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# The *ex vivo* toll-like receptor 7 tolerance induction in donor lymphocytes prevents murine acute graft-versus-host disease

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**Abstract**

*Background aims.* Acute graft-versus-host disease (aGVHD) remains a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation, mediated by alloreactive donor T cells. Toll-like receptors (TLRs), a family of conserved pattern-recognition receptors (PRRs), represent key players in donors' T-cell activation during aGVHD; however, a regulatory, tolerogenic role for certain TLRs has been recognized in a different context. We investigated whether the *ex vivo*–induced TLR-2,-4,-7 tolerance in donor cells could prevent alloreactivity in a mismatched transplantation model. *Methods.* TLR-2,-4,-7 tolerance was induced in mouse splenocytes, after stimulation with low doses of corresponding ligands. Cellular and molecular changes of the TLR-tolerant splenocytes and purified T cells were assessed by immunophenotypic and gene expression analyses. Incidence of aGVHD was evaluated by the clinical score and survival as well as histopathology of target tissues. *Results.* Only the R848-induced TLR7 tolerance prevented aGVHD. The TLR7 ligand–induced tolerance lasted for a critical post-transplant period and was associated with distinct cellular and molecular signatures characterized by induction of regulatory T cells, reduced alloreactivity and balanced regulation of inflammatory signaling and innate immune responses. The TLR7-tolerant T cells preserved the immunological memory and generated *in vitro* virus-specific T cells upon antigen stimulation. The anti-aGVHD tolerization effect was direct and specific to TLR7 and required the receptor–ligand interaction; $TLR7^{-/-}$ T cells isolated from B6 $TLR7^{-/-}$ mice presented a distinct gene expression profile but failed to prevent aGVHD. *Discussion.* We propose an effective and clinically applicable *ex vivo* approach for aGVHD prevention through a transient and reversible immune reprogramming exerted by TLR7-tolerant donor lymphocytes.

**Key Words:** *allogeneic hematopoietic stem cell transplantation, acute graft-versus-host disease, toll-like receptors, TLR7 tolerance*

## Introduction

Acute graft-versus-host disease (aGVHD) represents a leading cause of morbidity and mortality among patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) [1] and the major barrier to its more widespread and successful application. Interactions between donor T cells and activated host and donor antigen-presenting cells (APCs) induce aGVHD [2,3] that ultimately results in severe clinical sequelae from organ damage and impaired immunity [4]. With standard prophylaxis, approximately 40% of patients undergoing human leukocyte antigen–matched HSCT will develop aGVHD [1,5], whereas up to 60% of affected patients will present resistance or suboptimal response to corticosteroids [6]. Given the increasing use of HSCT, the dismal outcome of severe aGVHD and the limitations inherent to its current treatments, the development of alternative approaches to effectively prevent or treat aGVHD is a challenging and largely unmet demand.

During aGVHD development, donor T-cell activation is mediated through two main signaling events: binding to the T-cell receptor of an allogeneic peptide presented by the host's major histocompatibility complex and co-stimulatory signals delivered by APCs. The latter process involves innate immune mechanisms in which toll-like receptors (TLRs) are key

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players [7]. TLRs represent highly conserved pattern-recognition receptors that are broadly expressed in hematopoietic and non-hematopoietic cells [8]. They interact with exogenous pathogen-associated molecular patterns and also recognize endogenous damage-associated molecular patterns generated during "sterile" tissue injury [9,10].

TLR activation by microbial products of the intestinal flora after conditioning-induced interruption of the epithelial barrier was recognized as a key event in the initiation of inflammation during aGVHD [11], and the intestinal decontamination during conditioning to generate a "protective environment" has become a common practice in HSCT [12]. Nevertheless, the role of TLRs in aGVHD development has not been elucidated, and published reports remain controversial on whether specific TLR blockade or deficiency promotes or prevents aGVHD [13–20].

In addition to TLR involvement in proinflammatory responses, TLRs also contribute to tolerance; whereas a single administration of a high-dose TLR agonist can generate a profound inflammatory response, repeated exposure to low doses of TLR ligands results in TLR "desensitization," expressed as reduction in the magnitude of response to subsequent proinflammatory cytokine challenge, a phenomenon described as TLR tolerance [21]. TLR tolerance induced by repeated low-dose *in vivo* administration of TLR2 or TLR7 agonists has been experimentally exploited in the context of autoimmunity [22,23]. Conflicting reports exist, however, on whether the *in vivo* administration of TLR4, TLR5 and TLR7/8 agonists in allogeneic HSCT models results in either reduced incidence of aGVHD and improved survival [20,24,25] or, in sharp contrast, acceleration of aGVHD and low survival rates [19].

In this study, we sought to explore, for first time, whether an *ex vivo* intervention, by inducing TLR-2, -4, and -7 tolerance to donor cells, instead of the *in vivo* conditioning of the host with TLR agonists, could prevent alloreactivity in a mismatched transplantation model. Our data demonstrate that the *ex vivo*–induced TLR7 tolerance in donor lymphocytes confers a striking protective effect on aGVHD incidence and lethality and may serve as an effective and clinically translatable approach for aGVHD prophylaxis.

## Methods

Additional information on methods is provided in the supplemental Methods.

### *Mice and bone marrow transplantation*

C57BL/6J (H-2K[b]), BALB/c (H-2K[d]) and B6 TLR7$^{-/-}$ (B6.129S1-Tlr7tm1Flv/j strain) mice (Jackson Laboratory), between 12 and 16 weeks of age, were used in the experiments. Male and female BALB/c recipients irradiated with a single dose of 7 Gy ($^{137}$Cs source) were equally distributed between experimental cohorts and transplanted with a 1:1 mixture of male and female, fully mismatched, C57BL/6J or B6 TLR7$^{-/-}$ donor cells. All animal experiments were approved by the Animal Care and Use Committee of the Regional Veterinary Health Authority.

### *TLR tolerance induction*

TLR-2, -4, -7 tolerance was induced in splenocytes (SCs) of C57BL/6J mice. Cells were seeded at $3 \times 10^6$ cells/mL in "SC medium" consisted of RPMI 1640 (Gibco) and supplemented with 10% (v/v) heat-inactivated FBS (Gibco), 2 mmol/L L-glutamine (Lonza), 100 U/mL penicillin-100 µg/mL streptomycin (Gibco) and 50 µmol/L 2-ME (Sigma-Aldrich). To determine the optimal conditions of TLR-2, -4, -7 "desensitization", SCs were pretreated with different doses of their specific ligands [Pam3CSK4 (0.25–1.0 µg/mL, InvivoGen), high pure lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (5–100 ng/ml, Sigma-Aldrich), R848 (1–5 µg/mL, InvivoGen)] and for three exposure periods ($1 \times 24$, $2 \times 24$ and $3 \times 24$ h). At the end of each day, the culture was re-supplemented with the specific ligand dose. The optimal doses and exposure times for SC "desensitization" (maximum TLR ligand concentration, $3 \times 24$ h exposure time) were determined by enzyme-linked immunosorbent assay (ELISA) on the basis of the highest tumor necrosis factor (TNF)-α reduction (see supplementary Methods) and used in all experiments thereafter. To confirm tolerance, treated SCs were challenged (day 0) with 5 µg/mL Pam3CSK4, 1 µg/mL LPS and 25 µg/mL R848 for 2 h, and supernatants were analyzed for TNF-α levels by ELISA. In indicated experiments, CD3$^+$ T cells were immunomagnetically purified from whole SCs using CD3ε-biotin-conjugated magnetic beads (MACS, Miltenyi Biotec) according to the manufacturer's instructions and subsequently pretreated with either R848 (TLR7 ligand-tolerized T cells) or phosphate-buffered saline (PBS).

### *Assessment of aGVHD*

aGVHD was assessed using a previously described five-parameter clinical score [26]. Mice were monitored daily for survival and scored (scale 0–2) twice weekly for weight loss, posture (hunching), fur texture, skin integrity and activity, yielding a maximum index score of 10. Endpoints for survival were death, moribund status or weight loss >35%.