoform that regulates oxygen homeostasis and cell metabolism in the short-term hypoxia response. Both HIF1α and NO help to restore energy metabolism at a low oxygen concentration[73]. High NO has been described to induce HIF1α expression under normoxic conditions in a mitochondria-dependent manner, but the effect of low NO under normoxia is unknown[27]. It is very important for us to evaluate this effect because we have evidence that low NO under normoxia induces HIF1α and can activate a similar hypoxia response.

NO and cell metabolism in pluripotent stem cells

The increase in anaerobic respiration and the decrease in oxidative phosphorylation in the presence of available oxygen is known as the Warburg effect[74]. This effect was considered a particular feature of cancer cells due to the typical hypoxic environment of tumours, but currently, it is considered a metabolic shift that permits cells to divide and proliferate[75]. Because of this, reduction of the ROS levels by the reduction of oxidative phosphorylation permits the activity of proliferative kinases, such as ERK1/2 and Akt, which inhibits the activation of the apoptotic machinery *via* activation of anti-apoptotic control mechanisms and the non-activation of pro-apoptotic kinases, such as c-Jun-NH_2-terminal kinase and p38 mitogen-activated protein kinase[76].

It has been reported that somatic cells require a shift from oxidative to glycolytic metabolism for the reprogramming process. Both HIF1α and HIF2α are necessary in the early state of the reprogramming for this metabolic change and for the recovery of the pluripotent state[77]. The bioenergetics of pluripotent cells can vary depending on their developmental stage. hECSs present highly glycolytic metabolism and share this feature with cancer cells (Warburg effect)[78]. Because the stem cell niche presents an hypoxic environment, the glycolytic metabolism of undifferentiated cells could be an adaptation to low oxygen concentrations *in vivo*[79]. In addition, the efficiency of the reprogramming process is reduced by glycolysis inhibition and an increase in glycolytic potency during the generation of inducible PSCs (iPSCs)[80]. Furthermore, it has been described that hypoxia enhances the generation of iPSCs. HIF1α and HIF2α are essential for the metabolic changes required for early iPSC generation in humans. However, HIF2α is detrimental at later stages of reprogramming because of the upregulation of TNF-related apoptosis-inducing ligand[77].

Cell metabolism is remarkably important for determining the stem cell fate and the role of NO in the regulation of metabolism. Almeida *et al*[28] described that NO activates glycolysis in astrocytes *via* the phosphorylation of AMP-activated protein kinase (AMPK), which activates Phosphofructokinase 2 and protects cells from apoptosis.

The relationship between NO and cell metabolism could be vital for the expansion of pluripotent cells when NO is used as a supplement in the design of culture medium, and it seems reasonable that NO may be used as a pluripotency inducer (Figure 1).

HIGH NO INDUCES ERS

The endoplasmic reticulum (ER) is the organelle designated for the synthesis and folding of proteins that are directed for secretion or to the Golgi apparatus. Because proper protein synthesis and protein folding are the key functions of the ER, the interruption of this physiological process ends in a complex ERS response, with a goal of recovering physiological function. The ER also functions as a store for Ca^{2+} and regulates its homeostasis through Ca^{2+}-pumping and Ca^{2+}-releasing proteins located in its membrane. Ca^{2+} is also an essential ion for ER function. Many chaperones, such as calreticulin or protein disulphide isomerase, are dependent on Ca^{2+} concentration, and therefore, any variation in ER intra-organelle Ca^{2+} concentration leads to changes in ER function[81,82].

There are many states that can lead to an unfolded protein response (UPR), the primary role of which is

to recover internal homeostasis and adapt to the new conditions in the ER. Any alterations in Ca^{2+} homeostasis can involve the UPR due to the malfunctioning of proteins responsible for protein folding in the ER lumen; nevertheless, the possible causes of the UPR are not restricted to this process, as, for example, glucose deprivation can also lead to ERS[83].

The first stage in the UPR implies an attempt at adaptation triggered by the release of chaperones anchored in membrane proteins, mainly glucose regulate protein 78 (Grp78, also known as BiP), to prevent the accumulation of misfolded proteins in the ER lumen. This release of Grp78 entails the aggregation of membrane proteins to which Grp78 was attached as well as Grp78 auto phosphorylation. Among these proteins, we primarily found protein kinase-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (Ire-1) and transcription factor 6 (ATF6)[84] (Figure 1).

PERK is a protein kinase that is able to auto phosphorylate when it oligomerizes, activating its kinase domain and inactivating eukaryotic initiation factor 2 α (eIF2α) after its phosphorylation. This leads to a decrease of mRNA translation with the aim of reducing the protein mass in the ER. In turn, genes related with the UPR are preferentially translated, as is the case of the transcription factor ATF4[85].

ATF4 is a CHOP activator and an apoptotic enhancer[86]. CHOP has been involved in many processes that lead to cellular apoptosis through the regulation of genes related with the cell cycle and ROS generation[87]. CHOP dimerizes with C/EBP, preventing its binding to gene promoters and blocking genes such as Bcl2, apoptotic suppressor, or peroxisome proliferator-activated receptor gamma, which promotes cell proliferation. This uniformly activates the expression of genes such as IL-6 and GADD34, which promotes cell differentiation and ROS synthesis[82,86].

ATF6 is a transcription factor that acts in the ERS response. ATF6 is cleaved when unfolded proteins are accumulated in the ER, releasing its cytoplasmic domain, which translocates to the nucleus. The inactive form of ATF6 (p90ATF6) is transported to the Golgi and is activated *via* two-step cleavage by Site-1 protease and Site-2 protease. Then, the active form of ATF6 is transported to nucleus[88] and functions as a transcriptional activator for ERS-related genes such as CHOP[89] (Figure 1).

Ire1 is a protein that shares similar pathways with PERK. Upon its oligomerization after Grp78 is released, its kinase activity is activated, and Ire1 is able to process an intron from Xbox protein-1 (XBP-1) mRNA to trigger its translation. XBP-1 activates genes related to protein transport from the ER to the cytosol, resulting in its degradation[82]. XBP-1 has also been correlated with apoptotic processes[84] and lymphocyte differentiation[83].

The release of Grp78 from the end of ATF6 also leads to its release towards the Golgi apparatus, where proteases are in charge of its cleavage, after which ATF6 is able to migrate to the nucleus and regulate genes, including the activation of XBP-1, which, as mentioned before, is an upregulator of CHOP[85].

Despite previous research on the ERS response and the effects of NO in cell regulation, the connection between these two processes was not clarified until 2001, when the first relationships between NO and CHOP were established through Ca^{2+} release and the subsequent release of ER Ca^{2+} to the cytosol induced by NO. Increases in the cell NO concentration lead to Ca^{2+} release from the mitochondria due to Cyt c inhibition, and this unleashes Ca^{2+} release in the ER. This response to NO is triggered as a result of protein misfolding in the ER because many chaperones are Ca^{2+}-dependent, which causes a UPR that over time ends in an apoptotic response[90-92] (Figure 1).

Endoplasmic reticulum stress and cell differentiation

High concentrations of NO have been frequently associated with differentiation and apoptotic processes; nevertheless, the genetic mechanisms that initiate these processes are barely defined[93]. Recently, many reports have suggested the relevance of the ERS response in the differentiation of several cell lines, which suggests the possibility of studying this response as a possible differentiation mechanism triggered by high concentrations of NO (Figure 1).

Previous studies have demonstrated the importance of ERS for the differentiation of chondrocytes[94-96], plasma cells[95,97], adipocytes[98] and myoblasts[18]. Nonetheless, recently, the differentiating potential of ERS has been identified in many other cell lines. Saito *et al*[99] demonstrated the effect of ERS in the terminal differentiation of osteoblasts through the activation of the PERK-eIF2α-ATF4 pathway. The results concluded that PERK activation triggered by the ERS response is required for ATF4 activation in osteoblast differentiation and for the proper expression of genes that are essential for osteogenesis, such as Ocn and Bsp[99]. Heijmans *et al*[100] studied the loss of pluripotency in epithelial intestinal cells upon activation of the UPR. They proposed that the ERS response causes the loss of stemness in a manner dependent on Perk-eIF2α-ATF4, a pathway activated in the UPR. Their findings demonstrate that inhibition of this signalling pathway results in stem cell accumulation in the epithelium, suggesting that the ER stress response is a key factor in the differentiation of this cell type[101,102]. More recently, Matsuzaki *et al*[101] evaluated the effect of physiological ER stress in fibroblast differentiation, suggesting that fibroblasts subjected to RER are more susceptible to differentiation signals and implying that UPR signalling could be essential for this differentiation. This is significant, considering that these cells are subject to a high protein load in the ER lumen in their final differentiation state due to the high amount of protein directed to secretion[96,100].

These studies suggest the possibility of studying the effect of NO in differentiation through this response

because the precise control of this molecule in this cellular process has been at least partially characterized.

Physiological concentrations of NO and RER

Although NO is a molecule that in high concentrations can lead to apoptosis and differentiation processes, some studies have suggested its protective effect and have demonstrated that endogenous levels of NO produced by different types of NOS are necessary for proper cell function and pluripotency maintenance. Previous studies have shown the need for physiological levels of NO to avoid the generation of free radicals or the activity of many proteases or to increase the antioxidant potential of GSH[103].

In 2007, Kitiphongspattana *et al*[97] demonstrated that the constitutive production of NO by cNOS was essential for a proper ER response in β pancreatic cells. Its inhibition repressed the expression of genes involved in protein folding and antioxidant defence, such as Gclc, Grp78, Prx-1 or Gpx-1[88,97]. Moreover, a protective effect of physiological NO in β-cells through the activation of Nrf2, a transcription factor for antioxidant proteins, in the RER has been reported previously, as has the importance of the UPR in resistance to oxidative stress[104-106].

Hypoxia, ERS and AMPK protective effect

ERS activates apoptosis through several stimuli, including hypoxia, oxidative stress and Ca^{2+} depletion. AMPK activation has been shown to protect cardiomyocytes against hypoxic injury through the attenuation of ERS[107].

ERS promotes apoptotic signalling or cell survival in different cell types. Therefore, the decision between survival and apoptosis may depend on the balance between survival signalling and apoptotic signalling.

Three apoptotic pathways are known to be related to ERS. Among others, the induction of the gene for CHOP promotes apoptosis, and CHOP deficiency can protect cells from ER stress-induced apoptosis, suggesting that CHOP is involved in the process of cell death caused by ERS. Other authors proposed that the mechanism of ERS induction during hypoxia is the alteration of the Ca^{2+} balance *via* the inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase pump due to intracellular ATP depletion[108]. Because of this, Terai *et al*[107] hypothesized that conservation of the intracellular ATP content during hypoxia would protect cardiomyocytes from ERS-induced cell death. They concluded that AMPK would be a cell protector against ERS-induced cell death during hypoxia because AMPK acts as an intracellular energy sensor, maintaining the energy balance within cells during ischaemia. The mechanism by which AMPK protects cells during hypoxia is attributed to the suppression of protein synthesis due to the phosphorylation of eEF2. This protective effect mediated by AMPK has also been described in another recent study. Hwang *et al*[109] reported that AMPK mediated cell survival induced by resveratrol in H9c2 cells, and this may exert a novel therapeutic effect on the oxidative stress induced in cardiac disorders.

It is interesting to consider the effects of AMPK with regard to the role of NO in the activation of this kinase. NO has a protective role against ROS and ERS, and this action could be mediated by AMPK. As we have demonstrated, NO regulates cell metabolism, and this effect can help to maintain pluripotency. NO has become known as a potent molecule that regulates cell protection.

CONCLUSION

In this literature review, we would like to highlight the role of NO as a regulator of stem cell properties. Low NO concentrations have been shown to help to maintain pluripotency, but the molecular mechanisms of this effect are not yet known.

We have analysed the role of NO in mitochondrial function and ERS because this pathway is interrelated with stem cell fate and could be an explanation for the mechanism by which NO regulates embryonic development.

We have described that NO has an important role in mitochondrial biogenesis and the induction of PGC1α expression. Notably, PGC1α has a neuro-protective effect against certain levels of different toxins. Due to the relationship between NO and PGC1α, the information in this review suggests the importance of studying the potential protective role of NO in cells.

NO is highly involved in the ERS response through its effect on mitochondria initiation of an ERS response, initially triggered by the release of Ca^{2+} in the mitochondria. This ERS response originates differentiation processes in many cell lines. This suggests the importance of studying the effect of NO in the ERS response to clarify its different effects through this pathway.

Likewise, NO in the ER is also relevant at physiological concentrations because many studies have shown that a proper ERS response is not feasible without sufficient physiological intracellular NO synthesis.

NO is involved in the regulation of cell respiration at a physiological level. NO inhibits cytochrome c and induces glycolysis, which can help to regulate stem cell fate. Pluripotent cells have been reported to have a highly glycolytic metabolism. In the reprogramming process, it is necessary that restoration of glycolytic metabolism is mediated by hypoxia-inducible factors. The induction of glycolysis by NO is vital for using this molecule as an inducer of pluripotency. NO is a strong tool for culturing pluripotent cell lines in an undifferentiated state. Moreover, NO can be used for biotechnology applications in the design of a culture medium for pluripotent cell expansion and for the creation of a cell therapy programme.

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MINIREVIEWS

Impact of T cells on hematopoietic stem and progenitor cell function: Good guys or bad guys?

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Abstract

When hematopoietic stem and progenitor cells (HSPC) are harvested for transplantation, either from the bone marrow or from mobilized blood, the graft contains a significant number of T cells. It is these T cells that are the major drivers of graft-*vs*-host disease (GvHD). The risk for GvHD can simply be reduced by the removal of these T cells from the graft. However, this is not always desirable, as this procedure also decreases the engraftment of the transplanted HSPCs and, if applicable, a graft-*vs*-tumor effect. This poses an important conundrum in the field: T cells act as a double-edged sword upon allogeneic HSPC transplantation, as they support engraftment of HSPCs and provide anti-tumor activity, but can also cause GvHD. It has recently been suggested that T cells also enhance the engraftment of autologous HSPCs, thus supporting the notion that T cells and HSPCs have an important functional interaction that is highly beneficial, in particular during transplantation. The underlying reason on why and how T cells contribute to HSPC engraftment is still poorly understood. Therefore, we evaluate in this review the studies that have examined the role of T cells during HSPC transplantation and the possible mechanisms involved in their supporting function. Understanding the underlying cellular and molecular mechanisms can provide new insight into improving HSPC engraftment and thus lower the number of HSPCs required during transplantation. Moreover, it could provide new avenues to limit the development of severe GvHD, thus making HSPC transplantations more efficient and ultimately safer.

Key words: Hematopoietic stem cells; Hematopoietic stem and progenitor cells; CD8 T cells; Transplantation; Engraftment; Memory T cells; Facilitating cells; Bone marrow

Core tip: T cells act as a double-edged sword upon allogeneic hematopoietic stem and progenitor cells (HSPC) transplantation, as they support engraftment of HSPCs and provide anti-tumor activity, but are also the cause

of graft-*vs*-host disease (GvHD). Here, we discuss the findings from several studies that have addressed the still enigmatic role of T cells during HSPC transplantation, either in an allogeneic or autologous setting, in mice or men, and with HSPCs derived from bone marrow, peripheral blood or cord blood. We anticipate that a better comprehension of how T cells support HSPC engraftment may lead to new strategies to optimize HSPCs transplantations and prevent GvHD.

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INTRODUCTION

Over the past 60 years, hematopoietic stem cell transplantation (HSCT) has developed into routine treatment for several hematological and immunological malignancies and deficiencies. Hematopoietic stem cells (HSCs) are the rare, self-renewing progenitor cells at the top of the hematopoietic hierarchy that can give rise to all blood cell lineages, and are thus able to restore hematopoiesis and the immune system in transplanted recipients. Hematopoietic stem and progenitor cells (HSPCs) are a mixture of HSCs and more differentiated progenitor cells, which provide respectively long and short-term repopulation of blood cells following transplantation. In the past, HSPCs required for transplantation were solely acquired from the bone marrow (BM), typically from the hipbone. This is an invasive procedure and if the BM harvest was not optimal, this often resulted in reduced engraftment of HSPCs and limited hematopoietic/immunologic recovery. Transplantation of higher doses resolved the majority of engraftment problems. Nowadays, this is facilitated by more favorable and less invasive HSPC harvesting procedures from mobilized peripheral blood (MPB) or umbilical cord blood (UCB). In the case of MPB HSPCs, donors are usually pretreated with granulocyte colony-stimulating factor (G-CSF), which mobilizes the HSPCs from the BM into the blood stream[1,2]. Subsequently, donors undergo one or two apheresis sessions to reach the desired HSPC dose for transplantation, normally 2-10 $\times$ 10^6 $CD34^+$ cells/kg recipient body weight[3-5]. In case of UCB HSPCs, the UCB is collected from the postpartum placenta, as this is a rich source of HSPCs. Typically, two grafts are required to reach sufficient numbers of UCB HSPCs when transplanting an adult. UCB HSPC transplants are generally used in an allogeneic transplantation setting, whereas mobilized HSPCs are used both during autologous and allogeneic HSCT. Autologous HSCT following high-dose chemotherapy has become routine treatment for many types of lymphomas[6]. It is considered a relatively low risk treatment, as the recipients receive their own HSPCs that were harvested before the chemotherapy was started. Allogeneic HSCT is regarded as a more precarious procedure, as HSPCs from a non-self origin are transplanted, which are thus subject to rejection by the host. In addition, allogeneic HSCT is combined with conditioning regimes of different intensity, leading to more variable transplantation outcomes. As with any allogeneic transplantation, matching of the human leukocyte antigen (HLA)-profile between donor and patient is instrumental to lower the risks of graft rejection. The first step in finding a suitable donor is to search within the immediate family. Related siblings have a 25% chance of being perfect donors, *i.e.,* matching 10 out of the 10 HLA antigens. Unrelated donors can also match the HLA antigens perfectly, although the chance of finding a perfectly HLA-matched, unrelated donor in a timely manner is much lower. Transplantation with partially matched (haploidentical), related donors (parent, child or sibling) is now considered as a viable alternative. Although these donors are usually more readily available, haploidentical donors have more HLA-disparities when compared to the recipient. This increases the risk of graft rejection, but also the development of graft-*vs*-host disease (GvHD)[7]. GvHD is a complicating side effect of an allogeneic HSPC transplantation that can develop early or late after the transplantation, in which transplanted donor T cells mount an extensive immune response against the recipient's tissues. A mild degree of GvHD is considered beneficial when the HSPC transplantation is part of an anti-cancer treatment, as the allo-responsive donor T cells in the graft can also eradicate the remaining tumor cells present in the recipient. However, severe forms of GvHD are very hazardous and hence the predominant cause of the high morbidity and mortality rate associated with allogeneic HSPC transplantations. A highly efficient procedure to minimize the risk of developing acute and chronic GvHD is T cell depletion (TCD) from donor grafts[8]. Unfortunately, transplantations with TCD grafts also revealed major pitfalls, such as graft failure and disease relapse. This was observed in BM transplants with grafts from HLA-identical siblings and HLA-non-identical (but related) donors with minimal mismatch[8,9]. What was apparent from these studies is that conditioning regimes and post-transplant immune suppression treatments can all affect the outcome of transplantation with TCD grafts. To this day, it is still unclear how the depletion of T cells leads to increased risk of graft failure. What is certain is that T cells can aid the engraftment of HSPCs in the damaged BM environment in which they find themselves post-transplant. Here, we review what is currently known about the contribution of T cells on HSPC engraftment in different transplantation settings, as this knowledge can be used to improve both the efficiency and safety of this important clinical procedure.

FACILITATING T CELLS

While grafts depleted of T cells minimize the risk of developing GvHD, T cell depletion also compromises the engraftment of transplanted HSPCs. This unwanted effect

has been attributed to the loss of specific cell subsets that assist the engraftment, the so-called facilitating cells (FC) that were depleted during the T cell depletion process. In 1994, Kaufman *et al*[10] showed for the first time that engraftment of murine BM HSPCs was facilitated by BM cells that were positive for CD8, CD45, CD45R, CD3, dull/intermediate for MHC class II and negative for the TCR. The absence of the TCR indicates that these cells are not T cells. However, some discrepancy does exist regarding this finding. Gandy *et al*[11] observed increased survival of mice transplanted with allogeneic HSPCs together with BM $CD8^+$ cells. When separated into $TCR\beta^+$ and $TCR\beta^-$ fractions, both $CD8^+$ $TCR\beta^+$ and $CD8^+$ $TCR\beta^-$ cells had the ability to enhance survival of transplanted mice. The $TCR\beta^+$ population displayed morphology resembling T lymphocytes. The majority of the $TCR\beta^-$ cells had granular cytoplasm and low nuclear to cytoplasm ratio with a lymphoid dendritic cell phenotype. Further examination on the $CD8^+$ $TCR\beta^+$ cells, which are predominately present in the BM, revealed that they express CD44[11]. This indicates that these T cells have a memory phenotype. In the BM, three different subsets of $CD8^+$ T cells co-exist; effector memory (T_{EM}; $CD44^+$ $CD62L^-$), central memory (T_{CM}; $CD44^+$ $CD62L^+$) and naïve T cells (T_{NV}; $CD44^-$ $CD62L^+$)[12]. We recently showed that the frequency of total $CD8^+$ T cells is similar within the different bones found throughout the murine body. Additionally, we observed that during steady state conditions, T_{NV} cells are the dominant subset and that this quickly changes after an acute infection with lymphocytic choriomeningitis virus, as the T_{EM} cells replace the T_{NV} cells[13]. Also in humans, it has been shown that $CD8^+$ cells are important for HSPC engraftment. Martin *et al*[14] demonstrated that removal of $CD8^+$ cells but not $CD4^+$ cells from donor bone marrow grafts results in graft failure. Interestingly, they also showed that the dose of the $CD8^+$ cells in the grafts is of major importance as more than half of the patients that received grafts containing less than 3.9×10^6 $CD8^+$ cells/kg experienced graft failure. They further observed that the high dose of $CD8^+$ cells required to prevent graft rejection also increases the risk for acute and chronic GvHD[14]. Taken together, many studies in mice and human have quite clearly shown that $CD8^+$ T cells have a beneficial effect during HSCT. This in itself is surprising given the fact that T cells, depending on their activation state, are known to have a strong skewing impact on hematopoiesis. We and many others have shown that activated T cells can directly affect HSC function by increasing differentiation and limiting self-renewal[15]. Interferon-gamma (IFN-γ) is one of the pro-inflammatory cytokines produced by activated T cells, which can inhibit HSC self-renewal and enhance their differentiation in a direct manner[16,17], but also indirectly by acting on surrounding niche cells[18,19]. This indicates that the impact that T cells can have on the behavior and function of HSPCs is complex and not only dependent on the T cell subset, but also on the activation status of the T cells and the niche cells they interact with.

THE INFLUENCE OF T CELLS ON HSPC ENGRAFTMENT DURING ALLOGENEIC AND AUTOLOGOUS TRANSPLANTATION

Although early studies demonstrated a clear contribution of $CD8^+$ T cells on HSPC engraftment, this concept received surprisingly limited follow-up, both scientifically and clinically. This is most likely due to the fact that current transfusion protocols are performed with high numbers of HSPCs, which compensates for any sub-optimal engraftment condition[14]. Nonetheless, understanding how to optimize the engraftment potential of HSPCs is still desirable, especially when HSPC numbers are limited. We therefore also took into account studies that investigated which T cell subsets are involved in the development of GvHD. The majority of these studies did not examine HSPC engraftment *per se*, but do provide interesting clues on which T cell subsets may be beneficial for this process, as they did assess immune reconstitution after transplantation. Chen *et al*[20] depleted $CD62L^+$ T cells (T_{NV} and T_{CM}) from murine BM grafts and found that $CD62L^-$ T cells (T_{EM}) accelerated the recovery of $CD4^+$ and $CD8^+$ T cells after transplantation, which could indicate enhanced hematopoietic engraftment. In addition, transplantation of the T_{EM} subset alone did not result in GvHD, while grafts that still included T_{NV} and T_{CM} did. Another interesting observation made is that transplantation of $CD62L^-$ T cells led to increased donor chimerism, as it lowered the numbers of residual recipient T cells[20]. Similar results were obtained in human studies. Naïve human T cells express CD45RA and switch to the CD45RO isoform upon antigen encounter and develop into memory T cells. Touzot *et al*[21] demonstrated that allogeneic HLA-mismatched HSCT with CD45RA-depleted BM grafts were successful and did not lead to severe GvHD, suggesting that this could be a potential approach to treat patients with primary combined immuno–deficiencies. Importantly, they also observed that viruses detected prior to the HSCT were rapidly cleared post-HSCT, indicating that the CD45RA-depleted graft contained $CD8^+$ T cells functionally active against pathogens. Furthermore, Triplett *et al*[22] found similar results when transplanting patients suffering from hematological malignancy with haploidentical CD45RA-depleted grafts. Here, patients received on day 0 G-CSF MPB grafts that was highly enriched for $CD34^+$ cells and thus depleted of $CD3^+$ cells, with a median dose of 11.2 and 0.012×10^6/kg, respectively). On day 1 patients received MPB grafts depleted of $CD45RA^+$ T cells (and thus enriched for memory T cells), and on day 6, they received an infusion with purified NK cells from the same donor[22]. The authors demonstrated that this combination of differently prepared grafts led to rapid neutrophil engraftment, quick conversion to full donor chimerism and fast reconstitution of innate and adaptive immunity. These three clinical parameters signify rapid HSPC engraftment, which may well have been the result of the co-injected $CD45RO^+$ memory T cells on day 1, although this was not formally tested in this study.

Importantly, none of the patients developed acute GvHD. Several patients did show signs of chronic oral GvHD, but not of severe nature[22]. Next to removing specific subsets that might cause GvHD, many studies also focused on adding back T cells after transplantation. The transfer of donor T cells after transplantation has in fact become a frequently applied clinical procedure, called donor lymphocyte infusion (DLI). This is usually performed to cause a milder and more controllable degree of GvHD, aiming for the donor T cells to eliminate residual recipient cells and thereby improve donor chimerism and/or remove residual tumor cells (graft-*vs*-tumor effect). For DLI, peripheral blood is donated by the same HSPC-donor, this time without G-CSF pre-treatment. Next, $CD3^+$ cells are isolated and given to the patient after a brief recovery period after the initial HSCT[23]. A study performed in mice found that DLI was able to improve engraftment of HSC without resulting in GvHD[24]. They observed that mice, which had received low (sub-lethal) dose of irradiation, rejected allogeneic donor BM cells, unless this procedure was combined with an injection of peripheral blood mononuclear cells. Further analysis revealed that $CD8^+$ cells were facilitating the engraftment. Importantly, this beneficial effect was only observed when the DLI was given on the same or the following day, but not on third or seventh day of the HSCT[24]. This suggests that early DLI may be sufficient to counteract the beginning of an anti-donor response by boosting HSPC engraftment. Interestingly, a study in humans did not show similar effect of DLI; Kreiter *et al*[25] found that minimal conditioning prior to T cell-reduced allogeneic HSCT combined with subsequent DLI was insufficient to sustain long-term engraftment. This study, similar to Nakamura's study, gave DLI on the same day as the HSCT. However, the fact that none of the transplanted subjects reached complete donor T cell chimerism indicates that this specific combination of minimal conditioning and DLI dose was not optimal[25]. In contrast to the mice in Nakamura's study, these human subjects were patients suffering from hematological malignancies. It remains to be determined if and how the conditioning regimes and the presence of disease affect the possible facilitating role of DLI on HSPC engraftment. Interestingly, also in a DLI setting, infusion of T cells depleted of naïve T cells was shown to favor engraftment without causing GvHD[26]. Additional investigation on the murine $CD62L^-$ T cell subset originally described by Chen *et al*[20] revealed that addition of 1×10^6 $CD62L^-$ T cells could rescue 90% of graft rejection that developed after transplantation with TCD grafts in mice. The $CD62L^-$ T cell subset was also shown to prevent tumor growth and help combat viral infection[26]. More importantly, addition of $CD62L^-$ T cells resulted in 100% donor chimerism within 30 d and was maintained long-term. Similar results were obtained in human studies, as Shook *et al*[27] transplanted patients with $CD3^+$-depleted haploidentical grafts and infused CD45RA-depleted cells the following day. They observed that all patients reached complete donor chimerism. In this study patients received myeloablative conditioning. Remarkably, 3 mo after the HSCT, the majority of $CD4^+$ and $CD8^+$ T cells were still $CD45RA^-$, indicating that the T cells were derived from the CD45RA-depleted grafts[27]. DLIs can also be given to patients not responding to anti-viral medication during an infection post HSCT. This is particularly important after HSCT with T cell-depleted grafts, as protective immunity is not transferred in this setting and engraftment, and thus immune reconstitution is delayed. Stemberger *et al*[28] demonstrated that as few as 3750 antigen-specific T cells per kg body weight was sufficient to decrease viral load in a patient suffering from systemic CMV infection after CD3-depleted HSCT[28]. In this study, the impact on HSPC engraftment was not reported. Nevertheless, this study highlights a feasible strategy to combat persisting infections post HSCT. So far, the facilitating role of $CD8^+$ T cells, especially memory T cells in HSPC engraftment has been well established in allogeneic HSCT setting. Less explored is if these cells have similar effects in autologous HSCT. Interestingly, Rutella *et al*[29] demonstrated that patients undergoing autologous transplantation with selected $CD34^+$ cells instead of unmanipulated PBMCs experienced delayed repopulation of the T cell lineage. This suggests that the lack of T cells might also affect engraftment in an autologous setting. Furthermore, Russell *et al*[30] assessed mobilization and engraftment in autologous donors. They observed that grafts with low numbers of $CD34^+$ cells (poor mobilizers) contain more $CD8^+$ T cells than grafts from moderate or high mobilizers. Subsequently, they assessed if the $CD8^+$ T cell content was associated with time to neutrophil engraftment after HSPCT. The results obtained suggest that engraftment occurs faster when there are more $CD8^+$ T cells present in the grafts. This was limited to grafts that contain low numbers of $CD34^+$ cells[30]. Taken together, these studies show that when HSPC numbers are limiting, memory $CD8^+$ T cells have a beneficial effect on HSPC engraftment, both in allogeneic and autologous transplantation. The effects of different (graft) treatments on HSCT are summarized in Table 1.

THE ROLE OF T CELLS UPON TRANSPLANTATION OF HSPCS FROM UMBILICAL CORD BLOOD

In the clinic, when an HLA-matched sibling and unrelated matched donor are not available, the next best option is HSCT with UCB. It is now well established that transplantation with UCB grafts results in lower rates of GvHD disease when compared to HSCT from BM or MPB. This in itself is very interesting, especially as the majority of the T cells present in a UCB graft are $CD45RA^+$[31], and thus potentially capable of inducing GvHD. However, these $CD45RA^+$ UCB T cells are functionally distinct and less mature than $CD45RA^+$ naïve T cells found in

Table 1 Effects of (graft) treatments on hematopoietic stem cell transplantation

Treatment	Removed	Remaining	Effect
Complete T cell depletion	All T cells	N/A	GvHD ↓[8,14,44] Disease relapse ↑[8,9,44] Graft failure ↑[8,9,44] Immune reconstitution ↑[44]
Partial T cell depletion	CD45RA (T_{NV}) $CD62L^+$ (T_{NV}, T_{CM})[1]	CD45RO (T_{MEM}) $CD62L^-$ (T_{EM})[1]	GvHD ↓[20,21,22] Neutrophil engraftment ↑[22] Immune reconstitution ↑[20] Protective immunity ↑[21,22] Donor chimerism ↑[20,22]
Donor lymphocyte infusion	CD45RA (T_{NV}) $CD62L^+$ (T_{NV}, T_{CM})[1]	CD45RO (T_{MEM}) $CD62L^-$ (T_{EM})[1]	GvHD ↓[26] Tumor growth ↓[26] Engraftment ↑[26] Graft failure ↓[26] Immune reconstitution ↑[26] Protective immunity ↑[26,28] Donor chimerism ↑[26,27]

[1]Signifies the murine equivalent of the human T cell subset described above. Here, we summarize the impact that either full or partial T cell depletion of an HSPC graft, or selective donor lymphocyte infusion, can have on the clinical outcome of a HSPC transplantation. Indicated are the T cell subsets that have either been removed or that remain, and the biological or clinical effects that have been reported following this treatment. N/A: Not available; HSPC: Hematopoietic stem and progenitor cell; GvHD: Graft-*vs*-host disease.

adults[32], explaining why these cells are less related to the development of GvHD. T cells in UCB have the ability to respond to allogeneic stimulation, but the response generated is less cytotoxic than that of adult T cells. Additionally, dendritic cells present in UBC have been found to be in an immature state and thus limiting the activation of UCB T cells[32]. Nevertheless, while the development of GvHD is reduced, patients who undergo UCBT are subjected to high incidence of infection, as immune reconstitution is slow. It is believed that the low numbers of HSPCs and downstream progenitors transplanted during UCBT compared to MPB HSCT are the cause of the delay in hematopoietic reconstitution, while the absence of memory T cells would render the recipient more sensitive to viral infections. However, following our line of reasoning, the lack of memory T cells during UCBT might contribute to impaired HSPC engraftment. Currently, the focus on improving engraftment has been on reducing conditioning regimes, performing double UCB transplantation, *ex vivo* expansion of UCB HSPCs and intra-bone infusion of UCB grafts[31]. An interesting approach is the combination of UCB grafts with $CD34^+$ cells isolated from haploidentical grafts[33]. The idea behind this concept is that the haploidentical graft will provide early engraftment, while the UCB graft provides long-term engraftment. Indeed, the authors observed fast engraftment of neutrophils and platelets post haplocord SCT. Furthermore, UCB cells replaced this first wave of hematopoiesis by the haploidentical $CD34^+$ cells within 100 d. An important future aspect of this approach is that not the cell number of the UCB graft but rather the matching of the HLA type to that of the patient will take priority when finding suitable UCB grafts for UCBT. This by itself will provide more options for adult patients lacking related and unrelated HLA matched donors.

POSSIBLE UNDERLYING MECHANISMS ON HOW $CD8^+$ T CELLS IMPROVE ENGRAFTMENT OF HSPCS

The underlying reasons on why and how $CD8^+$ T cells contribute to HSPC engraftment are still poorly understood. Currently, there are more questions than answers, which we will address here; the mechanisms discussed below are depicted in Figure 1. For example, do donor T cells contribute to engraftment by killing residual host HSPC and thus eliminating the competition? This is unlikely, as Gandy *et al*[11] showed that $CD8^+$ T cells did not facilitate HSPC engraftment *via* their lytic potential, as $CD8^+$ T cells deficient in their lytic activity were still able to assist engraftment. However, one of the most important observations made by several studies is that the addition of donor T cells eliminates residual host T cells in mice[20,24,26]. These observations suggest that removal of residual host T cells is an essential part in eliminating any type of resistance from the host to allow engraftment. Furthermore, is it possible that donor T cells somehow directly affect the function of HSPCs? An interesting observation made by Adams and colleagues is that in the $\beta2m^{-/-}$NOD/SCID mice, $CD8^+$ cells augmented homing and engraftment of $CD34^+$ cells by modulating their response towards CXCL12 by affecting their phosphotyrosine-mediated signaling. *Ex vivo*, this modulated response towards CXCL12 resulted in increased migration through a BM endothelial cell line[34]. Further analysis revealed that this was not the result of factors secreted by $CD8^+$ cells, though an active cytoskeleton in the $CD8^+$ cells was required for the increased transmigration of $CD34^+$ cells[34]. Moreover, it could also be that $CD8^+$ T cells can affect the HSC niche by making the environment more favorable for engraftment of the newly arrived HSPCs.

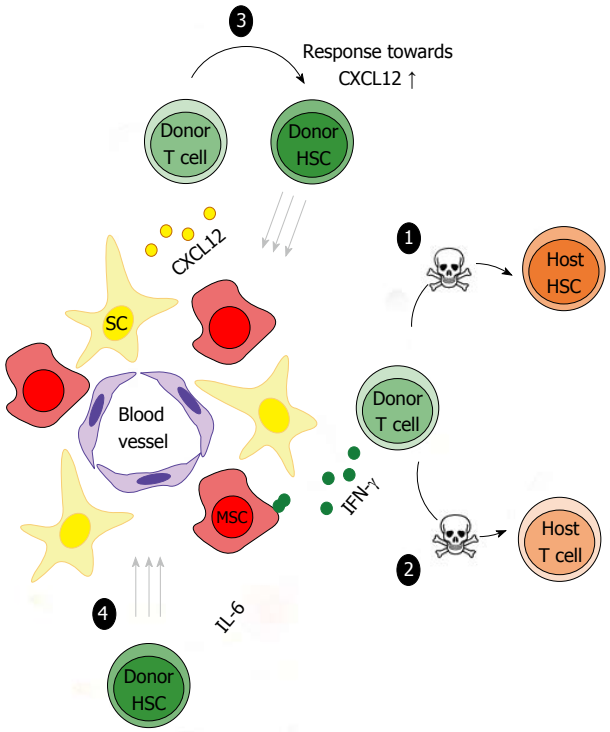

Figure 1 Potential mechanisms on how CD8$^+$ T cells improve hematopoietic stem and progenitor cells engraftment. The following modes of action have been described or suggested by which donor T cells can support the engraftment of HSPCs upon transplantation: (1) killing of residual host HSPCs; (2) killing of residual host T cells; (3) augmented homing of HSPCs to CXCL12 produced by reticular SC or MSCs; (4) increased HSPC differentiation by IFN-γ-induced production of IL-6 by MSCs. HSPCs: Hematopoietic stem and progenitor cells; SC: Stromal cells; MSCs: Mesenchymal stromal cells; IFN-γ: Interferon-gamma; IL-6: Interleukin-6.

The production of IFN-γ by CD8$^+$ T cells was shown to promote the release of interleukin-6 from mesenchymal stromal cells (MSCs), an essential component of the HSC niche[18]. This enhanced myeloid differentiation of more committed progenitors, though the impact of T cell-modulated MSCs on HSPC engraftment has not been examined. Lastly, an intriguing question on the functional impact of CD8$^+$ T cells on HSCT is whether CD8$^+$ T cells and HSCs co-localize in the BM. It has been shown that memory CD8$^+$ T cells co-localize with VCAM-1-expressing stromal cells in BM[35], whereas HSC-supporting MSCs also express VCAM-1[36,37]. These findings are compatible with the hypothesis that HSCs and CD8$^+$ memory T cells share the same niche, though actual co-localization between these cells has not yet been experimentally demonstrated.

THE IMPACT OF OTHER FACILITATING CELL TYPES ON HSPC ENGRAFTMENT

Although the positive impact of CD8$^+$ T cells on HSPC engraftment has been addressed most extensively, there is evidence that other cell types in the BM can also have this effect. As mentioned before, not all CD8$^+$ cells that can facilitate HSPC engraftment also express the TCR[10]. In follow-up of these findings, Grimes *et al*[38] found that CD8$^+$TCR$^-$ FC do not express TCR gene transcripts (TCRα and TCRβ), clearly distinguishing them from conventional T cells. Furthermore, they showed that CD8$^+$TCR$^-$ FC do express CD3ε and that this complex is important for the beneficial effect of these cells during allogeneic transplantation[38]. Further gene expression analysis on the CD8$^+$TCR$^-$ FC revealed that the DOCK2 gene was most significantly different between functional and functionally impaired FC cells. Indeed, FCs lacking the expression of DOCK2 do not enhance engraftment and do not promote homing and lodgment of HSPCs in the bone marrow[39]. Additionally, also human CD3$^+$CD8$^+$TCR$^-$ cells have been shown have facilitating potential when co-transplanted with suboptimal doses (3-5 × 10^4) of UCB CD34$^+$ cells in NOD/SCID mice[40].

Next to CD8$^+$ T cells, multiple studies have shown that CD4$^+$ T cells, especially regulatory T cells (Tregs) can also support HSPC engraftment. Danby and colleagues showed that higher proportions of Tregs in MPB grafts improve recovery and clinical outcomes[41]. It has also been demonstrated that host Tregs co-localize with transplanted allo-HSPCs in BM[42], indicating that these cells may provide an immune privilege site for HSPCs in the BM. Furthermore, also TCRγδ$^+$ T cells have gained recognition for their facilitating role in engraftment of HSPCs. Kawanishi *et al*[43] found that engraftment was associated with the dose of TCRγδ$^+$ T cells present in BM grafts. Importantly, they found no association between the TCRγδ$^+$ T cells dose and an increased risk for the development of acute GvHD in patients that received grafts from related donors[43]. In conclusion, it is clear that the BM contains multiple cell types that can enhance HSPC engraftment. This is highly relevant from a clinical perspective, though it remains unclear to what extent these cell types also support the function or maintenance of HSPCs in the BM under physiological conditions.

FUTURE PERSPECTIVES

After decades of development in the allogeneic HSCT field, GvHD is still a major complication. To this day, the best approach for decreasing the risk for GvHD is the removal of T cells from the graft. After it was apparent that TCD procedure led to poor engraftment, delayed immune reconstitution and increased disease relapse[44], TCD procedures were dismayed as a reliable method to safely and efficiently combat GvHD. However, several studies discussed here suggest that future of TCD HSCT may lie in partial instead of complete depletion of T cells. The aim of the variety of the approaches attempted so far was to remove T cells that contribute to GvHD while maintaining T cells that provide immediate but also long-term immune protection. The focus has never necessarily been to improve engraftment of HSPCs, as this potential problem is covered by the immense amounts of HSPCs transplanted. Nevertheless, identifying T cell subsets that specifically favor HSPC engraftment and the underlying mechanism may be more beneficial in the long run, as more efficient and rapid engraftment will be required if

less and less intense conditioning regimes are used in the future. Moreover, efficiency of HSPC transplantation is significantly reduced when donor HSPCs numbers are limited or when HSPCs are genetically modified, which thus requires protocols in which their engraftment is fully optimized. More in depth studies are required to determine whether the future of allogeneic HSCT can/will develop into one where patients receive minimal conditioning with a low dose of HSPCs followed by multiple infusions of different T cells subsets; these could be chosen based on their ability to improve HSPC engraftment, to provide the first wave of protective immunity and/or to induce a low-grade level of GvHD to boost an anti-tumor response. Unpublished work from our group suggests that *ex vivo* expanded $CD8^+$ T cells with a memory phenotype have facilitating potential at the level of HSPC engraftment. It is hence interesting to speculate that the future of transplantation may include the addition of *ex vivo* expanded T cells from the graft in order to enhance engraftment and immune reconstitution. Hopefully, the developments in HSCT treatment in the coming years will results in faster recovery, decrease disease relapse and overall shorter hospital stays.

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