Alloreactivity-Based Medical Conditions

Guest Editors: Stanislav Vukmanovic, Margaret G. Petroff, Anne M. Stevens, and Daniel Rukavina
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Clinical and Developmental Immunology

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Alloreactivity is a response of the immune system to individual antigenic differences within species. These responses in general occur following exposure to alloantigens as a consequence of medical intervention (such as transfusion or tissue transplantation) or during pregnancy. Responses to alloantigens form a basis of a broad spectrum of medical conditions, such as graft rejection, graft versus host disease, reaction to blood (products), or biopharmaceuticals and fetal and neonatal diseases (thrombocytopenia, hemolytic anemia, hemochromatosis, biliary atresia, and glomerulopathy). On the other hand, normal pregnancies are often accompanied by strongly tolerogenic responses to fetal alloantigens. Diverse immunologic components, including T cells, antibodies (B cells), and NK cells, promote alloreactivity, and this complexity makes the pathogenesis and tolerogenesis of these conditions distinct and unique. This special issue contains seven manuscripts touching on various aspects of alloreactivity in medicine.

Blocking CD40-CD40L interaction can induce acceptance of cardiac allografts. However, the blockade is less efficient under inflammatory conditions. In “Glucocorticoid-induced TNFR-related protein reverses cardiac allograft acceptance induced by CD40-CD40L blockade,” Krill et al. demonstrate that stimulation of GITR (a cell surface molecule displayed by effector and regulatory T cells) can initiate cardiac graft rejection irrespective of the presence or absence of CD40-CD40L blockade, defining thus at least one possible molecular mechanism of resistance to CD40-CD40L suppression. The balance between the effector and regulatory T cells is critical for development of graft-specific immune responses in general. Franzese et al. review the usefulness of this balance in predicting or establishing the diagnosis of graft rejection in “Regulatory T cells in the immunodiagnosis and outcome of kidney allograft rejection.”

Allogeneic hematopoietic stem cell transplantation is used for treatment of autoimmune diseases, multiple organ transplantation, consequences of supralethal irradiation and advanced malignancies and other serious conditions refractory to conventional methods. In “New allogeneic hematopoietic cell transplantation method: hematopoietic cell transplantation plus thymus transplantation for intractable diseases,” Hosaka demonstrates that allogeneic hematopoietic stem cell transplantation combined with the same donor thymus transplantation has a superior effect producing more efficient T cell function and reduced graft versus host disease.

Redzovic et al., in “Mucins help to avoid alloreactivity at the maternal fetal interface,” review roles of mucin-1 and tumor associated glycoprotein-72 in regulating immune responses. Parallels are drawn between the ability of these molecules to promote tumor growth, invasion, and metastasis and to prevent trophoblast invasion. Removing glycoproteins TAG-72 and Muc 1 from the eutopic implantation site likely contributes to better control of trophoblast invasion by T cells and NK cells. This enables tolerance to paternal antigens of the fetus and normal course and outcome of the pregnancy.

Three manuscripts explore distinct aspects of allosantibody formation. Preformed donor HLA class I-specific antibodies are a risk factor for rejection of kidney, heart, and lung grafts, but the role of these antibodies is less clear in liver transplantation, where rejection rates are lower. Yoshizawa et al., in “Significance of semiquantitative assessment of preformed donor-specific antibody using luminex single bead assay...”
describe a quantitative assay for donor specific antibodies that has a predictive value for living donor liver transplantation. In “Nuclear antigens and auto/alloantibody responses: friend or foe in transplant immunology,” Nakano et al. review current thinking about the role of nuclear antigens and antibodies specific for nuclear antigens in initiation and regulation of immune responses and graft rejection. Finally, transfusion of allogeneic red blood cells may in some recipients induce alloantibodies. Tatari-Calderone et al. in “The association of CD81 polymorphisms with alloimmunization in sickle cell disease” suggest that genetic elements in the gene encoding B-cell molecule CD81 may be predictive markers of alloimmunization.

The huge breadth of the field of alloreactivity-based medical conditions allows the special issue only to scratch under the surface of any specific topic. Our hope, however, is that this issue will help raise awareness of the immunological basis of these conditions and will foster further efforts to better understand their pathogenesis. Ultimately, this should help us design better treatments or prevent the development of these medical conditions altogether.

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Review Article

Mucins Help to Avoid Alloreactivity at the Maternal Fetal Interface

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During gestation, many different mechanisms act to render the maternal immune system tolerant to semi-allogeneic trophoblast cells of foetal origin, including those mediated via mucins that are expressed during the peri-implantation period in the uterus. Tumour-associated glycoprotein-72 (TAG-72) enhances the already established tolerogenic features of decidual dendritic cells with the inability to progress towards Th1 immune orientation due to lowered interferon (IFN)-γ and interleukin (IL)-15 expression. Mucine 1 (Muc 1) supports alternative activation of decidual macrophages, restricts the proliferation of decidual regulatory CD56+ bright natural killer (NK) cells, and downregulates their cytotoxic potential, including cytotoxic mediator protein expression. Removing TAG-72 and Muc 1 from the eutopic implantation site likely contributes to better control of trophoblast invasion by T cells and NK cells and appears to have important immunologic advantages for successful implantation, in addition to mechanical advantages. However, these processes may lead to uncontrolled trophoblast growth after implantation, inefficient defence against infection or tumours, and elimination of unwanted immunocompetent cells at the maternal-foetal interface. The use of mucins by tumour cells to affect the local microenvironment in order to avoid the host immune response and to promote local tumour growth, invasion, and metastasis confirms this postulation.

1. Introduction

The mother and conceptus are engaged in a chemical conversation throughout pregnancy [1]. Cycling endometrium provides a microenvironment in which molecules secreted by uterine cells, including glycoproteins mucin-1- (Muc 1-) and tumour-associated glycoprotein-72 (TAG-72), are transported into the uterine lumen, where they represent histotrophs required for blastocyst growth and development [2]. An active embryonic stimulus is required to initiate implantation in terms of the removal of epithelial surface glycoproteins from directly beneath the implantation site [2]. The blastocyst forms directly under uterine influence after its apposition and adhesion to the receptive endometrium [2, 3]. Cytokines, chemokines, and growth factors expressed by decidual cells, with their pleiotropic and redundant functions, are broadly involved in stimulating growth, differentiation, and the function of uterine and trophoblast cells, as well as in their mutual coordination and synchronization [2, 4]. Blois et al. [5] showed that pregnancy-specific glycoproteins influence trophoblast immune evasion. Additionally, angiogenesis in mice and its predictive value for the pregnancy outcome have been considered [5]. Recently, the broad and complex role(s) of Muc 1 and TAG-72 was evaluated at the maternal-foetal interface at the beginning of pregnancy, when the dynamic exchange of cytokine orientations was indispensable.
for immunoadherence [6]. However, many studies in animal and human models failed to completely elucidate mucin-mediated immunophysiological and immunopathological pathways involved in establishing pregnancy.

2. Alloreactivity at the Maternal-Foetal Interface

During gestation, many different mechanisms act to render the maternal immune system tolerant to the foetus. Induction of the adaptive immune response of an allograft begins with the recognition of the alloantigen by the mother’s T cells. This generally occurs through 3 main processes, including the direct, indirect, and semidirect pathways, depending on the antigen presented within major histocompatibility complex (MHC) molecules [7]. However, "the first signal" by itself induces T-cell anergy, which is followed by an active immune response, representing immunomodulation in the absence of "the second signal" [8]. The second signal represents engagement of costimulatory molecules on antigen-presenting cells (APCs) with CD28 or CTLA4 on the surface of T cells, allowing selective, activating, or inhibiting T-cell responses [8]. As the second signal providers, decidual dendritic cells (DCs) as well as macrophages play very important roles in immune adjustment during pregnancy [9, 10]. Human early pregnancy decidua harbours primarily myeloid mature CD83+ DCs [11], a very small population of intermediate CD205+ DCs [12] and CD1a+ cells [11, 13]. In humans, the number of mature CD83+ cells decreases during early pregnancy compared to that in the late secretory phase, whereas immature CD209+ DCs dominate in the decidua [14, 15]. Decidual T cells are hypo-reactive to trophoblast alloantigens [16], possibly because of their interaction with cognate immune DCs. Therefore, it has been hypothesized that antigen presentation by immature DCs induces tolerance, whereas antigen presentation by mature DCs induces immunity [17, 18]. However, mature decidual DCs induce CD4+ T-cell tolerance in mice [19]. Lung tissue-specific DCs under steady-state conditions are sufficiently mature to express intermediate levels of MHC class II and costimulatory molecules, but these cells polarize T cells towards the Th2 helper pathway [20].

Thus, the assumption that different maturation states or different subsets have varying tolerogenic functions can no longer be used to distinguish between the tolerogenic and immunogenic properties of DCs [21]. Additionally, freshly isolated human myeloic decidual mature DCs show a reduced capacity to produce IL-12 p70 compared to peripheral blood DCs [22]. Naïve allogeneic human CD4+ T cells primed with these DCs led to a higher percentage of Th2 cells than peripheral myeloid DCs [22]. However, the assumption that decidual DCs have tolerogenic functions must be further investigated because natural killer (NK) cells and macrophages may stimulate DCs to secrete IL12 and may cause further induction of Th1 cytokine production by lymphocytes, resulting in apoptosis of trophoblast cells and a Th1-type immune bias of abortion [23]. This finding strongly suggests that the microenvironment is a potent, or perhaps the most important, modulator of DC function [24], but the substances responsible for these actions, including mucins, remain to be elucidated.

3. Accessory Regulators of Alloreactivity

Distinct profiles of cytokine and chemokine secretion by appropriately stimulated DCs induce and orientate T cells [25], representing "the third signal" in T-cell activation. DCs use T-cell-mediated responses by stimulating "naïve" T cells via the Th2-promoting cytokine IL-10 to induce tolerance at the maternal-foetal interface and via the Th1-promoting cytokine IL-12 to stimulate T-cell activity [10], in the absence of appropriate tolerogenic stimuli. Prostaglandin E2, supported by progesterone, polarizes the maturation of myeloid DCs into Th2-promoting DCs, and transforming growth factor beta promotes tolerogenic DCs [26]. Both mediators are present at the maternal-foetal interface during normal pregnancy and may be utilized by DCs to regulate decidual health. Progesterone enhances the expression of leukaemia inhibitory factor (LIF) [27], which is one of the most important factors involved in fertilization and implantation [28]. In humans, maximum expression of LIF occurs between 19 and 25 days of the menstrual cycle, corresponding to the opening of the “implantation window” [2]. However, significant thinning of the glycoprotein layer (glycocalyx) beneath the trophoblast implantation site, including disappearance of its constituent Muc 1, is indispensable for successful implantation in many species [29]. Muc 1 acts as a physical barrier between the cell surface and external environment, thus protecting cells from microorganisms, toxins, or pathogenic factors, as well as preventing trophoblast invasion with equal efficiency [30, 31]. We recently demonstrated the absence of tumour-associated TAG-72 in uterine decidua of normal and pathological human pregnancies (nonembryonic pregnancy and missed abortion) at the implantation sites although TAG-72 was present in epithelial cells at and away from the tubal implantation site of an ectopic pregnancy [32]. Moreover, our recent in vitro studies confirmed the interaction between Muc 1 [33] and TAG-72 [32] with APCs at the maternal-foetal interface [32]. Our experiments suggested that they participate in the maturation of CD1a+ DCs and macrophages and critically influence their phenotype, cytokine and chemokine production, and functions, as shown in Figure 1(a). This method of controlling immune reactions, including trophoblastic invasion, occurs at the maternal-foetal interface [34].

4. Regulation of Mucin Expression

Mucins are a family of heavily glycosylated proteins [35]. Numerous groups of transmembrane mucins include Muc 1, which harbours a transmembrane domain, a short cytoplasmic tail, and an extensive extracellular domain [33]. Mucins are ubiquitously present in diverse mucous membranes and are localized at the apical surface of polarized epithelial cells in the lungs, stomach, intestines, and eyes [36, 37].
Figure 1: Proposed model for antigen-presenting cells and decidual lymphocytes interactions at the maternal-foetal interface in the presence (a) and absence (b) of mucin-1 (Muc 1-) and tumour-associated glycoprotein-72 (TAG-72). The functions of dendritic cells (DCs) and macrophages (Mfgs) may be influenced by Muc 1 and TAG-72 that bind to the mannose receptor (MR) and CD209. TAG-72-shaped DCs may produce less CD83, resulting in lower proliferation and selective apoptosis of cognate cytotoxic T cells to allow survival of Th2-oriented T cells with low production of IFN-gamma (IFN-γ), attracted by CC chemokine ligand- (CCL-)19 and CCL22. Mfgs in the presence of TAG-72 produced higher levels of interleukin (IL)-10 and IL-1 receptor antagonist (IL-1RA), but significantly decreased levels of IL-12 and CCL3, supporting a Th2 bias. Muc1-shaped Mfgs increased IL-1 receptor type II (IL-1R type II) expression, whereas a D6 decoy, CD80, CD86, and human leukocyte antigen (HLA)DR remain relatively unchanged. Muc1-shaped Mfgs and TAG-72-treated DCs appear to decrease IL-15 production and cannot support the proliferation of CD56 bright NK cells and expression of cytotoxic mediators. Low IFN-γ expression by TAG-treated DCs does not support decidual vessel remodelling. During normal eutopic implantation, removing surface epithelial glycoproteins (b) allows antigen-presenting cells to support mild proinflammatory reactions by increasing IL-15 and IFN-γ production and amplifying NK cells, which are rich in cytotoxic mediators.

Most studies examining the role of mucins in reproduction have focused on human Muc 1 because of its predominant expression in the human endometrium [38]. Muc 1 is present at the apical surface of epithelial cells in the endometrium throughout the menstrual cycle [39]. Human Muc 1 expression is high during the peri-implantation period [40]. It is likely the first molecule that the embryo encounters before adhering to the endometrium [30, 41] (Figure 1(a)). In the uterus, Muc 1 suppresses the interaction between the implanting embryo and maternal endometrial adhesion molecules, thus creating a barrier to implantation in humans [30] as well as many other mammalian species [31]. This theory has been confirmed by experimental data that show that mice lacking the gene for Muc-1 have a persistently receptive endometrium [38, 42]. Its removal is necessary for successful implantation in humans [43]. Indeed, Muc-1 is not present beneath the embryo implantation site [29, 30]. It is thought that embryos send paracrine signals and participate in Muc-1 clearance [43]. Muc 1 may participate in selectin-dependent interactions in the uterus, thus promoting...
cell-cell interactions in some contexts [44]. Muc 1 can be also removed from the site of implantation because of paracrine signals from local milieu [43], including different bacterial and viral products [45]. It could be overexpressed by hypoxia at the maternal-foetal interface and contributes to hypoxia-driven angiogenesis, as it was seen previously in the pancreatic cancer cells [46]. TNF-α is a cytokine secreted by endometrium and blastsocyst, which probably mediated binding of nuclear factor kappa β to its binding site in the promoter of Muc-1 gene [45]. On the other hand, TNF-α leads to the removal of Muc-1 in human uterine epithelial cells by increasing expression of sheddase TACE (tumour necrosis factor-alpha-converting enzyme)/ADAM17 (a dis-integrin and metalloprotease-like 17) [47]. Membrane-type matrix metalloprotease (MT1-MMP) also contributes eliminating the external part of Muc-1 molecule independently of TACE/ADAM17, and its expression increases during the receptive phase in the endometrial biopsy [48]. Furthermore, immunohistochemistry demonstrated the colocalization of MUC-1 and MT1-MMP in human uterine epithelium during implantation [48].

Moreover, Muc 1 is transcriptionally regulated by oestrogen, progesterone, and glucocorticoids [49, 50]. Sex hormones control gene transcription of Muc 1 either by direct interaction with the Muc 1 promoter or indirectly by stimulating or suppressing other transcription factors in the uterus [49]. Progesterone increases the synthesis and secretion of Muc 1 at the systemic level during early pregnancy, but cells—both beneath and immediately adjacent to attached embryos—do not contain detectable levels of Muc 1, whereas the cells further away in the epithelial layer remain unaffacted [49, 50]. Uterine tissue macrophage-derived factors (LIF) and cytokines (IL-1β) promote uterine receptivity by regulating surface glycan structures in epithelial cells [51]. Furthermore, immunohistochemistry demonstrated the colocalization of MUC-1 and MT1-MMP in human uterine epithelium during implantation [48].

Osteen et al. observed TAG-72 in the normal postovulatory, secretory phase endometrium, but not in the proliferative phase [53]. TAG-72+ cells were rarely observed in the lower uterine segment using immunohistochemistry [53]. We recently showed that TAG-72 was not present in the decidual tissue of healthy and eutopic pathological early pregnancies, including missed abortion and blighted ovum [54]. In contrast, TAG-72 was present in the tubal mucosa at and away from the implantation site during ectopic tubal pregnancy, but not in the uterine mucosa of the same woman [54]. To our knowledge, in the recent scientific literature there is little data that speaks about TAG-72 regulation; however, TAG-72 appears to be hormonally dependent. Estradiol can inhibit TAG-72 expression, whereas progesterone does not directly stimulate TAG-72 [55]. Additionally, TAG-72 downregulates oestrogen and progesterone receptors in endometriotic lesions [55]. Both autocrine and paracrine mechanisms are involved in regulating TAG-72 expression [56].

Glycoproteins, Muc 1 [30, 40] and TAG-72 [53, 54], are present in peri-implantation period in uterus and participate certainly in highly controlled process of trophoblast invasion, which resembles tumor invasion by deep decidual invasion, modifying of cellular morphology, and an epithelial-to-mesenchymal transition [3, 57]. Muc 1 overexpression suppresses extravilious trophoblast invasion mainly via modulating β1-integrin signaling in severe preclamptic placentas [58]. β1-integrin-silenced cells show a defective activation of the epithelial growth factor receptor-signaling cascade, leading to decreased in vitro proliferation, impaired migration, and invasive behavior [58]. However, the modification of cellular glycosylation is a common phenotypic change of cancer cells that mainly affects the outer part of glycans, leading to the expression of tumour-associated carbohydrate antigens [59]. The transmembrane mucins, in particular, are overexpressed and aberrantly glycosylated in most cases of adenocarcinoma and are also associated with constitutive activation of a growth factor signalling, invasive proliferation of tumors, possibility of metastatic spread, and activation of a programme of tumor cell repair and survival (immortality) [3, 59]. The tumor mucins interact with monocytes, dendritic cells, and T and NK cells supporting anti-inflammatory and tolerogenic immune response toward tumor cells, favoring in that way the tumor growth with a patient’s poor outcome [3, 59].

5. Mucins Suppress Alloreactivity at the Maternal-Foetal Interface

Trophoblast cells express mucins throughout gestation and thereby contribute to increased mucin concentrations at the maternal-foetal interface during pregnancy [60]. Whether Muc 1 affects decidual NK and T-cell functions during later pregnancy is unknown. In vitro Muc 1 competes with the PAM-1 monoclonal antibody, which is directed towards the carbohydrate recognition domain of the mannose receptor (MR) [25], followed by binding and internalization of MR in early decidual CD14+ cells in a dose-dependent manner [33]. This finding presents a possible mechanism for Muc 1 to influence the phenotype and functional properties of decidual CD14+ cells in in vivo, as illustrated in Figure 1(a). In in vitro experiments, Muc 1 stimulated macrophages to increase the surface expression of IL1-R type II (Figure 1(a)), which binds to IL-1 but does not transduce signals in the cell, quenching the IL-1 proinflammatory signal [61]. In contrast, a D6 decoy for proinflammatory chemokines, as well as co-stimulatory CD80 and CD86 molecules, HLA-DR and CD83, remains relatively unchanged after Muc 1 stimulation [33]. Muc 1 significantly decreases IL-15 in decidual macrophages, whereas IFN-γ, IL-18, IL-10, CCL3, and CCL17 are essentially unchanged [33].

Immature decidual CD14+ cells express CD209 [15] and a high number of MRs on the surface of single cells [62]. MR contains 8 and CD209 contains 7 extracellular carbohydrate recognition domains [63], indicating that these cells can bind to highly glycosylated molecules such as TAG-72 [64] (Figure 1(a)). Indeed, TAG-72 competes with mannan, which is a primordial ligand for the carbohydrate recognition domain [64], and it binds MR and CD209 on decidual CD14+ cells in vitro [32]. This binding is important because
ligands binding the carbohydrate recognition domain of MR can activate an anti-inflammatory and a tolerogenic response in monocyte-derived DCs [25] such as decidual DCs [32]. Indeed, after TAG-72 stimulation, decidual CD1α+ cells show significantly decreased CD83 molecules, likely due to its active secretion [65]. CD83 molecules have tolerogenic properties owing to the significantly reduced DC-mediated T-cell stimulation [65], which may contribute to immune tolerance at the maternal-foetal interface in the presence of TAG-72 (Figure 1(a)).

6. Effects of Mucins on Decidual T-Cell Functions

Hiltbold et al. showed that DCs present various glycosylated or nonglycosylated forms of Muc 1 using MHC class I molecules [66]. The efficiency of processing and the resulting strength of CD8α T-cell activities inversely correlated with the degree of glycosylation of the antigen [66]. Agrawal et al. also suggested that Muc-1-derived peptides are processed and presented in the context of MHC class I molecules on the surface of tumour cells [67]. CD8α T cells could detect Muc 1 peptides associated with MHC class I. Most Muc 1 epitopes did not contain a consensus motif for a particular MHC class I allele and bound with low “affinity,” compared with known high-affinity peptides [68]. MHC-restricted antigen recognition of mucins indicates that a given T-cell will recognize a peptide antigen only when it is bound to a host body’s self-MHC molecule [68]. Normally, T cells are stimulated only in the presence of self-MHC molecules; hence, the antigen is recognized only when peptides are bound to these self-MHC molecules. At the maternal-foetal interface, CD8α T cells are nearly depleted [68]. Magarian-Blander et al. showed that direct recognition of the Muc 1 peptide epitope by a T-cell receptor in the absence of presentation by MHCs induces a partial signal that is completed by further interactions with other receptor/ligand pairs on the surface of the CTL and their target cells [69].

TAG-72-treated decidual CD1α+ cells were unable to stimulate the proliferation of syngeneic decidual T cells [32], mostly of the CD45+ phenotype [70] (Figure 1(a)), although they significantly stimulated the proliferation of allogeneic, naïve cord blood T cells [32]. Furthermore, allogeneic T-cells cocultured with monocyte-derived DCs, which were matured using an antimannose receptor monoclonal antibody of the PAM-1 clone in vitro, initially proliferated but later became anergic and behaved as suppressor/regulatory cells [25]. Thus, poor proliferation that is observed upon rechallenge with cognate TAG-72-treated CD1α+ DCs suggests some degree of decidual T-cell tolerance. This finding agrees with the previous finding that TAG-72-treated CD1α+ cells decreased CD83 expression, which is considered a DC-activation marker [32] and is responsible for specific support of the expansion of newly primed naïve CD8α T-cells and long-term survival of antigen-specific T cells by inducing proliferation and inhibiting apoptosis [71].

Furthermore, TAG-72-treated CD1α+ cells decreased IFN-γ production in syngeneic decidual and allogeneic cord blood T cells, even in the presence of lipopolysaccharide (LPS) [32] (Figure 1(a)). However, IL-4 is not likely to be increased in decidual T cells after close contact with TAG-72-primed CD1α+ DCs [32]. However, intracellular IL-4 expression was increased in allogeneic cord blood T cells, even in the presence of TAG-72 and LPS pretreated CD1α+ cells [32]. This powerful tolerogenic feature of TAG-72 is not observed in freshly isolated decidual T cells, which appear to be oriented towards the Th2 pathway.

Accordingly, PAM-1-treated monocyte-derived DCs could not polarize Th1 effector cells and did not secrete proinflammatory chemokines, CXCL10 and CCL19 [25]. In contrast, they produced large amounts of anti-inflammatory CCL22 and CCL17 chemokines [72]. These findings emphasize the anti-inflammatory properties of CD1α+ cells treated with ligands for the carbohydrate recognition domain, particularly TAG-72, in terms of its interaction with T cells. Additionally, mucins can potentiate selective survival of naïve T cells, which may be modified using mucins [39].

7. Effects of Mucins on Decidual NK Cell Functions

The addition of Muc 1 in a suspension of decidual mononuclear cells substantially reduced the percentage of IL-15- and IFN-γ-expressing CD14+ cells [33], as illustrated in Figure 1. Through contact with MUC-1-treated macrophages, decidual cognate NK cells proliferated less efficiently and showed significantly decreased expression of perforin, Fas Ligand, and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) based on decreased IL-15 levels in decidual CD14+ cells in the presence of MUC1 [33] (Figure 1(a)). These NK cells are likely inefficient in the control of trophoblast invasion owing to their low numbers and weak cytotoxic potential. However, in the presence of Muc 1, trophoblast invasion does not occur at the eutopic implantation site. At the implantation site in tubal ectopic pregnancy, TAG-72 is present and appears to disturb trophoblast growth control because of diminished intracellular IL-15 and IFN-γ cytokine production in DCs [32]. The lower proportion of IL-15-expressing CD1α+ DCs isolated from the tubal implantation site than that isolated from the lining of the uterus of the same woman [32] supports the hypothesis and may explain deeper trophoblast invasion in the tubal wall during ectopic pregnancy; this may also be the main reason for tubal rupture and tubal pregnancy termination [73]. Similarly, tumour-associated macrophages isolated from human ovarian cancer express MR, which can bind TAG-72 to modulate cytokine production towards an unwanted immune-suppressive profile with an increased IL-10, not IL-12, and decreased Th1-attracting chemokine CCL3 expression [74]. Furthermore, TAG-72 inhibits lipopolysaccharide-mediated intracellular signalling cascades in tumour-associated macrophages induced by infection, leading the orientation of T cells with no experience, toward a Th2 response [75]. This result suggests a mechanism of alternative activation, which allows faster tumour progression by promoting tumour cell invasion, migration, and metastasis [75]. It may also explain why
higher expression of TAG-72 occurs in tumour tissues during advanced tumour stages compared to early stages in ovarian [76], breast [77], and gastric [78] tumours.

In the absence of tolerogenic mucin stimulation [25, 79] at the eutopic implantation site, DCs appear to support mild proinflammatory reactions through IL-15 and IFN-γ production [32], as shown in Figure 1(b). IL-15-shaped decidual NK cells allow trophoblast infiltration during normal pregnancy, but they can also kill these cells in the presence of IL-15, causing termination of pregnancy [80], likely through perforin- and granulysin-mediated cell killing involving the necrotic and apoptotic pathways [81]. Perforin and granulysin expression is abundant in freshly isolated normal human decidual NK cells [82]. These apoptotic molecules can be upregulated upon cocultivation with DCs and macrophages [83], which are the main sources of IL-15. Decidual NK cells proliferate and secrete more IFN-γ, which plays critical roles in angiogenesis, immune modulation at the implantation site, and maintenance of the decidual (maternal) component of the placenta [84].

8. Conclusion

During pregnancy in mammalian maternal tissues, immunocompetent cells are in direct and intimate contact with trophoblast cells of the foeto-placental unit. Successful survival of the foetal allograft can be explained using numerous immunoregulatory mechanisms acting at the maternal-foetal interface. According to our data, the main roles of Muc 1 and TAG-72 include regulation of maturation as well as expression of chemokines and cytokines by decidual APCs. Their effects are primarily mediated by C-type lectin endocytic receptors (CD209 and MR), which direct Th2 immune responses and alternative activation of APCs [85]. Thus, decidual mucins restrain strong anti-inflammatory local milieu. Similarly, studies examining tumour models have shown that mucin overexpression, aberrant intracellular localization, and changes in glycosylation are consistently associated with stronger invasion [86]. The removal of mucins during the first trimester of pregnancy is necessary to create a moderate “inflammatory” microenvironment, successful implantation, and tissue remodelling.

Acknowledgment

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References


Clinical and Developmental Immunology


Regulatory T Cells in the Immunodiagnosis and Outcome of Kidney Allograft Rejection

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Acute rejection (AR) is responsible for up to 12% of graft loss with the highest risk generally occurring during the first six months after transplantation. AR may be broadly classified into humoral as well as cellular rejection. Cellular rejection develops when donor alloantigens, presented by antigen-presenting cells (APCs) through class I or class II HLA molecules, activate the immune response against the allograft, resulting in activation of naive T cells that differentiate into subsets including cytotoxic CD8+ and helper CD4+ T cells type 1 (TH1) and TH2 cells or into cytoprotective immunoregulatory T cells (Tregs). The immune reaction directed against a renal allograft has been suggested to be characterized by two major components: a destructive one, mediated by CD4+ helper and CD8+ cytotoxic T cells, and a protective response, mediated by Tregs. The balance between these two opposite immune responses can significantly affect the graft survival. Many studies have been performed in order to define the role of Tregs either in the immunodiagnosis of transplant rejection or as predictor of the clinical outcome. However, information available from the literature shows a contradictory picture that deserves further investigation.

1. Introduction

Acute rejection (AR) is responsible for up to 12% of graft loss with the highest risk generally occurring during the first six months after transplantation [1]. Patient monitoring following the transplant includes physical examination, blood and urine tests, and tissue biopsy.

Rejection can often be histologically diagnosed before any variation of results obtained with laboratory tests. Many centers have introduced periodic biopsy surveillance protocols; however, to date, the clinical impact of a monitoring strategy based on biopsies is not clear [2, 3].

AR may be broadly classified into humoral and cellular rejections. In particular, antibody-mediated rejection is characterized by the presence of an antibody infiltration into the transplanted kidney, targeting HLA antigens on the peritubular and glomerular capillary endothelia, which results in complement activation, cytokine and chemokine release, and induction of adhesion molecules. This inflammatory response leads to platelet aggregation and leukocyte infiltration, which eventually contribute to the pathogenesis of acute lesions such as glomerulitis, peritubular capillaritis, microthrombi, and vessel necrosis [4].

New insights are now available into the mechanisms responsible for the immune response directed against a transplanted organ. Cellular rejection develops when donor alloantigens, presented by antigen-presenting cells (APCs) through class I or class II HLA molecules, activate the immune response against the allograft, resulting in activation of naive T cells that differentiate into subsets including cytotoxic CD8+ and helper CD4+ T cells type 1 (TH1) and TH2 cells or into cytoprotective immunoregulatory T cells (Tregs) [5]. CD4+ and CD8+ T cells infiltrate into the transplanted kidney, where they release cytokines and chemokines, causing cell death either directly or indirectly [6].

The immune reaction directed against a renal allograft has been suggested to be characterized by two major components: a destructive one mediated by CD4+ helper and CD8+...
cytotoxic T cells and a protective response mediated by Tregs. The balance between these two opposite immune responses can significantly affect the graft survival [7].

Many studies have been performed in order to define the role of Tregs either in the immunodiagnosis of transplant rejection or as predictor of the clinical outcome. However, information available from the literature shows a contradictory picture that deserves further investigation.

In this paper, we will analyze the possible role of Tregs in T-cell-mediated transplant rejection as useful biomarker for the immunological monitoring of the kidney transplantation outcome.

2. Principal Mechanisms of T-Cell-Mediated AR

Transplant rejection is the consequence of the recipient's alloimmune response and consists of manifested deterioration or complete function loss of the transplanted organ. From a physiopathological point of view, AR involves both cell-mediated and antibody-mediated immunities. Both cellular and humoral responses result in the allorecognition of foreign antigens which leads to immunocompetent cell activation and the orchestration of an effector response. This process ultimately results in the damage of the transplanted organ and the graft loss, both of which can show an early or late onset, as well as a striking or gradual development.

Different cell types are involved in the graft rejection including T and B cells, macrophages, plasma cells, eosinophils, and neutrophils. T cells play a crucial role either in mounting and/or regulating alloreactive responses. The main targets of cell-mediated damage are the tubular epithelium and the endothelium.

Generally acute allograft rejection starts (origins?) when the recipient's T cells recognize the donor alloantigens presented by APCs. In particular, donor antigens are carried by immature dendritic cells from the transplanted organ to the recipient's draining lymph nodes and spleen, in a journey which induces their transformation into mature APCs [8]. After the homing of APCs to lymphoid organs, the allore cognition of foreign antigens leads to T cell activation followed by differentiation into different subpopulations (Figure 1) and the return to the graft, where they play a fundamental role in destroying the transplanted organ.

T cell infiltration into the graft is mainly at the level of postcapillary venule endothelium. Three main steps can be identified: tethering, adhesion, and transmigration [9].

Tethering consists in the attachment and subsequent rolling of T cells along the endothelium, a process mediated by endothelial selectins that not only operate as a “conveyor belt,” but can also slow down cellular movement, thus prolonging T cell interaction with the endothelium itself. This initial step is followed by T cell activation as a consequence of exposure to locally produced chemokines which induce the expression of integrins including LFA-1, thus resulting in T cell adhesion to the endothelium. In the following step of transmigration, a diapedesis-mediated T cell infiltration into the endothelial gap junctions occurs. Once T cells reach the interstitium, the induced production of metalloproteases permits the digestion of the extracellular matrix allowing T cells to move along the tissue following a chemokine-dependent gradient (chemotaxis).

The main target of T cell activity is represented by the tubular epithelium and by the endothelium.

CD4+ T cells can induce cell damage either indirectly through the activation of cytotoxic CD8+ T cells and macrophages and/or directly through the production of inflammatory cytokines including TNF and IFN-γ. CD8+ T cells can induce damage at tubular and endothelial levels either through the release of cytotoxic molecules including perforin, granzyme B, and granulolysins or through the involvement of Fas molecule and induction of apoptosis [10]. Activated macrophages can in turn play their damaging role versus the tubular epithelium and endothelium by producing TNF-alpha and reactive oxygen species including NO.

3. Treg and Tolerance

Tregs play a critical role in the maintenance of T cell homeostasis under different immune conditions. They prevent the activation of autoreactive immune responses, contribute to maintaining self-tolerance and homeostasis of the microbial flora of the gut, and promote the immunogenic escape of cancer cells [11–13].

3.1. Origin of Tregs. Tregs were identified as a CD4+ T cell subpopulation expressing CD25 [14] molecule and “cytotoxic
T-lymphocyte antigen 4” (CTLA-4) at a similar extent to that displayed by activated T cells [15, 16]. The presence of CTLA-4 and the release of inhibitory cytokines including IL-10 and IL-35 [17,18] suggested a suppressor phenotype for these cells and critical role in controlling the activation and function of T lymphocytes as well as of APC and NK cells.

Tregs originate mainly from the thymus (natural, nTregs) and from the peripheral conversion of naive CD4+ T cells under appropriate stimulus conditions (induced, iTregs) [19,20].

Following exposure to antigens and activation of costimulatory molecules, peripheral naive CD4+ T cells can differentiate into different subpopulations (Figure 1): T helper 17 (Th17), Th1, Th2, and iTregs [21]. Several transcription factors contribute to the functional specialization of these subsets, including Foxp3, RORγt, T-bet, and GATA3, which activate genes involved in the control of T cell function [22–25].

3.2. Circulating Pool and Activation of Tregs. Circulating Tregs represent 3% of total lymphocytes in blood. Tregs are essential for maintaining peripheral tolerance; nevertheless, they show a quiescent phenotype when isolated from a non-inflammatory environment and require functional activation for the acquisition of Treg full functional suppressive activity [26] that can be achieved following exposure to self-antigens or to antigens presented at mucosal surfaces where they can be recruited. Tregs can also be functionally activated while migrating through inflamed tissues, or by exposure to environmental conditions such as those produced by tumors [27,28].

Inflammation plays an important role in driving the local cytokine milieu. In particular TGF-β, IL-10, and IL-2 have been shown to be critical in regulating activation and/or maintenance of the immunosuppressive functions of Tregs [29].

3.3. Regulation of Immune Responses by Tregs. Several mechanisms have been proposed to explain the role of Tregs in the control of immune responses in lymphoid and nonlymphoid tissues. Tregs produce IL-10, which is able to inhibit, either directly or indirectly, effector T cell activity during infection, autoimmunity, and cancer [30,31]. Selective deletion of IL-10 in Tregs results in the development of spontaneous colitis and exaggerated immune responses at the skin level and lung interfaces [32] while the role of CTLA-4 has been suggested by the observation that its loss results in severe lymphoproliferative disease and spontaneous multiorgan autoimmunity [33].

Regulation of immune functions mediated by CTLA-4-expressing Tregs depends on the ability of CTLA-4 molecule to downregulate the expression of costimulatory molecules CD80 and CD86 on dendritic cells (DCs) of lymphoid tissues resulting in impaired costimulation via CD28 and defective T cell stimulation [42]. Indeed, studies have confirmed stable contacts between Tregs and DCs, confirming Treg-mediated inhibition of these cells [43,44]. Tregs can also induce perforin-dependent cytolysis of DCs in tumour-draining lymph nodes [45]. Therefore, Tregs can control DC activity by multiple mechanisms, and this results in the inhibition of effector T cell activation and promoting of functional tolerance.

4. Regulatory T Cells in the Immunodiagnosis and Outcome of Kidney Allograft Rejection

The introduction of modern immunosuppressive therapies has improved the functional prognosis of the transplanted kidney. In particular, the existing immunosuppressant drugs have been shown to decrease the progression of renal damage at 5 years towards a framework of interstitial fibrosis/tubular atrophy [46]. However, there is still much to be done in order to further decrease the percentage of graft loss.

A current research challenge is the definition of biochemical and/or histological markers which can be considered as early signs or predictive of rejection. An ideal indicator should have the ability of discriminating between rejection and other causes of inflammation as well as to correlate with long-term prognosis and therapy efficacy.

In the search of biomarkers for the diagnosis of cell-mediated AR and prognosis of renal transplant, an increasing attention has been paid to the role of Tregs.

The role of Tregs in inducing tolerance to allogeneic grafts was demonstrated in tolerated skin allografts [47,48]. The induction of peripheral Tregs with specificity for non-self-peptides suggested a way for obtaining antigen-specific Treg ex vivo [49]. However, although a direct and active involvement of Treg-mediated T cell suppression at the site of the tolerated transplants has been demonstrated, the specificity for donor antigens has not been fully evidenced [50]. Noteworthy, the induction of dominant allograft tolerance dependent on regulatory T cells does not necessarily result in a reduced capacity to respond to environmental pathogens [51] providing support for the development of tolerance induction protocols in clinical transplantation.

One of the most important studies dealing with the role of Tregs in renal transplantation is the work of Muthukumar et al. [34]. Urine samples from 83 kidney-transplant recipients were analyzed. Among the patients considered in the study, 36 subjects showed graft dysfunction and biopsy-confirmed AR, 29 subjects had stable allograft function and normal allograft biopsy, and 18 subjects presented allograft dysfunction and biopsies indicating chronic allograft nephropathy. The levels of Foxp3 transcripts, as a specific marker of Tregs [22], in cells obtained from urine samples of the 36 subjects with AR were higher as compared with those observed in the other 2 groups analyzed. This result contrasted with the general expectation that Foxp3 should be lower in rejection. Among the 36 episodes of AR, 26 successfully reversed, while 10 patients lost their grafts within 6 months following the acute episode of rejection. In this case a combination of Foxp3 transcripts and creatinine levels proved to be a better prognosticator of rejection reversal (90 percent sensitivity and 96 percent specificity) than Foxp3 transcripts (90 percent sensitivity and 73 percent specificity) or serum creatinine levels alone (85 percent sensitivity and 90 percent specificity).
Banff histologic grade of the subjects in this case was not able to predict graft failure outcome, because there was no difference in the histological grade between the 2 groups (5 patients with IA and 5 with >IA in the group showing graft loss versus 11 patients with IA and 15 with >IA in the group with a functional graft, $P = 0.68$). Authors explained these results by suggesting that cells infiltrating the transplanted kidney would include both graft-destructive cells such as cytotoxic T cells and graft-protective Foxp3-expressing Tregs. Indeed the transient expression of Foxp3 can be a normal consequence of T cell activation without the acquisition of a Treg phenotype [52]. Consequently, graft dysfunction and response to therapy may be predicted more accurately when the heterogeneous nature of the cellular components is taken into account.

In addition, patients who displayed both rejection and higher levels of urinary Foxp3 showed better responsiveness to steroid treatment together with significantly lower risk for graft failure as compared with subjects with lower levels of the transcription factor.

In 2008, Aquino-Dias et al. analyzed the expression of some of the molecules mainly involved in the cytolytic attack to the graft (perforin, granzyme B, and fas-ligand), together with Foxp3 using real-time PCR from urinary cells, peripheral blood mononuclear cells, and 48 surveillance kidney biopsies from 35 patients with delayed graft functions, 20 of which showed histopathological features of AR and 28 of acute tubular necrosis [37]. All analyzed transcripts were higher in AR as compared with acute tubular necrosis. Similar results and significant correlations were observed in kidney tissue, peripheral blood leukocytes, and urinary cells for all genes analyzed. Although all correlations reached statistical significance, results concerning Foxp3 showed highest significance (94 percent sensitivity and 95 percent specificity). In a very recent study from Muthukumar et al., the urinary cell mRNA profiles for Foxp3 and other molecules where able to associate an early steroid withdrawal regimen with antithymocyte globulin induction, with excellent graft and patient outcomes in HIV-infected recipients of kidney allografts [53].

Mansour et al. [38] measured mRNA levels of Foxp3, Granzyme B, IFN-γ, IL-23, and RORyt in renal tissue obtained from 46 untreated subjects with renal allografts with borderline lymphocytic infiltrates according to Banff scheme (changes insufficient for diagnosis of AR, including foci of tubulitis with mild to moderate cortical infiltration and without intimal arteritis). Twenty-five patients were considered “nonprogressive,” as defined by serum creatinine level below 110% of baseline during the 40 days following biopsy. In contrast, 21 patients were considered “progressive,” as defined by an increase in serum creatinine level more than 110% of baseline and by repeated histologic examinations showing AR. In general, higher levels of Foxp3 mRNA were found in the nonprogressive group as compared with those observed in the progressive group.

In a retrospective study, Xu et al. [39] analysed 125 surveillance biopsies displaying interstitial T-lymphocyte infiltration between nonatrophic tubules in the cortex, 14 with subclinical rejection, 32 with borderline change, and 79 showing interstitial T-lymphocyte infiltration without obvious pathological abnormalities according to Banff criteria.

All previously described cases were classified into two groups: a regulatory phenotype (RP) group, characterized by Foxp3+ infiltrating T lymphocytes in biopsies, and a cytotoxic phenotype (CP) group, which was dominated by Granzyme B+ T lymphocytes. No patient of the RP group developed any AR during nearly 5 years of followup, while subjects of the CP group developed biopsy-proven or clinical diagnostic AR within 1 year after biopsy.

The clinical significance of the ratio between IL-17-secreting cells and Treg infiltration in renal allograft tissues with acute T-cell-mediated rejection (ATCMR) was investigated by Chung et al. [40] on 56 patients with biopsy-proven ATCMR, who were divided into the Foxp3-high group (with Log Foxp3/IL-17 > 0.45) and the IL-17-high group (with Log Foxp3/IL-17 < 0.45).

The IL-17-high group showed a stronger correlation significantly decreased as compared with that displayed by the Foxp3-high group, together with higher severity of interstitial and tubular injuries and lower 1-year (54% versus 90%, $P < 0.05$) and 5-year (38% versus 85%, $P < 0.05$) allograft survival rates. Multivariate analysis revealed that the Foxp3/IL-17 ratio was a significant predictor for allograft outcome.

The level of circulating Tregs at peripheral blood level and the association with long-term graft survival were analyzed by flow cytometry in 90 kidney transplant recipients [41]. Patients who maintained high Treg levels (above 70%) at both 6 and 12 months displayed a better long-term graft survival at 4 and 5 years followup.

The previously mentioned study would suggest that Tregs may play a role in antagonizing the inflammatory state associated with kidney transplantation and may possibly be considered as a prognostic factor of outcome. However, several studies show divergent data potentially contrasting with this vision.

Veronese et al. [35] analyzed 73 renal transplant biopsies selected for the diagnosis of acute cellular rejection (ACR) type I or type II, acute humoral rejection (AHR), or calcineurin inhibitor toxicity (CNI). The number of Tregs was found to be significantly higher in ACR type I and type II, as compared with that observed in AHR and CNI toxicity; 96% Foxp3+ cells were CD4+ T lymphocytes aggregated within renal tubules. However, Kaplan-Meier analysis of 2-year graft survival in patients with ACR type I or type II showed a lower survival rate in patients with higher Foxp3 scores as compared with the other group.

Bunnag et al. [36] analyzed Foxp3 mRNA expression in 83 renal transplant biopsies for causes linked to histopathology. Kidneys with T-cell-mediated rejection, antibody-mediated rejection, and mixed rejection showed higher Foxp3 expression as compared with kidneys without rejection. According to Banff classification, higher Foxp3 expression was associated with higher levels of interstitial inflammation and tubulitis. In their multivariate analysis, CD4 positivity, and not Foxp3 mRNA expression, was independently associated with graft survival.
Table 1: Characteristics of the described studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Type of patient</th>
<th>Patients* (n*)</th>
<th>Treg identification</th>
<th>Treg or Foxp3 expression</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muthukumar et al. (2005) [34]</td>
<td>KT with AR</td>
<td>36</td>
<td>Foxp3 mRNA in urine</td>
<td>Elevated</td>
<td>Survival rate graft</td>
</tr>
<tr>
<td>Veronese et al. (2007) [35]</td>
<td>KT with AR</td>
<td>59</td>
<td>Foxp3 CD4+ in kt</td>
<td>Elevated</td>
<td>Survival rate graft</td>
</tr>
<tr>
<td>Bunnag et al. (2008) [36]</td>
<td>KT with AR</td>
<td>31</td>
<td>Foxp3 mRNA in kt</td>
<td>Elevated</td>
<td>Survival rate graft</td>
</tr>
<tr>
<td>Aquino-Dias et al. (2008) [37]</td>
<td>KT with AR</td>
<td>20</td>
<td>Foxp3 mRNA in kt, PBL, urine</td>
<td>Elevated</td>
<td>Diagnosis of AR</td>
</tr>
<tr>
<td>Mansour et al. (2008) [38]</td>
<td>KT with BL changes</td>
<td>46</td>
<td>Foxp3 mRNA in kt</td>
<td>Reduced in PG</td>
<td>Outcome of BL changes</td>
</tr>
<tr>
<td>Xu et al. (2012) [39]</td>
<td>KT</td>
<td>125</td>
<td>Foxp3 CD4+ T lymphocytes in kt</td>
<td>Elevated in RPG</td>
<td>Outcome of graft</td>
</tr>
<tr>
<td>Chung et al. (2012) [40]</td>
<td>KT with AR</td>
<td>56</td>
<td>Foxp3/IL-17 secreting cells ratio in kt</td>
<td>Elevated (in SRGa)</td>
<td>Survival rate graft</td>
</tr>
<tr>
<td>San Segundo et al. (2012) [41]</td>
<td>KT</td>
<td>90</td>
<td>Foxp3 CD4+ CD25+ in PBL</td>
<td>Elevated (in SRGa)</td>
<td>Survival rate graft</td>
</tr>
</tbody>
</table>

*The number of patients does not refer to the total number of patients in each study, but to the subpopulation considered.

KT: kidney transplantation; AR: acute rejection; kt: kidney tissue; PBL: peripheral blood lymphocytes; BL changes: borderline changes; PG: progressive group; RPG: regulatory phenotype group; SRGa: patients with augmented survival rate graft.

Batsford et al. [54] found no association between Foxp3 T cell expression and graft function one year after transplantation. However, this study was affected by the reduced number of samples and the choice of excluding patients with a degree of rejection higher than type 1 TCMR.

Dummer et al. [55] have recently observed that intragraft expression of both Foxp3 mRNA and protein was not associated with a better allograft outcome, analysed in terms of graft function and survival at 5 years after transplantation in 96 kidney transplants.

5. Concluding Remarks

The analysis of some of the most important recent studies dealing with Tregs used as possible biomarker of acute kidney transplant rejection and/or prognostic factor related to the graft survival (see Table 1) inspires some critical observations.

First of all it must be pointed out that most of the times the final assessment of the studies is affected by the modest statistical validity of the analyzed sample due to the small number of patients included.

The consideration that analysis of Foxp3 mRNA and Foxp3+ CD4+ T cells is often performed on biotic samples is a critical element to take into account. The use of a graft survival biomarker could be able to improve the prognostic validity of the procedure, also in terms of evaluation of response to immunosuppressive therapies. On the other hand, it must be considered that renal biopsying for the diagnosing of allograft rejection is an invasive and time-consuming procedure with some risk of complications and not easily manageable for all patients. Therefore, in the general followup of transplanted patients, a noninvasive test could be more advantageous, although this option still needs to be confirmed on additional cohorts taking into account all the aspects considered earlier.

Lastly, the different immunosuppressive therapies employed in the available studies and the potential effects on Treg expression and function constitute another critical variable to take into account in the evaluation of Treg function in the allograft outcome. Recent clinical studies have demonstrated how different immunosuppressive drugs can influence differently the number and function of Tregs, by inducing stimulation, inhibition, or even noninterference. In particular, while corticosteroids and rapamycin have been shown to improve the suppressive activity and survival of Tregs, other treatments as calcineurin inhibitors (CIs) have been shown to affect Treg function [56]. However, it is difficult to differentiate the effects of different immunosuppressants, although the use of selective pharmacological treatments able to regulate the suppressive function of Tregs would be attractive in organ transplantation.

References


Clinical Study

Significance of Semiquantitative Assessment of Preformed Donor-Specific Antibody Using Luminex Single Bead Assay in Living Related Liver Transplantation

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Aim. To analyze the risks of preoperatively produced donor-specific antibody (DSA) in liver transplantation. Methods. DSA was assessed using direct complement-dependent cytotoxicity (CDC) and anti-human globulin- (AHG-) CDC tests, as well as the Luminex Single Antigen assay. Among 616 patients undergoing blood type identical or compatible living donor liver transplantation (LDLT), 21 patients were positive for CDC or AHG-CDC tests, and the preserved serum from 18 patients was examined to determine targeted Class I and II antigens. The relationships between the mean fluorescence intensity (MFI) of DSA and the clinical outcomes were analyzed. Results. Patients were divided into 3 groups according to the MFI of anti-Class I DSA: high (11 patients with MFI > 10,000), low (2 patients with MFI < 10,000), and negative (5 patients) MFI groups. Six of 11 patients with high Class-I DSA showed positive Class-II DSA. Hospital death occurred in 7 patients of the high MFI group. High MFI was a significant risk factor for mortality ($P = 0.0155$). Univariate analysis showed a significant correlation between MFI strength and C4d deposition ($P = 0.0498$). Conclusions. HLA Class I DSA with MFI > 10,000 had a significant negative effect on the clinical outcome of patients with preformed DSA in LDLT.

1. Introduction

The effect of preformed antibodies targeting human leukocyte antigens (HLAs) on the outcome of organ transplantation has been demonstrated in kidney, heart, and lung transplantation, and a lymphocyte crossmatch test (LCT) is considered mandatory. On the other hand, such effect is controversial in liver transplantation, even in living donor liver transplantation (LDLT) [1, 2]. We previously reported the negative effect of preformed donor-specific antibody (DSA) in LDLT and demonstrated that the risk factors for mortality were an adult recipient and a female gender [2, 3]. However, our previous studies did not include determination of the HLA targeted by the preformed DSA as well as analysis of the relationship between the amount of HLA-specific DSA and clinical outcome. Recently, a single antigen bead assay using the Luminex analyzer has enabled the determination of targeted HLA [4]. Furthermore, the mean fluorescence intensity (MFI) generated by the Luminex analyzer might enable the measurement of the amount of DSA. Musat et al. reported the significance of DSA in rejection after liver transplantation using the Luminex...
analyses and the histological examination of C4d deposition [5].

In the present study, we retrospectively analyzed the targeted HLA and the amount of DSA in the preserved serum of patients with a positive LCT prior to LDLT to clarify the relationship between the amount of DSA and the clinical outcome.

2. Methods

2.1. Patients. Between January 2000 and March 2008, 616 patients underwent blood type identical or compatible LDLT. Among them, 21 recipients (3.4%) were LCT positive preoperatively. Pretransplant sera from 18 of these 21 recipients were preserved and available for examination in the present study. These 18 patients were enrolled in this study and their characteristics are shown in Table 1. Their ages ranged from 6 months to 67 years (median, 48.0 years). There were 2 men and 16 women, 10 of whom had a history of pregnancy. Eleven patients had histories of blood transfusion, 5 had none, and 2 had no record. Nine patients had upper abdominal surgeries possibly leading to operative difficulty in 11 patients with histories of abdominal surgeries. In 2 patients, the second transplantations were investigated. The donors were 6 parents, 6 sons or daughters, 2 siblings, and 4 unrelated spouses.

This study was approved by the Ethics Committee of Kyoto University Hospital according to the Declaration of Helsinki of 1975 as revised in 2008.

2.2. Liver Transplantation and Initial Immunosuppression. All recipients underwent LDLT employing our standard methods. In our protocol, the target trough level of tacrolimus was 10 to 15 ng/mL for the first 2 weeks, and then it was tapered and adjusted individually depending on each patient's condition [6]. Intravenous methylprednisolone was used immediately after reperfusion, which was tapered, and then followed by oral prednisolone on day 8, which was stopped at 3 months.

2.3. Histological Evaluation. Liver specimens were fixed in 10% buffered formalin, processed routinely, and cut into 3 μm-thick paraffin sections. The routine staining methods included hematoxylin and eosin, Masson trichrome, and cytokeratin 7 (CK-7, OV-TL 12/30, Dako, Denmark; dilution 1:200) staining. Acute cellular and chronic rejections were evaluated according to the Banff Schema [7]. Each evaluation was blindly conducted by 2 pathologists (A. Miyagawa-Hayashino and H. Haga).

2.4. C4d Staining. Polyclonal antibody against C4d complement (BI-RC4D; Biomedia, Vienna, Austria; dilution 1:50) was used for immunostaining with an automated immunostainer (BENCHMARK XT, Ventana Medical Systems, Tucson, AZ). For antigen retrieval, deparaffinized and rehydrated sections were treated with protease I (Ventana Medical Systems; 0.5 U/mL) at 37°C for 20 minutes [8, 9].

Biopsy specimens in which only the vascular endothelium was stained were evaluated as endothelial positive. Biopsy specimens in which both the vascular endothelium and the stroma were stained were evaluated as endothelial and stromal positive (E&S positive). Any C4d staining on elastic fibers within the arteries and stroma was regarded as a nonspecific finding without clinical significance [10].

2.5. Lymphocyte Crossmatch Test. Pretransplant LCT was performed using both direct complement-dependent cytotoxicity (CDC) and CDC with added anti-human globulin (AHG-CDC) tests. Incubation was conducted using 1 milliliter of donor lymphocyte suspension and 5 milliliters of recipient serum in a Terasaki plate (Nunc, Roskilde, Denmark) at room temperature for 30 min. In the AHG-CDC test, AHG (Goat IgG k and l light chains) was added and incubated at room temperature for 3 min. Five microliters of rabbit complement were added to each well and the mixture was incubated at room temperature for 60 min. Two microliters of 5% eosin solution were added and the mixture was examined using phase-contrast microscopy (IMT-2; Olympus, Tokyo, Japan). The results were considered positive when more than 20% of the donor lymphocytes were killed by the recipient’s serum in either test. Dithiothreitol was not used for the inactivation of IgM antibodies.

2.6. HLA DNA Typing. Tissue typing was performed in patients and donors for HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ for class I and II loci using WAKFlow (Wakunaga Corp., Hiroshima, Japan) and Luminex xMAP technology (Luminex Corp., Austin, TX) [11].

2.7. Antibody Screening Employing LABScreen Mixed Assay. Pretransplant sera were retrospectively analyzed for HLA antibodies employing a multiplexed microsphere-based suspension array from Luminex xMAP technology (Luminex Corp.). In brief, 5 microliters of LABScreen Mixed (One Lambda, Canoga Park, CA) color-coded microbeads coated with purified HLA were incubated in the dark for 30 min at 20°C to 25°C with 20 microliters of test serum. Any HLA antibodies present in the sera were bound to the LABScreen Mixed surface antigens coating the microbeads and were subsequently labeled with R-phycocerythrin-conjugated goat anti-human IgG. The microbead fluorescent emission of R-phycocerythrin was then detected and quantified using the LABScan 100 flow analyzer (One Lambda).

The determination of positive and negative sera was performed with One Lambda software (LABScreen PRA software, One Lambda) according to the manufacturer's protocol. Sera reactivity was assessed based on the fluorescent signal for each HLA-coated microbead following correction for nonspecific binding to the negative control microbead. In the LABScreen Mixed assay, the normalized fluorescent signal is equal to the value of the antigen-coated microbead minus the value of the negative control microbead. If any
Table 1: Profiles of crossmatch-positive recipients.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Primary disease</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Donor</th>
<th>Blood type compatibility</th>
<th>Graft type</th>
<th>GRWR (%)*</th>
<th>History of pregnancy</th>
<th>History of blood transfusion</th>
<th>History of abdominal surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBC†</td>
<td>67</td>
<td>F</td>
<td>Son</td>
<td>Identical</td>
<td>Left lobe</td>
<td>1.43</td>
<td>Yes</td>
<td>Yes</td>
<td>Cholecystectomy, Hassab operation</td>
</tr>
<tr>
<td>2</td>
<td>PBC</td>
<td>47</td>
<td>F</td>
<td>Son</td>
<td>Compatible</td>
<td>Right lobe</td>
<td>1.29</td>
<td>Yes</td>
<td>Yes</td>
<td>Cholecystectomy, choledocojejunostomy, hepaticojejunostomy</td>
</tr>
<tr>
<td>3</td>
<td>Biliary atresia</td>
<td>19</td>
<td>F</td>
<td>Mother</td>
<td>Identical</td>
<td>Right lobe</td>
<td>1.16</td>
<td>No</td>
<td>Yes</td>
<td>Kasai operation</td>
</tr>
<tr>
<td>4</td>
<td>HCV-LC‡</td>
<td>49</td>
<td>F</td>
<td>Husband</td>
<td>Identical</td>
<td>Right lobe</td>
<td>1.41</td>
<td>Yes</td>
<td>Yes</td>
<td>Appendectomy</td>
</tr>
<tr>
<td>5</td>
<td>PBC</td>
<td>53</td>
<td>F</td>
<td>Daughter</td>
<td>Identical</td>
<td>Right lobe</td>
<td>0.98</td>
<td>Yes</td>
<td>Unknown</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>Metastatic neuroendocrine tumor of pancreas</td>
<td>62</td>
<td>F</td>
<td>Daughter</td>
<td>Identical</td>
<td>Left lobe</td>
<td>0.77</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>HCV-LC</td>
<td>47</td>
<td>F</td>
<td>Husband</td>
<td>Compatible</td>
<td>Left lobe</td>
<td>1.00</td>
<td>Yes</td>
<td>Yes</td>
<td>Distal pancreatectomy and splenectomy and repair of portal vein injury</td>
</tr>
<tr>
<td>8</td>
<td>Graft failure due to portal vein thrombosis after LDLT^A</td>
<td>53</td>
<td>F</td>
<td>Father</td>
<td>Identical</td>
<td>Right lobe</td>
<td>0.75</td>
<td>Yes</td>
<td>Unknown</td>
<td>Hysterectomy</td>
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<tr>
<td>9</td>
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<td>25</td>
<td>F</td>
<td>Sister</td>
<td>Identical</td>
<td>Right lobe</td>
<td>1.66</td>
<td>No</td>
<td>Yes</td>
<td>Kasai operation, splenectomy, distal splenorenal shunt, 1st LDLT</td>
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<td>10</td>
<td>LC</td>
<td>44</td>
<td>F</td>
<td>Husband</td>
<td>Identical</td>
<td>Right lobe</td>
<td>1.26</td>
<td>Yes</td>
<td>Yes</td>
<td>Splenectomy, esophageal transection, cholecystectomy, subtotal gastrectomy</td>
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<tr>
<td>11</td>
<td>LC</td>
<td>55</td>
<td>F</td>
<td>Son</td>
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<td>Right lobe</td>
<td>1.16</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
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<td>12</td>
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<td>50</td>
<td>F</td>
<td>Daughter</td>
<td>Identical</td>
<td>Right lobe</td>
<td>0.96</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>Cryptogenic LC (AIH^F)</td>
<td>51</td>
<td>F</td>
<td>Sister</td>
<td>Identical</td>
<td>Left lobe</td>
<td>0.72</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>Biliary atresia</td>
<td>0 (6 M)</td>
<td>F</td>
<td>Mother</td>
<td>Identical</td>
<td>Lateral segment</td>
<td>3.19</td>
<td>No</td>
<td>No</td>
<td>Kasai operation, revision</td>
</tr>
<tr>
<td>15</td>
<td>Biliary atresia</td>
<td>30</td>
<td>F</td>
<td>Mother</td>
<td>Identical</td>
<td>Left lobe</td>
<td>0.91</td>
<td>No</td>
<td>No</td>
<td>Kasai operation</td>
</tr>
<tr>
<td>16</td>
<td>Wilson's disease</td>
<td>50</td>
<td>M</td>
<td>Wife</td>
<td>Compatible</td>
<td>Right lobe</td>
<td>0.82</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>Wilson's disease</td>
<td>8</td>
<td>M</td>
<td>Mother</td>
<td>Identical</td>
<td>Lateral segment</td>
<td>1.34</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>18</td>
<td>Wilson's disease</td>
<td>0 (11 M)</td>
<td>F</td>
<td>Mother</td>
<td>Compatible</td>
<td>Lateral segment</td>
<td>2.94</td>
<td>No</td>
<td>Yes</td>
<td>Kasai operation, 1st LDLT</td>
</tr>
</tbody>
</table>

*Graft recipient weight ratio; †primary biliary cirrhosis; ‡hepatitis C virus-related liver cirrhosis; §hepatitis B virus-related fulminant hepatic failure; ^living donor liver transplantation; #autoimmune hepatitis; **hepatocellular carcinoma.

one microbead in the mixed assay is positive, the result is considered positive.

2.8. Single Antigen Bead Assay. The Single Antigen bead assay is essentially the same as the assay outlined earlier according to manufacturer's protocol. In brief, 20 microliters of test serum were incubated with 5 microliters of the selected single beads and 5 microliters of LABScreen Singles control beads. Samples were read on the LABScan 100 flow analyzer (One Lambda). Raw trimmed MFIs were obtained from the output file generated by the flow analyzer and normalized.

2.9. Statistical Analysis. A $P < 0.05$ was used for variable selection and was considered to indicate a statistically significant difference. SAS version 9.2 (SAS Institute Inc., Cary, NC) was used for statistical analysis. The log rank test was employed to estimate significance.

3. Results

3.1. Patient Survival. The patient survival rate was 72% on postoperative day (POD) 60, 67% on POD 90, and 61% on POD 180 until 10 years (Figure 1).
AHG-CDC tests showed negative DSA on the Single Antigen assay.

Regarding the relationship between high DSA or high non-DSA and possible backgrounds, there was no significant relationship between history of blood transfusion and high DSA ($P = 0.306$) and between history of blood transfusion and high non-DSA ($P = 0.464$). On the other hand, there was a significant relationship between history of pregnancy and high DSA ($P = 0.003$).

3.3. Histological Examination. The MFI of DSA, the histological findings of the first liver biopsy after transplantation, and the clinical outcomes of the 18 patients are shown in Table 2. Twelve patients underwent liver biopsy after transplantation: 9 within 90 days and 3 after 90 days. The major histological diagnosis was cholangitis in 5 patients, as reported by Takaya et al. [12].

Eight of 12 initial biopsy specimens showed positive C4d staining: stromal and endothelial deposition in 4 patients and endothelial deposition in 4 patients. All 4 cases with endothelial C4d staining only showed focal staining (portal C4d immunolabeling of fewer than 50% of portal tracts). All 4 cases with endothelial and stromal C4d staining showed diffuse staining pattern (C4d deposition in the hepatic artery, portal vein, or capillary endothelium of more than 50% of portal tracts). There were cases showing sinusoidal C4d staining. Three of the 4 patients with stromal and endothelial deposition (75%) and 3 of the 4 patients with endothelial deposition (75%) showed positive DSA with high MFI. All C4d-negative patients showed negative DSA on the Single Antigen assay. A significant correlation between MFI strength and C4d deposition was found on univariate analysis ($P = 0.0498$). Two patients with negative DSA and positive non-DSA showed negative C4d staining.

3.4. Clinical Courses and Risk Factors of Mortality. Seven patients died within 4 months after transplantation. The causes of death were sepsis in 5 and vascular complications in 2.

All of the 7 patients who died early had DSA with high MFI prior to LDLT. The risk factors for mortality were analyzed and a high level of Class-I DSA was found to be a significant risk factor (Fisher exact test, $P = 0.015$) (Table 3). The 1-year patient survival rate was 36% in the high MFI DSA group and 100% in the low and negative MFI DSA groups (Log-rank test, $P = 0.042$). Non-DSA or Class-II DSA was not a significant risk factor. History of blood transfusion and histories of abdominal surgery were not either.

Eleven patients are alive and the follow-up period ranged from 777 to 3,479 days. All but one showed normal hepatic chemistries and their performance status was 0. Case 2 showed an AST of 21 U/L, an ALP of 2,140 U/L, and a total bilirubin level of 4.4 mg/dL, and her performance status was 2 at 3,479 days after transplantation. Histological findings from the last liver biopsy specimens on postoperative days (PODs) 339 to 2,360 of 9 out of 11 alive patients are shown in Table 2. Three patients showed mild portal inflammation,
Table 2: Results of lymphocyte crossmatch test, Mixed assay, and Single Antigen assay and histological findings and clinical outcomes in crossmatch-positive recipients.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>LCT*</th>
<th>Mixed assay HLA-Ab antibody</th>
<th>MFI$ of anti-Class I HLA antibody</th>
<th>Mixed assay HLA-Ab antibody</th>
<th>MFI$ of anti-Class II HLA antibody</th>
<th>Histological findings of the first liver biopsy (POD)</th>
<th>C4d deposition</th>
<th>Outcome Alive/dead (POD)</th>
<th>Causes of death</th>
<th>Histological findings of the last liver biopsy in alive patients (POD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Cholangitis (40)</td>
<td>E</td>
<td>Dead (118)</td>
<td>Sepsis</td>
<td>Chronic cholangitis, possible PBC** recurrence (2360)</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Cholangitis (183)</td>
<td>E &amp; S**</td>
<td>Alive (3479)</td>
<td>Hepatic artery rupture due to biliary leakage Sepsis after repeated steroid pulse therapy for refractory ACR Sepsis and fungal infection after steroid pulse therapy for refractory ACR</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>Negative</td>
<td>No biopsy</td>
<td>–</td>
<td>Dead (28)</td>
<td>Chronic cholangitis, possible PBC** recurrence (2360)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Cholestasis (18)</td>
<td>E &amp; S</td>
<td>Dead (69)</td>
<td>Intra-abdominal hemorrhage, sepsis after steroid pulse therapy for refractory ACR</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>No biopsy</td>
<td>–</td>
<td>Dead (26)</td>
<td>Intra-abdominal hemorrhage, sepsis after steroid pulse therapy for refractory ACR</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>±</td>
<td>High</td>
<td>High</td>
<td>Negative</td>
<td>No biopsy</td>
<td>–</td>
<td>Alive (1286)</td>
<td>No biopsy</td>
<td>Chronic cholangitis, HCV** recurrence (339)</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>Negative</td>
<td>Lobular inflammation (3)</td>
<td>E &amp; S</td>
<td>Dead (49)</td>
<td>Sepsis of intestinal perforation Intra-abdominal hemorrhage and hepatic failure</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Acute cholangitis (18)</td>
<td>E</td>
<td>Alive (1160)</td>
<td>Chronic cholangitis, HCV** recurrence (339)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>–</td>
<td>No biopsy</td>
<td>–</td>
<td>Dead (72)</td>
<td>Sepsis of intestinal perforation Intra-abdominal hemorrhage and hepatic failure</td>
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</tr>
<tr>
<td>10</td>
<td>–</td>
<td>+</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>No biopsy</td>
<td>–</td>
<td>Dead (12)</td>
<td>Chronic cholangitis, HCV** recurrence (339)</td>
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</tr>
<tr>
<td>Patient no.</td>
<td>LCT*</td>
<td>Mixed assay HLA-Ab class I</td>
<td>MFI† of anti-Class I HLA antibody</td>
<td>Mixed assay HLA-Ab class II</td>
<td>MFI† of anti-Class II HLA antibody</td>
<td>Histological findings of the first liver biopsy (POD)</td>
<td>C4d deposition</td>
<td>Outcome Alive/dead (POD)</td>
<td>Causes of death</td>
<td>Histological findings of the last liver biopsy in alive patients (POD)</td>
</tr>
<tr>
<td>------------</td>
<td>------</td>
<td>---------------------------</td>
<td>-------------------------------</td>
<td>---------------------------</td>
<td>-------------------------------</td>
<td>---------------------------------------------</td>
<td>----------------</td>
<td>------------------------</td>
<td>----------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>11</td>
<td>−</td>
<td>+</td>
<td>High</td>
<td>High</td>
<td>−</td>
<td>Negative Steatosis (32)</td>
<td>E</td>
<td>Alive (1407)</td>
<td></td>
<td>Mild portal inflammation (1377) Resolving cholangitis (2286)</td>
</tr>
<tr>
<td>12</td>
<td>−</td>
<td>+</td>
<td>Low</td>
<td>High</td>
<td>+</td>
<td>Negative Cholangitis (183)</td>
<td>E &amp; S</td>
<td>Alive (2660)</td>
<td></td>
<td>Steatohepatitis (613) Mild portal inflammation (913)</td>
</tr>
<tr>
<td>13</td>
<td>−</td>
<td>+</td>
<td>Low</td>
<td>Low</td>
<td>−</td>
<td>Negative Steatosis (61)</td>
<td>E</td>
<td>Alive (777)</td>
<td></td>
<td>ACR (468)</td>
</tr>
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<td>+</td>
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<td>Low</td>
<td>−</td>
<td>Negative Cholangitis (28)</td>
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<td>Alive (1885)</td>
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<td>No biopsy</td>
</tr>
<tr>
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<td>−</td>
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<td>Negative</td>
<td>−</td>
<td>Negative Cholestasis (20)</td>
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<td>Alive (1490)</td>
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<tr>
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<td>Negative</td>
<td>Negative</td>
<td>−</td>
<td>Negative No biopsy</td>
<td>−</td>
<td>Alive (1095)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>+</td>
<td>−</td>
<td>Negative</td>
<td>Negative</td>
<td>−</td>
<td>Negative Severe ACR†† (8)</td>
<td>N</td>
<td>Alive (3318)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>−</td>
<td>Negative</td>
<td>Low</td>
<td>−</td>
<td>Negative Centrilobular hemorrhage and congestion (228)</td>
<td>N</td>
<td>Alive (2765)</td>
<td></td>
<td>Mild portal inflammation and fibrosis (2273)</td>
</tr>
</tbody>
</table>

*Lymphocyte crossmatch test; †direct complement-dependent cytotoxicity; ‡anti-human globulin with added CDC; ††mean fluorescence intensity (high, >10,000; low, <10,000); ∆donor-specific antibody; *
endothelial deposition; ** endothelial and stromal deposition; ††acute cellular rejection; ‡‡primary biliary cirrhosis; †††hepatitis C.
Table 3: Risk factors for mortality.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number</th>
<th>%</th>
<th>Mortality</th>
<th>P value*</th>
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<td>0.245</td>
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<td></td>
<td></td>
</tr>
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<td>47</td>
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<td>History of pregnancy</td>
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<td>50</td>
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<td>History of blood transfusion</td>
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<td>55</td>
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<td>Husband-son-daughter</td>
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<td></td>
</tr>
<tr>
<td>Lateral segment</td>
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<tr>
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<td></td>
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<tr>
<td>&lt;0.8</td>
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<td>0</td>
<td></td>
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<tr>
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<td></td>
<td></td>
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<tr>
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<td></td>
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<tr>
<td>High</td>
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<td>64</td>
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<tr>
<td>FI of anti-Class I-non-DSA</td>
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<td>Low</td>
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<tr>
<td>High</td>
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<td>Mix assay Class I</td>
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<td>FI of anti-Class I-DSA^</td>
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<td>0.057</td>
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<td>25</td>
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<tr>
<td>Low</td>
<td>3</td>
<td>100</td>
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</tr>
<tr>
<td>High</td>
<td>3</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>FI of anti-Class I-non-DSA</td>
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<td></td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td></td>
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</tr>
</tbody>
</table>
The incidence in this LDLT series including pediatric patients in deceased donor liver transplantation in Western counties is approximately 10% in deceased donor liver transplantation in Western counties [12,13]. The incidence in this LDLT series including pediatric patients was 3.4% (21 in 616 patients), whereas that in the Ashihara series consisting of adult patients in our center was 3.0% [2]. With the assumption that the sensitivity of LCT is the same, recipients in Western countries show a higher chance of developing preformed DSA based on LCT. In the present study, a significant relationship between history of pregnancy and high DSA was found. The chance of husbands, sons, and daughters becoming donors in LDLT for female patients was high, but this could lead to unfavorable outcomes owing to a large amount of DSA secondary to strong sensitization during their pregnancy. Therefore, information on the specificity and amount of DSA is very important in LDLT.

All DSA-positive patients showed positive non-DSA. It can be considered that positive DSA is part of the phenomena of sensitization against HLA including donor-specific antigens. However, in this study, high DSA positivity was found to be a significant risk factor for mortality. Blood transfusion is theoretically the most important contributing factor for sensitization. However, blood transfusion was found to be independent of the high positivity of DSA and non-DSA. Therefore, we analyzed the combined effect of high DSA or high non-DSA and history of blood transfusion. A significant difference in the incidence of mortality between positive history of blood transfusion and high DSA (n = 7, 6/7) and others (n = 9, 0/9) was found (Fisher exact test, P = 0.001). Moreover, there was a significant difference in the incidence of mortality between positive history of blood transfusion and high non-DSA (n = 8, 6/8) and others (n = 8, 0/8) (Fisher exact test, P = 0.007). Taken together, when patients who had histories of blood transfusion were highly sensitized, the mortality increased. This phenomenon might be related to unfavorable immune regulation leading to postoperative fatal infections.

Based on these results, we changed our policy of donor selection. A donor candidate to whom a recipient is highly sensitized with MFI > 10,000 is rejected. A donor to whom a recipient is sensitized with MFI < 10,000 is not considered when other donors are available; however, such a donor can be accepted after B cell desensitization using our protocol for ABO incompatible transplantation, which involves administration of rituximab, plasma exchange, and intravenous immunoglobulin. During 1 year from December 2009, 100 patients were evaluated regarding their LDLT and 12 patients were found to be positive for anti-class I antibodies. Five of them had DSA against donor candidates. Only 1 patient was highly sensitized and another family member to whom the patient had no DSA donated the graft. Another patient could change the donor, but the remaining 3 could not. All 5 patients survived after the transplantation.

A limitation of this study is that it did not reveal the relationship between the specific HLA and the clinical outcome in recipients with preformed DSA. Although the significance of MFI generated by the LumineX analyzer for the DSA assay has not yet been established, this study showed that DSA-MFI > 10,000 had a significant effect on the clinical outcome and a significant relationship with LCT. Further studies to clarify the meaning of low MFI and postoperative changes in DSA using the Single Antigen assay are required.

### Table 3: Continued.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number</th>
<th>%</th>
<th>Mortality</th>
<th>P value*</th>
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<td>C4d staining</td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
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<td>0</td>
<td></td>
<td>0.709</td>
</tr>
<tr>
<td>Endothelial</td>
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</tr>
<tr>
<td>Endothelial and stromal</td>
<td>4</td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Fisher exact test; † graft recipient weight ratio; ‡ compliment-dependent cytotoxicity; § anti-human globulin with added compliment-dependent cytotoxicity; ¶ fluorescence intensity of single antigen assay for anti-Class I donor-specific antibody (high, >10,000; low, <10,000).
Abbreviations

DSA: Donor-specific antibody  
LDLT: Living donor liver transplantation  
HLA: Human leukocyte antigens  
LCT: Lymphocyte cross-match test  
CDC: Direct complement-dependent cytotoxicity  
AHG: Anti-human globulin  
MFI: Mean fluorescence intensity.

Disclosure

Atsushi Yoshizawa and Hiroto Egawa wrote the paper. Hiroto Egawa organized the study. Aya Miyagawa-Hayashino and Hironori Haga performed the pathological analysis. Kimiko Yurugi, Rie Hishida, Hiroaki Tsuji, Eishi Ashihara, and Taira Maekawa conducted the HLA analysis, LCT, and Luminex assays. Satoshi Teramukai carried out the statistical analysis. Shinji Uemoto supervised the study.

Conflict of Interests

All authors of this paper reported no biomedical financial interests or potential conflict of interests.

References


Clinical Study

The Association of CD81 Polymorphisms with Alloimmunization in Sickle Cell Disease

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The goal of the present work was to identify the candidate genetic markers predictive of alloimmunization in sickle cell disease (SCD). Red blood cell (RBC) transfusion is indicated for acute treatment, prevention, and abrogation of some complications of SCD. A well-known consequence of multiple RBC transfusions is alloimmunization. Given that a subset of SCD patients develop multiple RBC allo-/autoantibodies, while others do not in a similar multiple transfusional setting, we investigated a possible genetic basis for alloimmunization. Biomarker(s) which predicts (predict) susceptibility to alloimmunization could identify patients at risk before the onset of a transfusion program and thus may have important implications for clinical management. In addition, such markers could shed light on the mechanism(s) underlying alloimmunization. We genotyped 27 single nucleotide polymorphisms (SNPs) in the CD81, CHRNA10, and ARHG genes in two groups of SCD patients. One group (35) of patients developed alloantibodies, and another (40) had no alloantibodies despite having received multiple transfusions. Two SNPs in the CD81 gene, that encodes molecule involved in the signal modulation of B lymphocytes, show a strong association with alloimmunization. If confirmed in prospective studies with larger cohorts, the two SNPs identified in this retrospective study could serve as predictive biomarkers for alloimmunization.

1. Introduction

Transfusion of red blood cells (RBCs) is a key component of the comprehensive management of patients with SCD [1]. Transfusion increases the oxygen carrying capacity of blood by increasing hemoglobin A [2, 3] and decreasing hemoglobin S [4–7]. Further, transfusion decreases blood viscosity, improves blood flow, and suppresses endogenous erythropoiesis. Due to these benefits, most SCD patients receive multiple RBC transfusions in their lifetime. RBC transfusion therapy is complicated by development of antibodies specific for allelic (alloantibodies) or self (autoantibodies) RBC determinants. Alloantibodies are more frequent than autoantibodies, whose clinical significance remains questionable. The presence of anti-RBC antibodies in SCD patients may cause delay in finding suitable blood donors, which can result in life threatening anemia. In addition, anti-RBC antibodies may cause delayed hemolytic transfusion...
reactions, which resemble sickle cell crises and can be lethal [8–10]. Finally, anti-HLA antibodies promoting rejection of hematopoietic cell grafts are more frequent in patients with anti-RBC antibodies [11]. Identification of biomarker(s) predictive of susceptibility to alloimmunization could identify a subpopulation of SCD patients at risk before the onset of a transfusion program and thus has important implications for their clinical management. In addition, such markers could assist in enhancing our insight into the mechanism(s) underlying alloimmunization.

RBC alloantibodies develop in 18–47% of patients with SCD [3, 12–15]. These rates are higher than 0.2–2.8% found in transfused patients without SCD [16–19]. One possible explanation for these high rates is antigenic disparity—different blood group distribution between predominantly Caucasians blood donors and SCD patients who are of African or African-Caribbean descent [20]. This concept is supported by alloimmunization frequencies in SCD patients in Saudi Arabia (13.7%), Uganda (6.1%), Egypt (21.4%), and Tunisia (16.6%) where blood donors and SCD patients are from similar ethnic background [21–24]. Even lower alloimmunization rates (2.6%) were noted in a Jamaican patient cohort [25], but they might have second to very low number of units received (1-2 per patient). However, even these reduced rates are higher than the above mentioned “background” rate of 0.2–2.8%, suggesting a multifaceted etiology of alloimmunization.

The human population in Africa has been subject to selection pressures imposed by infectious agents. One of the best-documented examples is malaria which significantly shaped the genetic make-up in several regions of Africa [26]. The most prominent genetic trait is Hb\(\beta^\text{S}\) heterozygosity that confers about 10-fold increase in protection against life-threatening forms of Plasmodium falciparum malaria [27]. Malaria infection has also exerted pressure for stronger immune responsiveness as evidenced by association of polymorphic variants in HLA-B, HLA-DR, IL-4, CD40L, FcGR2A, and TNF\(\alpha\) which result in increased resistance to malaria [26]. We reasoned that there may be other as yet undiscovered malaria-selected polymorphisms that promote stronger immune responsiveness and that some of them may lie close to Hbb. Such neighboring immune response-modifying genetic markers are more likely to segregate with Hb\(\beta^\text{S}\) than those located farther away on the same chromosome, or those located on other chromosomes. We therefore hypothesized that two malaria-protective polymorphisms were coselected: the Hb\(\beta^\text{S}\) and an allele of a near-by gene, encoding a molecule with immunomodulatory function. The consequence of such coselection by heterozygosity would result in an undesired, exacerbated immune response in homozygous Hb\(\beta^\text{S}\) individuals, with an eventual increase in the incidence of alloantibody response following transfusion. The closest gene of immune interest to the HBB locus is the gene encoding Sjogren Syndrome Antigen 1. Although we have recently demonstrated an association of a SNP in this gene with tolerance induction in early childhood [28], we found no evidence of association with alloimmunization per se, which prompted us to continue our search for other genes in the neighborhood of Hb\(\beta\) gene. We herein identified a strong association of alloimmunization with two single nucleotide polymorphisms (SNPs) in the CD81 gene encoding for a molecule involved in the signal modulation of B lymphocytes which qualify them as predictive markers of alloimmunization.

2. Materials and Methods

2.1. Subjects. Seventy-five adult SCD patients (44 Females, median age 32 years, range 21–73; 31 males, median age 30 years, range 20–58) regularly followed at Tenon Hospital in Paris, France were recruited for the study. All specimens were belonging to the larger French repository of blood samples from recipients of chronic RBC transfusion, financially supported and initiated in 1987 by the Institut National de la Transfusion Sanguine. Patients are first or second generation immigrants from Sub-Saharan Africa, that is, Atlantic West Africa, Central West Africa, and Bantu-speaking Africa (62 patients), and 13 patients were from the French West Indies. Patients were unrelated to each other and were not part of the same family. Samples were obtained during a routine clinical consultation at the steady state; a clinical status characterized by the absence of any infectious process or acute complication (such as vasoocclusive crisis) in the 3 months preceding the consultation. Patients were enrolled in this observational cross-sectional study after obtaining their informed consent according to the ethical research committee of “Assistance Publique-Hopitaux de Paris (AP-HP)” at Tenon Hospital. The criteria for inclusion were: (1) the diagnosis of sickle cell anemia (made using standard laboratory procedures including complete blood count, hemoglobin electrophoresis and family studies, and by direct molecular identification of the Hb\(\beta\) mutation) and (2) having received at least five RBC transfusions matched for AB0 and RhD blood antigens. Alloimmunized patients were received antigen negative blood for identified alloantibodies [40]. All patients received RBC transfusions during their in- or outpatient treatment at Tenon Hospital. Leukoreduced RBC units were collected and distributed by “Etablissement Français du Sang (EFS).” Patients receiving hydroxyurea therapy (HU) were identified in each group to evaluate the influence of this therapy on the incidence of alloimmunization (Table 1). The mean ages of the patients at the time of the study were 35.1 ± 8.4 years (mean ± SD) and 30.9 ± 8.7 years (mean ± SD) for patients with and without alloimmunization, respectively (Table 1). The mean of age, gender, number of RBC transfusions and exposure to hydroxyurea did not vary significantly between alloimmunized and nonalloimmunized patients.

2.2. SNP Detection. Genotyping was performed using a TaqMan allelic discrimination assay that employs the 5’ nuclease activity of Taq polymerase to detect a fluorescent reporter signal. Allele-specific oligonucleotides designed by Applied Biosystems, (Foster City, CA, USA) were labeled with different fluorophores in order to detect both alleles simultaneously. Genotypes were determined by the ratio of the two fluorophores used. The PCR for each SNP contained
Table 1: Clinical and demographic characteristics of SCD patients.

<table>
<thead>
<tr>
<th></th>
<th>Alloimmunized</th>
<th>Non-alloimmunized</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(N = 40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mean age ± SD</td>
<td>35.1 ± 14</td>
<td>30.9 ± 8.7</td>
<td>NS</td>
</tr>
<tr>
<td>Range</td>
<td>(21 y–73 y)</td>
<td>(20 y–58 y)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>14</td>
<td>17</td>
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</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>RBC transfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 30 unit</td>
<td>25</td>
<td>25</td>
<td>NS</td>
</tr>
<tr>
<td>&gt; 30 unit</td>
<td>10</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>17</td>
<td>NS</td>
</tr>
<tr>
<td>No</td>
<td>24</td>
<td>23</td>
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</tbody>
</table>

Table 2: Distribution and specificities of alloantibodies.

<table>
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<tr>
<th>Blood group system</th>
<th>Alloantibody specificity</th>
<th>Number of alloantibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh</td>
<td>D, D partial, C, C partial, C, C\text{w}, E, rh, and V</td>
<td>24</td>
</tr>
<tr>
<td>Kell</td>
<td>K and Kp\text{a}</td>
<td>4</td>
</tr>
<tr>
<td>Duffy</td>
<td>Fy\text{a}, Fy\text{ab}, and Fy5</td>
<td>9</td>
</tr>
<tr>
<td>Lewis</td>
<td>Le\text{a} and Le\text{b}</td>
<td>1</td>
</tr>
<tr>
<td>Kidd</td>
<td>Jk\text{a} and Jk\text{b}</td>
<td>7</td>
</tr>
<tr>
<td>MNS</td>
<td>M and S</td>
<td>4</td>
</tr>
<tr>
<td>Dombrock</td>
<td>H\text{y}</td>
<td>1</td>
</tr>
<tr>
<td>Colton</td>
<td>Co\text{b}</td>
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</tr>
<tr>
<td>Cartwright</td>
<td>Yt\text{b}</td>
<td>1</td>
</tr>
<tr>
<td>Other blood group systems</td>
<td>Kn\text{a}, Kn\text{b}, and Bg\text{a}</td>
<td>3</td>
</tr>
</tbody>
</table>

20 ng of DNA, 900 nM primers, 200 nM probes, and TaqMan Universal PCR Master Mix, in a final volume of 15 \(\mu\)L. PCR was performed on an MJ Research Tetrad thermal cycler (Waltham, MA, USA). The PCR profile was 10 minutes at 95°C (denaturation), 44 cycles of 15 seconds at 92°C, and 1 minute at an annealing temperature of 60°C. Reactions were set up using a MWG robot, and fluorescence ratios and allele calling were done using an ABI 7900. For the quality control of our genotyping, 10% of samples were randomly tested in three independent setup, and genotypes were compared. There was 100% concordance of genotypes in all three replicates.

2.3. \(\beta\)-Globin Gene Locus Genotype. Beta-globin gene haplotypes were determined by PCR-RFLP as previously described [43].

2.4. Statistical Analysis. The association of SNPs with alloimmunization was performed using the Chi-square testing with Yate’s correction or Fisher’s exact test when appropriate. After Bonferroni adjustment for multiple testing, the findings were considered statistically significant if \(P\) value was equal or less than 0.00185.

3. Results

3.1. Alloantibodies. Thirty-five of 75 adult patients (47%) had clinically significant RBC alloantibodies, listed in Table 2 (except for the Le\text{a} and Le\text{b}-specific antibodies that were considered clinically not significant). The incidence of alloimmunization in males (14 of 31 males who received transfusion were 45%) and females (21 of 44 females who received transfusion were 48%) was not significantly different. The number of alloantibodies per patient ranged from 1 to 9. Twenty patients had one alloantibody, eight patients had two, and nine patients had three or more antibodies. In twenty-eight patients (80%) who developed significant alloantibodies, the specificity was directed against antigens in the Rh or Kell systems. Five patients developed antibodies in the Rh system with uncommon specificity (Cw\text{p}, partial D, partial C, V, and rh\text{i}). However, twenty one patients (60%) developed additional alloantibodies to antigens in the Duffy, Kidd, Lewis, and MNS systems, and six patients (17%) developed additional antibodies to antigens in uncommon blood group systems.

3.2. Selection of SNPs for Analysis. There are several genes located in the vicinity of the \(HBB\) locus that can potentially impact the immune system (Table 3), with literally hundreds of SNPs identified in each. Given the plethora of candidate SNPs, we identified two sets of criteria to establish the priority for SNP analysis as following.

(1) Criteria related to the selection of genes:

(a) closeness of the gene to the \(Hb\beta\) globin gene (the closer the genes are, the more likely the alleles are in linkage disequilibrium (LD));
(b) involvement of the gene product in immune response;
(c) preserved homology of the genes between species (the less homologous genes are likely to produce a “high background” of genetic variation against which it is more difficult to detect genetic associations).

(2) Criteria related to the SNPs selection:

(a) known association of a polymorphism with an immune phenomenon;
(b) SNP frequency difference (HapMap database) between Yoruba (YRI) in Ibadan, Nigeria, and CEPH (Utah residents with ancestry from northern and western Europe: CEU) [44];
(c) characterization of SNPs by the HapMap database as informative for the linkage disequilibrium in haplotype analysis using the software
Table 3: List of genes in the proximity of HBB locus encoding molecules affecting the function of the immune system.

| Gene symbol | Distance from HBB locus (MB) | Molecule                      | Effects on the immune system                                                                 | Sequence homology | Haploview version 3.2 (http://www.broadinstitute.org/). The HapMap database provides information on millions of SNPs that determine the variation among human beings. This database provides the frequency for each SNP located in the noncoding region of the genome. Based on the above criteria, we examined HapMap-designated-informative SNPs in ARHG, CHRNA10, and CD81 genes. 3.3. Association of CD81 SNPs and Alloimmunization. Table 4 summarizes the results of association analysis of alloimmunization with 27 SNPs, all located in the noncoding regions of appropriate genes. Overall, allelic distribution was similar to that reported in the general population in Sub-Saharan region (HapMap; NCBI). For some patients, we were unable to establish a genotype despite repeated attempts. These patients may have previously undescribed/novel alleles or additional polymorphisms in the primer-binding regions. Therefore, the total number of patients for some SNPs do not equal the 35 (alloimmunized) or 40 (nonalloimmunized). Of the 27 SNPs studied, two had a statistically significant association with alloimmunization. Both SNPs are located in the CD81 gene and, based on the haploview analysis of disequilibrium linkage data, are in general not co-inherited. Consistent with this, the “informative” genotypes (defined as those that differ most between the alloimmunized and nonalloimmunized patients—CC in rs708564 and CC in rs2237863) were not co-inherited in most patients. Of the 28 patients with rs708564 C/C genotype, 9 also had the rs2237863 C/C genotype. The presence of at least one of the “informative” genotypes was present in 37 out of 75 patients (49%). The presence of “informative” genotypes correctly “predicted” alloimmunization status in 30 out of 40 SCD patients (75%) with no antibodies detected, and only in 6 of 35 patients (17%) with antibodies (Table 5). The absence of both two “informative” genotypes occurred in 29 out of 35 patients with antibodies (83%) and in 10 out of 40 patients without antibodies (25%). Thus, these SNPs may serve as a powerful predictor of alloimmunization. 3.4. Hemoglobin β Haplotypes. The existence of five Hbβ^S haplotypes suggested that the Hbβ^S mutation arose independently in different geographical regions [45, 46]. The co-inheritance of Hbβ^S neighboring DNA markers, including the ones affecting alloimmunization, could also have selectively occurred in one or several independently derived haplotypes. Therefore, we studied the Hbβ^S haplotypes in this patient cohort. Only patients homozygous for Bantu and Benin haplotypes were present in numbers sufficient to allow statistical analysis (Table 5). The predictive power of rs708564C/C and/or rs2237863C/C CD81 SNP genotypes was preserved in subgroups of patients with Bantu/Bantu, Benin/Benin, or all other Hbβ^S haplotypes grouped together. These data suggest that CD81 SNPs in our subject population were inherited independently of any particular Hbβ^S haplotype. 4. Discussion Blood and blood product transfusion most frequently either provoke no obvious immune reaction or induce immune suppression. The latter, known as transfusion-related immuno-
Table 4: SNP genotype association with alloimmunization in SCD.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene</th>
<th>Genotypes</th>
<th>Number of patients</th>
<th>Antibody positive</th>
<th>Antibody negative</th>
<th>P value</th>
<th>HapMap*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) rs1049388</td>
<td>ARHG</td>
<td>CC/CG/GG</td>
<td>21/8/0</td>
<td>21/11/0</td>
<td>0.593273</td>
<td>62/30/8</td>
<td></td>
</tr>
<tr>
<td>(2) rs1451724</td>
<td>ARHG</td>
<td>AA/AG/GG</td>
<td>1/3/27</td>
<td>0/3/29</td>
<td>0.833054</td>
<td>0/23/77</td>
<td></td>
</tr>
<tr>
<td>(3) rs4910852</td>
<td>ARHG</td>
<td>AA/AG/GG</td>
<td>25/7/0</td>
<td>18/10/0</td>
<td>0.264449</td>
<td>61/30/9</td>
<td></td>
</tr>
<tr>
<td>(4) rs7128013</td>
<td>ARHG</td>
<td>AA/AC/CC</td>
<td>13/12/5</td>
<td>11/18/4</td>
<td>0.543399</td>
<td>25/49/26</td>
<td></td>
</tr>
<tr>
<td>(5) rs7929197</td>
<td>ARHG</td>
<td>CC/CT/TT</td>
<td>7/13/12</td>
<td>8/12/5</td>
<td>0.429608</td>
<td>30/45/25</td>
<td></td>
</tr>
<tr>
<td>(6) rs10742177</td>
<td>ARHG</td>
<td>CC/CG/GG</td>
<td>10/14/7</td>
<td>18/3/5</td>
<td>0.008979</td>
<td>41/45/14</td>
<td></td>
</tr>
<tr>
<td>(7) rs10835182</td>
<td>ARHG</td>
<td>AA/AT/TT</td>
<td>0/14/15</td>
<td>0/12/20</td>
<td>0.444898</td>
<td>2/23/75</td>
<td></td>
</tr>
<tr>
<td>(8) rs10835184</td>
<td>ARHG</td>
<td>AA/AT/TT</td>
<td>25/2/0</td>
<td>26/2/2</td>
<td>0.550071</td>
<td>95/5/0</td>
<td></td>
</tr>
<tr>
<td>(9) rs10835187</td>
<td>ARHG</td>
<td>AA/AT/TT</td>
<td>7/13/14</td>
<td>8/12/11</td>
<td>0.817062</td>
<td>30/45/25</td>
<td></td>
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<tr>
<td>(10) rs10835187</td>
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<td>AA/AG/GG</td>
<td>30/2/0</td>
<td>25/1/0</td>
<td>0.999999</td>
<td>93/7/0</td>
<td></td>
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<tr>
<td>(11) rs2231529</td>
<td>CHRNA</td>
<td>CC/CT</td>
<td>27/3/1</td>
<td>35/2/0</td>
<td>0.493847</td>
<td>93/7/0</td>
<td></td>
</tr>
<tr>
<td>(12) rs2231532</td>
<td>CHRNA</td>
<td>AA/AG/GG</td>
<td>19/13/3</td>
<td>14/12/5</td>
<td>0.609984</td>
<td>63/35/2</td>
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<tr>
<td>(13) rs2271583</td>
<td>CHRNA</td>
<td>AA/AG/GG</td>
<td>4/14/15</td>
<td>2/9/18</td>
<td>0.422893</td>
<td>10/33/57</td>
<td></td>
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<tr>
<td>(14) rs2672213</td>
<td>CHRNA</td>
<td>CC/CT/TT</td>
<td>30/2/0</td>
<td>23/4/0</td>
<td>0.397881</td>
<td>97/3/0</td>
<td></td>
</tr>
<tr>
<td>(15) rs2672216</td>
<td>CHRNA</td>
<td>CC/CT/TT</td>
<td>32/2/0</td>
<td>25/8/1</td>
<td>0.062890</td>
<td>85/13/2</td>
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</tr>
<tr>
<td>(16) rs2741862</td>
<td>CHRNA</td>
<td>CC/CT/TT</td>
<td>5/25/4</td>
<td>14/14/5</td>
<td>0.022396</td>
<td>23/35/42</td>
<td></td>
</tr>
<tr>
<td>(17) rs12221525</td>
<td>CHRNA</td>
<td>CC/CG/GG</td>
<td>20/8/4</td>
<td>20/11/2</td>
<td>0.562563</td>
<td>50/37/13</td>
<td></td>
</tr>
</tbody>
</table>

* Patient numbers are displayed in the order shown in the column labeled genotypes. Relative distribution of genotypes in Sub-Saharan population, as reported by the HapMap. This research utilizes the NCBI SNP database [41], (http://www.ncbi.nih.gov/snp/). The Single Nucleotide Polymorphism database (dbSNP) is a public domain archive for a broad collection of simple genetic polymorphisms. dbSNP reports many cases of SNPs genotyped by HapMap and other projects which provide additional genotype and allele frequency information. ** NCBI database describes two additional alleles, A/G with very low frequencies (<0.5%) for rs708564. This might be due to a mutational mechanism that leads to the simultaneous creation of two new base pairs at the same site which is beyond the scope of this study [42]. Our results, however, are based on the two reference SNPs alleles (C/T) of rs708564. ## P values significant comparing SCD patients with and without alloimmunization.

Table 5: HbßS genotypes in SCD patients with or without antibodies or with “informative” SNP genotypes.

<table>
<thead>
<tr>
<th>HbßS haplotype</th>
<th>Total patients</th>
<th>Antibody positive</th>
<th>Antibody negative</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bantu/Bantu</td>
<td>15</td>
<td>2</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Benin/Benin</td>
<td>28</td>
<td>1</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Other**</td>
<td>33</td>
<td>3</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>6</td>
<td>29</td>
<td>10</td>
</tr>
</tbody>
</table>

* Patients were divided into groups that had rs708564C/C and/or rs2237863C/C genotypes (group one) or were double negative for these genotypes (group two).
** Includes any haplotypes found in 10 or less subjects (Senegal/Senegal; Benin/Cameroon; Senegal/Benin; Cameroon/Cameroon; Benin/Bantu; Bantu/Cameroon; Senegal/Cameroon; Benin/ßthal; and Senegal/ßthal).
modulation, may reduce transplant rejection rate and promote cancer recurrence, postoperative infections, and virus activation [47, 48]. So, what is (are) the factor(s) in some transfusion settings that converts an inert or tolerogenic event into an immunogenic one, leading to alloimmunization? And why is the alloimmunization rate higher in subjects with SCD? One theory is that discrepancies in RBC antigen protein structures between blood donors and recipients enhance the stimulation of B-cell clones to produce glycoprotein-specific alloantibodies. Blood group distribution is different between predominantly Caucasians blood donors and SCD patients who are of African or African-Caribbean descent. The SCD patients in the present study are mostly first/second generation Africans or West Indies whereas blood donors are mostly Caucasians. Hence, the antigen disparity between donor and recipient can explain at least in part the relatively high rate of alloimmunization (44%) in our patient population.

Transfusion-nonrelated preexisting recipient conditions that may lead to increased inflammation and activation of innate immunity are thought to contribute to alloantibody development [49, 50]. This has certainly been shown in a mouse model of alloimmunization [51]. SCD patients display increased inflammation and activation of innate immunity [52, 53] and increased levels of serum cytokines [54–57]. While antigenic disparity and proinflammatory milieu are important factors for higher rates of alloimmunization in SCD, it still remains unclear why the majority of RBC recipients never develop allo-/autoantibodies despite multiple transfusions. We therefore hypothesized that immune response to RBC alloantigens is additionally influenced by inheritable variations of immune function. We searched for genetic variations in the vicinity of Hbβ locus because one of the several susceptibility loci for development of autoimmune/inflammatory diseases is located on chromosome 11p15, in proximity of Hbβ [58]. Although the present work does not constitute a proof for the above hypothesis, it provides the first of several steps towards that goal. If confirmed in larger patient cohorts, the work may lead to the discovery of markers of alloimmunization-genetic traits that associate with alloimmunization, but do not necessarily cause or prevent alloimmunization. Although CD81 is in the relative vicinity of the Hbβ locus, the distance is large enough to suggest that the linkage disequilibrium is not a likely mechanism of association.

CD81 is a molecule which participates in the formation of so-called tetraspanin web that promotes cisassociations with different cell surface receptors including CD19/CD21 in B cells CD4 and CD8 in T cells [59, 60]. This association has functional consequences in B-cell-related pathways. Indeed, enhanced activation is observed in CD81 deficient B cells [32] and the use of antibodies to CD81 alter CD19/CD21-mediated signaling [33]. In addition, CD81 is a part of a complex involved in the uptake of exosomes by dendritic cells [61] and can modulate chemokine-induced dendritic cell migration [62]. Thus, CD81 can influence the immune system at multiple levels, and the identified SNPs may affect the function of CD81 protein at any of these steps. An alternative explanation is that markers may have an indirect effect on CD81, or may be associated with alloimmunization through linkage disequilibrium to a yet to be identified functional variant within CD81 gene or another gene.

SCD is caused by homozygous mutation in a single gene (Hbβ, glu6val), but it has exceptional phenotypic variability. This is explained by the interaction of environmental factors with a select group of polymorphic genes. Understanding whether there are genetic modifiers responsible for the phenotypic variability in clinical disease manifestations may provide novel approaches to treatment. The most studied genetic modifiers affecting the severity of SCD are the level of HbF expression, BCL11A, HBSIL-MYB, and coinheritance of α-thalassemia [63–65]. Other modifier genes include IL-4R, TGFβR3, adenylylate cyclase, and HLA-DPB1*0401 where certain allelic variants predispose SCD patients to large vessel stroke [26, 66, 67]. SNPs in bone morphogenetic protein receptor 2 (BMPR2) is associated with familial and sporadic pulmonary hypertension [68–70], and acute chest syndrome in women with SCD is associated with a SNP in the endothelial nitric oxide synthase gene (NOS3) [71]. Sickle cell adhesion to laminin may be partially dependent on SNPs in the β-adrenergic receptor gene (ADRB2) and adenylylate cyclase (ADCY6) gene [72]. Susceptibility to infection in SCD has been associated with polymorphism in HLA class II gene. The data shows a protective role of HLA-DRB1*15 against infection, in contrast to HLA-DQB1*03 which predisposes the SCD patients to a higher risk of infection such as meningitis, septicemia and osteomyelitis [73]. In contrast, some polymorphisms in TNFa, β-adrenergic receptor 2 (ADRB2) and HLA-DPB1*1701 were protective against stroke [74]. Given the plethora of studies addressing genetic influences on different aspects of SCD, it is surprising that genetic modulation of alloimmunization has been virtually unexplored. One study has reported the possible association between HLA-B35 and RBC alloantibodies [75]. However, the study was performed on a very small number of subjects, and the finding has not been replicated.

5. Conclusions
Our data suggest that SNPs in CD81 gene could potentially serve as predictors of RBC alloimmunization. The present study will have to be expanded in independent cohort of patients such as African-American patients with SCD. Furthermore, the impact of the SNPs on CD81 expression, and the role of CD81 in alloimmunization will need further investigation to gain full understanding of the mechanism underlying the cis-association of rs708564 and rs2237863 with alloimmunization.

Abbreviations
Hb: Hemoglobin
RBCs: Red blood cells
RFLP: Restriction fragment length polymorphism;
SCA: Sickle cell anemia
SCD: Sickle cell disease.
Acknowledgments

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References

[28] Z. Tatar-Calderone, C. P. Minniti, T. Kratovil et al., "rs660 polymorphism in Ro52 (SSA1; TRIM 21) is a marker for age-dependent tolerance induction and efficiency of alloimmunization in sickle cell disease," Molecular Immunology, vol. 47, no. 1, pp. 64–70, 2009.


New Allogeneic Hematopoietic Stem Cell Transplantation Method: Hematopoietic Stem Cell Transplantation Plus Thymus Transplantation for Intractable Diseases

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Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) has become a valuable strategy for some intractable diseases, a number of problems remain to be resolved. We have developed a new HSCT method, HSCT + thymus transplantation (TT) from the same donor, which induces elevated T cell function with mild graft-versus-host disease (GVHD) in comparison to conventional HSCT alone and HSCT + donor lymphocyte infusion (HSCT + DLI). This new method is effective in the treatment of several intractable diseases and conditions, such as autoimmune diseases in aging, advanced malignant tumors, exposure to supralethal irradiation, multiple organ transplantation from different donors, and type 2 diabetes mellitus, for which conventional methods are ineffective. Our findings suggest that allo-HSCT + TT is preferable to conventional allo-HSCT alone or allo-HSCT + DLI. This method may become a valuable next-generation HSCT technique.

1. Introduction

In recent years, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has become a valuable strategy for the treatment of hematological disorders (leukemia, lymphoma, and aplastic anemia), congenital immunodeficiencies, autoimmune diseases, metabolic diseases, and malignant tumors [1–7]. However, there are still a number of problems associated with these methods. Although T cells in the graft facilitate engraftment, they often induce graft-versus-host disease (GVHD) [8]. Conversely, if the antihost reaction is low, hematopoietic failure and/or primary disease recurrence may occur. In addition, the success rate of hematopoietic stem cell transplantation (HSCT) is low in elderly patients with low facility for engraftment and/or risk of severe complications [9–11]. Recent experience with radiation accidents also indicated that HSCT alone is ineffective for patients exposed to supralethal doses of irradiation with severe organ damage [12, 13]. Therefore, it is extremely important to overcome these problems of allo-HSCT.

The thymus is the organ in which T cells are differentiated and produced with induction of tolerance to the host. The thymus also regulates biological homeostasis using the cells with several cytokines and hormones [14]. Therefore, allo-HSCT with cotransplantation of the thymus from the same donor may be a beneficial method, as another site of allo-T cell production. Thymus transplantation (TT) itself has been used to treat immunodeficiency diseases, such as DiGeorge syndrome and HIV infection, and to increase T cell function [15, 16]. TT is also effective in age-related diseases with correction of immune dysfunctions in mice [17]. To our knowledge, however, there have been no previous studies regarding the efficacy of HSCT + TT in treatment of intractable diseases.

We recently developed a method of allo-HSCT in conjunction with TT from the same donor [18–27]. This method results in elevated T cell function with mild GVHD compared to HSCT alone or HSCT + donor lymphocyte infusion (HSCT + DLI) [18]. The mechanism underlying these effects involves not only CD4⁺ FoxP3⁺ effector T cells (Teff cells) but also CD4⁺ FoxP3⁺ regulatory T cells (Treg cells), which
prevent GVHD and autoimmunity [28, 29], produced by the allotransplanted thymus. The percentages of these cells are intermediate between HSCT alone and HSCT + DLI. The percentage of T<sub>reg</sub> cells in HSCT + TT is lower than that in HSCT alone, but higher than that in HSCT + DLI, while the opposite is true for the percentage of T<sub>eff</sub> cells [18]. We have examined application of this method for several intractable diseases. Our findings indicated that allo-HSCT + TT is preferable to the conventional allo-HSCT alone or allo-HSCT + DLI for several intractable diseases and conditions.

2. Results

2.1. Theory of Allo-HSCT + TT. First, we present the theory of allo-HSCT + TT (Figure 1). In the case of conventional allo-HSCT, allo-HSC is transferred into the host, and allo-T cells develop in the host thymus. The T cells show induced tolerance toward the host with thymic antigen-presenting cells and/or epithelial cells [30] and do not induce GVHD with normal T cell function (Figure 1, left). In contrast, nontolerant allo-T cells are externally supplied in the case of HSCT + DLI, resulting in strong GVHD, and the T cell number and function finally decrease (Figure 1, right). In the case of HSCT + TT, allo-T cells develop internally from the transplanted allo-thymus in the host. Interestingly, the T cells are partially tolerant to the host and induce low GVHD. In addition, the T cell function increases.

2.2. Application of Allo-HSCT + TT

2.2.1. Autoimmune Diseases. We examined the effects of HSCT + TT in treatment of several intractable diseases (Table 1). Female MRL/lpr mice develop systemic lupus erythematosus (SLE-) like lupus glomerular nephritis [31]. Bone marrow transplantation (BMT) alone is ineffective in these mice because of the radiosensitivity with Fas deficiency [32–34]. However, BMT + adult thymus transplantation (ATT) overcame these problems and induced donor-derived chimerism [19]. As a result, it also led to successful treatment of nephritis with reduction of serum autoantibodies and deposition of IgG in glomeruli. Aged female MRL/+ mice developed chronic pancreatitis with sialoadenitis [35]. HSCT (BMT or fetal liver cell transplantation (FLT)) is ineffective because of insufficient engraftment of donor cells with the involved host thymus (described below). However, HSCT (BMT or FLT) + fetal thymus transplantation (FTT) showed complete engraftment of donor cells and was effective in treating diseases with reduction of serum amylase [20]. These results suggest that these autoimmune diseases can be treated by replacement of the pathological hematopoietic system with HSCT + TT [36, 37].

2.2.2. Malignant Tumors. We next investigated the effects of HSCT + TT in tumor-bearing mice [18]. Mice treated with BMT alone showed significant tumor regression compared with untreated controls. Although mice treated with BMT + DLI showed greater tumor regression than those treated with BMT alone, strong GVHD also occurred and they died at an early stage. Interestingly, mice treated with BMT + ATT showed less GVHD than those treated with BMT + DLI, even with a comparable level of tumor regression.

We also examined the effects of HSCT + TT on leukemia [22]. In contrast to solid tumors, most mice treated with BMT + ATT or BMT + DLI showed almost complete remission of the tumor with long-term survival compared to those treated with BMT alone. However, the level of GVHD in those treated with BMT + ATT was significantly lower than that in those treated with BMT + DLI. These results suggest that BMT + ATT may be effective in treatment of not only solid tumors but also leukemia, without increased risk of GVHD.

The thymus atrophies with bone marrow suppression in hosts with advanced tumors, thus causing immunodeficiency, which is one of the major causes of death in such cases [38, 39]. We further examined the effects of HSCT + TT in mice bearing advanced tumors [21]. Although the thymus still atrophied in mice treated with allo-BMT + FTT, the transplanted fetal thymus had grown well. These mice showed longer-term survival than those treated with syngeneic (syn-) or allo-BMT alone, or syn-BMT + FTT with inhibition of lung metastasis. Interestingly, third-party FTT was also effective (described below as triple chimeras). These findings suggest that HSCT + TT may also be effective for long-term survival in advanced tumors.

2.2.3. Aging. Advanced age is one of the risk factors for unsuccessful BMT [9, 10]. The major reason for this is the thymic involution leading to insufficient T cell production and function, as described above. We examined the effects of BMT + FTT in aged models: senescence-accelerated mouse Pi strain (SAMP1) [40] and aged MRL/+ mice (same model as no. 1) [20, 23]. Both strains of mice treated with HSCT + TT showed significantly longer survival than those treated with HSCT alone. Although donor cells could not be engrafted into the host by BMT alone, resulting in hematopoietic failure and early death, the cells could be engrafted sufficiently by BMT + FTT. Interestingly, these hosts with long-term survival also showed elevated T cell function compared to either untreated mice or those treated with HSCT alone, and the level was comparable to that in normal mice. These results suggest that BMT + FTT is effective not only for engraftment of donor cells but also in restoring immune function in aged hosts.

2.2.4. Use of Third-Party Thymus Tissue. Transplantation of multiple organs from different donors is desirable in patients with several intractable diseases. Although grafting of the thymus itself can also induce tolerance [41], its effects on further tolerance at allo-HSCT have not been examined. Therefore, we examined triple chimeras consisting of lethally irradiated athymic (nu/nu) (X) mice transplanted with allo-BMC (Y) and third-party fetal thymus (Z) with a different major histocompatibility complex (MHC) type from both BMC and host type (Figure 2). The mice showed tolerance to all three MHC types—host, BMC, and the grafted thymus—but not to a fourth foreign MHC type [23]. We further examined the triple chimera with the above aged mouse
Table 1: Effective allo-HSCT + TT for various diseases and conditions compared with HSCT and HSCT + DLI.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mouse model</th>
<th>TT</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>(1) Autoimmune diseases</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(i) Lupus-like glomerulonephritis</td>
<td>MRL/lpr</td>
<td>ATT</td>
<td>Treatment of the glomerulonephritis</td>
<td>[19]</td>
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<td>(ii) Chronic pancreatitis</td>
<td>MRL/+</td>
<td>FTT</td>
<td>Treatment of the chronic pancreatitis and sialoadenitis</td>
<td>[20]</td>
</tr>
<tr>
<td>(2) Malignant tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) Early tumor</td>
<td>BALB/c with Meth-A sarcoma</td>
<td>ATT</td>
<td>Regression of tumor with low GVHD</td>
<td>[18]</td>
</tr>
<tr>
<td>(ii) Advanced tumor</td>
<td>BALB/c with Meth-A sarcoma</td>
<td>FTT</td>
<td>Long-term survival with inhibition of lung metastasis</td>
<td>[21]</td>
</tr>
<tr>
<td>(iii) Leukemia</td>
<td>B6 with EL-4 leukemia</td>
<td>ATT</td>
<td>Remission of tumor with low GVHD</td>
<td>[22]</td>
</tr>
<tr>
<td>(3) Aging</td>
<td>MRL/+</td>
<td>FTT</td>
<td>Long survival with elevation of T cell function</td>
<td>[20]</td>
</tr>
<tr>
<td>(4) Use of third-party thymus tissue</td>
<td>B6, BALB/c, C3H (triple chimera)</td>
<td>FTT</td>
<td>Same as above</td>
<td>[23]</td>
</tr>
<tr>
<td>(5) HSCT conditioning</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) Low numbers of BMC</td>
<td>BALB/c</td>
<td>ATT</td>
<td>Long survival with high donor chimerism and low GVHD</td>
<td>[24]</td>
</tr>
<tr>
<td>(ii) Low-dose irradiation</td>
<td>BALB/c</td>
<td>ATT</td>
<td>Same as above</td>
<td>[24]</td>
</tr>
<tr>
<td>(iii) Supralethal irradiation</td>
<td>BALB/c</td>
<td>NTT &gt; FTT &gt; ATT</td>
<td>Rescue with improvement of intestinal injury</td>
<td>[25]</td>
</tr>
<tr>
<td>(6) Type 2 diabetes mellitus</td>
<td>db/db</td>
<td>NTT</td>
<td>Treatment of the type 2 diabetes mellitus</td>
<td>[26]</td>
</tr>
</tbody>
</table>

HSCT: hematopoietic stem cell transplantation; TT: thymus transplantation; ATT: adult thymus transplantation; NTT: newborn thymus transplantation; FTT: fetal thymus transplantation.
Effects of allo-HSCT + TT for intractable diseases

**Figure 1:** Theory of allo-HSCT + TT. In the case of conventional allo-HSCT (left), the allo-T cells develop and are tolerated in the host thymus, and no GVHD occurs. In the case of allo-HSCT + DLI (right), nontolerated allo-T cells are externally supplied, and strong GVHD is induced with reduction of T cell function. In the case of allo-HSCT + TT, the allo-T cells develop internally in the allothymus. The T cells show partial tolerance to the host, and only mild GVHD occurs with elevation of T cell function (middle).

**Figure 2:** Use of third-party thymus tissue in triple chimera. Lethally irradiated athymic nu/nu mice (host: X) were transplanted with allogenic HSC (donor: Y) and third-party thymus (donor: Z) as triple chimeras.

Model, SAMP1 mice, and the same results of tolerance for three MHC types were obtained. Although this model is limited to hosts with low thymic function, these results suggest that HSCT + TT can also be applied for multiple organ transplantation from different donors using third-party TT.

2.2.5. HSCT Conditioning. Reduction of the intensity of the HSCT conditioning regimen will decrease the side effects for both host and donor. Therefore, we examined the effects of HSCT and TT for two conditions, that is, low-dose irradiation (sublethal irradiation (SubLI)), which attenuates host damage, and low numbers of BMC (injection of low numbers of BMC (ILNBMC)), which reduces the burden on the donor [24]. BMT + DLI showed the shortest survival due to severe GVHD under both conditions, and BMT alone also showed short survival due to hematological failure with insufficient engraftment in ILNBMC. However, BMT + ATT was preferable for long-term survival with reduction of GVHD to BMT + DLI or with donor-derived chimeraism to BMT alone. Next, we investigated the effects of HSCT and TT on supralethal radiation exposure [25]. HSCT alone, such as BMT, newborn liver cell transplantation (NLT), or FLT, was almost completely ineffective in mice exposed to supralethal irradiation with severe intestinal injury and weight loss, as reported in humans [12, 13]. However, NLT + newborn thymus transplantation (NTT) rescued the animals with the greatest efficacy showing improvement of the injury and prevention of weight loss among the three types of HSCT + TT (BMT + ATT, NLT + NTT, and FLT + FTT).

2.2.6. Type 2 Diabetes Mellitus. There is increasing evidence that both autoimmune and autoinflammatory mechanisms are involved in the development of not only type 1 diabetes mellitus (T1 DM) but also type 2 DM (T2 DM) [42, 43]. Therefore, we recently examined the effects of HSCT + TT in leptin receptor-deficient (db/db) mice, an animal model of T2 DM [44]. BMT + NTT could be used to treat diabetes with complete replacement of HSC showing normalized immune functions, although BMT alone showed insufficient treatment with incomplete replacement of HSC and dysregulated immune function [26]. These results suggest that BMT + NTT may be effective for treatment of T2 DM and that correction of the pathogenic immunological function is important for treatment.

3. Discussion

As described above, allo-HSCT + TT is functionally superior to either conventional HSCT alone or HSCT + DLI for several intractable diseases and conditions. Elevation of T
cell function with low GVHD facilitates engraftment of donor cells in HSCT + TT, and these allo-T cells may work more effectively and safely in treatment of several diseases compared to HSCT alone or HSCT + DLI.

The elevation of T cell function in HSCT + TT is due to the newly developed T cells from the transplanted thymus [18]. In addition, the low GVHD is due to the partial deletion and induction of host antigen-reactive T eff and T reg cells from the transplanted allo-thymus. Although the detailed mechanism is not yet clear, it suggests that thymic antigen-presenting cells such as dendritic cells (DC) and/or medullary thymic epithelial cells (mTEC) in the transplanted thymus may play important roles with the antigen [30, 45–49] (Figure 3). As these T cells develop continuously in vivo, they do not induce lethal “autoimmune-like” GVHD to maintain homeostasis [50, 51]. Therefore, TT initially appears to represent a simple method but may represent a significant approach to supplying the organ in which T cells are differentiated, produced, and functionally regulated.

As the thymus shows functional differences with age, we also compared the effects of the thymus at three different ages (adult, newborn, and fetus) [25, 27]. Although HSCT + TT was superior to HSCT alone at all ages, NLT + NTT showed better results than FLC + FTT and BMT + ATT in these experiments. Although further studies are needed, these findings suggest that young thymus, as close as possible to newborn, may be preferable.

However, it is both ethically and technically difficult to obtain adequate thymus tissue for clinical use. In this respect, grafts could be obtained from patients with congenital heart diseases or from aborted fetuses, as utilized previously [15, 16]. These materials are close to the preferred NT and may be used in third-party TT. In addition, a method for thymus regeneration and differentiation from stem cells has also been developed [52–57]. Therefore, HSCT + TT may become a viable strategy for the treatment of intractable diseases, conditions, or transplantation, and therefore this may become a valuable next-generation HSCT method.

4. Conclusion

The findings presented here indicate that allo-HSCT + TT is more effective against several intractable diseases compared with conventional allo-HSCT methods. This method may become a valuable strategy for the treatment of various diseases in humans.

Abbreviations

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation
ATT: Adult thymus transplantation
BMT: Bone marrow transplantation
DLI: Donor lymphocyte infusion
FLT: Fetal liver cell transplantation
FTT: Fetal thymus transplantation
GVHD: Graft-versus-host disease
HSCT: Hematopoietic stem cell transplantation
NLT: Newborn liver cell transplantation
NTT: Newborn thymus transplantation
T eff: Effector T cells
T reg: Regulatory T cells
TT: Thymus transplantation
mTEC: Medullary thymic epithelial cells
DC: Dendritic cells.
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References


Research Article

Glucocorticoid-Induced TNFR-Related Protein Stimulation Reverses Cardiac Allograft Acceptance Induced by CD40-CD40L Blockade

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CD40-CD40L blockade has potent immunosuppressive effects in cardiac allograft rejection but is less effective in the presence of inflammatory signals. To better understand the factors that mediate CD40-CD40L blockade-resistant rejection, we studied the effects of stimulation through glucocorticoid-induced TNFR-related protein (GITR), a costimulatory protein expressed by regulatory and effector T cells. Stimulation of CD40−/− or wild-type recipient mice treated with anti-CD40L mAb (WT+anti-CD40L) and with agonistic anti-GITR mAb resulted in cardiac allograft rejection. GITR stimulation did not induce rejection once long-term graft acceptance was established. In vitro, GITR stimulation increased proliferation of effector T cells and decreased regulatory T cell (Treg) differentiation in both treatment groups. GITR-stimulated CD40−/− recipients rejected their allografts more rapidly compared to GITR-stimulated WT+anti-CD40L recipients, and this rejection, characterized by a robust Th2 response and significant eosinophilic infiltrate, could be mediated by CD4+ T cells alone. In contrast, both CD4+ and CD8+ T cells were required to induce rejection in GITR-stimulated WT+anti-CD40L-treated recipients, and the pathology of rejection was less severe. Hence, early GITR stimulation could initiate graft rejection despite CD40 deficiency or anti-CD40L mAb treatment, though the recipient response was dependent on the mechanism of CD40-CD40L disruption.

1. Introduction

CD40-CD40L blockade has potent immunosuppressive effects in graft rejection, and an anti-CD40L mAb (MR1) has been shown to induce long-term graft acceptance in mouse cardiac allograft models [1, 2]. Similarly, host CD40 deficiency (CD40−/−) also allows for acceptance of cardiac allografts [3]. Although the mechanisms of allograft acceptance induced by CD40-CD40L blockade are not fully defined, evidence suggests a role for the generation of allograft-specific regulatory T cells (Treg) [4, 5]. However, CD40-CD40L blockade is less effective under certain conditions, possibly due to the actions of other costimulatory molecules or the presence of memory T cells [5, 6]. For example, C57BL/6 mice deficient in both CD28 and CD40L acutely reject skin grafts [7, 8], but this rejection can be prevented by blocking OX40-OX40L interactions [7]. Conversely, inductive OX40 stimulation under the cover of CD40-CD40L blockade induces acute cardiac graft rejection, which correlates with the induction of Th1 and Th2 responses as well as the deposition of IgG1 and IgG2a within the graft [9]. Of note, once graft acceptance is established following CD40-CD40L blockade, delayed OX40 stimulation does not induce acute allograft rejection despite priming of graft-reactive Th1 and Th2 cells. However, signs of chronic rejection are observed [9]. Hence, T cell costimulatory pathways other than CD40-CD40L play a role in transplant rejection, though the extent of their influence may be dependent on the
inflammatory state of the transplanted tissue (reviewed in [5]).

The glucocorticoid-induced TNFR-related protein (GITR) is a transmembrane receptor belonging to the TNF receptor superfamilly and is expressed constitutively at low levels on naive T cells (reviewed in [10]). Following TCR activation, GITR is upregulated on CD4+ and CD8+ T cells. In CD4+ T cells GITR expression may be dependent on CD28 engagement [11, 12], whereas the interplay between CD28 and GITR costimulatory pathways in CD8+ cells has not been fully defined. GITR is also expressed at high levels on Treg and was formerly assumed to be a specific marker for this subset [13]. Studies of agonistic anti-GITR mAb (DTA-I) stimulation showed strong proinflammatory effects in mouse models of autoimmunity, tumor immunity, and infection [11, 14]. The effects of GITR signaling appear to be multifactorial; stimulation through GITR has been demonstrated to increase activation and proliferation of effector T cells (Teff), render Teff less resistant to regulation, stimulate inflammatory cytokine secretion by innate immune cells, and increase leukocyte extravasation [11]. Interestingly, GITR stimulation also results in loss of Treg suppressor function, though this effect is transient and appears to be offset in part by the capacity of GITR-stimulated Treg to proliferate [13, 15]. In contrast, blocking GITR interactions through GITR-Fc treatment has been shown to reduce inflammation [16–18]. Therefore, activation through GITR may play a pivotal role in lymphocyte response to transplantation under early inflammatory conditions by affecting the balance between Teff and Treg responses [5].

We investigated the consequences of increased GITR activation on graft acceptance in mouse cardiac allograft models based on recipient CD40 deficiency (CD40−/−) or treatment of wild-type recipients with anti-CD40L mAb (WT+anti-CD40L). In vitro, evidence suggested that stimulation through GITR mediated graft rejection both by increasing proliferation of Teff and by inhibiting development of Treg. Stimulation through GITR reversed allograft acceptance in both of these models. Interestingly, CD40−/− recipients demonstrated a more severe graft rejection response that could be mediated by CD4+ cells alone, while both CD4+ and CD8+ cells were required to mediate rejection in GITR-stimulated WT+anti-CD40L recipients. Stimulation through GITR was unable to mediate transplant rejection once long-term acceptance of the graft was established. Together, these results demonstrate the capacity of peritransplant GITR stimulation to override the protective effects of CD40-CD40L blockade and highlight the differences in cellular responses caused by CD40 deficiency versus anti-CD40L mAb treatment.

2. Materials and Methods

2.1. Culture Medium. RPMI supplemented with 2% fetal calf serum, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.6 mM L-glutamine, 10 mM HEPES buffer (all from Invitroge, Carlsbad, CA, USA), 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 μM folic acid, and 50 μM 2-ME (all from Sigma-Aldrich, St. Louis, MO, USA).

2.2. Mice. WT C57BL/6 (H-2b) and BALB/c (H-2d) mice of age 6–12 weeks were obtained from Charles River Laboratories (Wilmington, MA, USA). CD40−/− C57BL/6 mice were procured from Jackson Laboratories (Bar Harbor, ME, USA). Breeder pairs of CD40−/− BALB/c mice were kindly provided by Dr. Randolph Noelle (Dartmouth Medical School, Hanover, NH, USA). Foxp3 green fluorescent protein (GFP) reporter knock-in mice bearing a Foxp3-GFP fusion construct were obtained from Dr. Xian Li (Harvard Medical School, Boston, MA, USA; [19]). All animals were maintained under a protocol approved by the University of Michigan Committee on Use and Care of Animals.

2.3. Heterotopic Cardiac Transplantation. WT C57BL/6 and CD40−/− C57BL/6 mice were transplanted with WT BALB/c or CD40−/− BALB/c cardiac allografts, respectively. Transplantation of cardiac allografts was performed as previously described [20]. Transplant function was monitored by daily palpation of the graft, and rejection was defined as the cessation of palpable contractions.

2.4. Antibodies. Anti-GITR mAb (DTA-I) was generously provided by Dr. Anita Chong (University of Chicago, IL, USA) with kind permission from Dr. Shimon Sakaguchi (Kyoto University, Japan). Purified rat IgG (Sigma-Aldrich) was utilized as an isotype control for anti-GITR. The anti-CD40L mAb producing hybridoma MRI was obtained from Dr. Randolph Noelle (Dartmouth Medical School). Hybridomas producing anti-CD4 mAb (GK1.5) and anti-CD8 mAb (2.43) were obtained from ATCC (Manassas, VA, USA). All antibodies were produced, purified, and suspended in PBS by LigoCyte Pharmaceuticals, Inc. (Bozeman, MT, USA). Animals receiving anti-CD40L were injected intraperitoneally (i.p.) with 1 mg of mAb on days 0, 1, and 2 relative to transplant. Animals receiving anti-GITR were injected i.p. with 1 mg mAb on days –2 and –1 relative to transplant. For delayed GITR stimulation, anti-GITR mAb was injected at days 29 and 30 after transplant. Anti-CD4 or anti-CD8 mAb were given i.p. on days –1, 0 and 7 relative to transplant at 1 mg/injection.

2.5. Enzyme-Linked Immunospot (ELISPOT) Assay. ELISPOT assays were performed as previously described [21]. Capture and detection of mAb specific for mouse IFNγ (R4-6A2, XMG1.2) and IL-4 (11B11, BVD6-24G2) were obtained from BD Biosciences (San Diego, CA, USA). Irradiated (1000 rad) WT or CD40−/− BALB/c splenocytes were added at 4 × 10^5 cells/well, followed by 1 × 10^6 recipient WT or CD40−/− splenocytes. After overnight incubation, plates were washed, and biotinylated detection mAb was added, followed by a 1/1000 dilution of polyclonal alkaline phosphatase-conjugated anti-biotin antibodies (Vector Laboratories, Burlingame, CA, USA) in the IFNγ wells and a 1/2000 dilution of horseradish peroxidase (HRP) conjugated streptavidin (Dako, Carpinteria, CA, USA) in the IL-4
well. Plates were developed with NBT/BCIP (IFNγ) or 3- amino-9-ethylcarbazole (IL-4). Developed plates were digitally scanned and analyzed using an ImmunoSpot ELISPOT analyzer (Cellular Technologies, Cleveland, OH, USA).

2.6. $[^3]$H Thymidine Proliferation Assay. Freshly isolated naïve C57BL/6 responder splenocytes were cultured with irradiated stimulator BALB/c splenocytes with or without 100 $\mu$g/mL anti-CD40L mAb (MR1), and naïve C57BL/6 CD40−/− responder splenocytes were cultured with irradiated stimulator BALB/c CD40−/− splenocytes for 5 days. Control rat IgG and anti-GITR mAb were added as indicated at a concentration of 100 $\mu$g/mL. 16 hours prior to harvest, cells were pulsed with 0.25 $\mu$Ci of $[^3]$H thymidine. $[^3]$H thymidine incorporation was determined via a Wallac BetaPlate scintillation counter (PerkinElmer, Waltham, MA, USA). Stimulation index was defined as the counts per minute (cpm) of responder cells + stimulator cells divided by the cpm of responder cells only.

2.7. In Vitro Generation of $T_{reg}$. Splenocytes from naïve Foxp3 GFP knock-in mice were isolated and cultured for 3 days with 10 U/mL recombinant IL-2, 10 ng/mL recombinant TGF-β, and 2% final volume of hybridoma supernatant containing anti-CD3 mAb (YCD3-1). In a modification of a previously published protocol, anti-GITR mAb or rat IgG control Ab was added at a concentration of 100 $\mu$g/mL [22]. Lymphocytes were isolated using Ficoll-Paque PLUS (Stemcell Technologies, Vancouver, BC, Canada), stained with phycoerythrin-conjugated anti-CD25 mAb (PC61), and analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.8. Statistical Analyses. Graft survival times were compared using a logrank comparison test. ELISPOT analyses were performed using a Student's $t$-test with Welch's correction (to account for different variances in treatment groups). Proliferation responses were compared using a paired Student's $t$-test. All analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Anti-GITR mAb Reverses Graft Acceptance Induced by CD40-CD40L Blockade. We hypothesized that stimulation through GITR by DTA-1 mAb would exacerbate acute graft rejection and override the protective effects of CD40-CD40L blockade. First, we determined the effects of GITR stimulation on an unmodified WT allogeneic rejection response. C57BL/6 recipients of BALB/c cardiac allografts rejected their grafts by day 7 after transplant, and GITR stimulation did not alter the rate of rejection (Figure 1(a)) or appreciably change allograft histopathology (data not shown). We then tested whether GITR stimulation could reverse allograft acceptance induced by CD40-CD40L blockade. As expected, recipients treated with control rat IgG and anti-CD40L mAb (WT+anti-CD40L) and control rat IgG-treated CD40+/− recipient mice accepted their allografts until the termination of the experiment at 35 days after transplant, demonstrating the effectiveness of CD40-CD40L blockade in inducing graft acceptance. Histology of these allografts was unremarkable and was characterized by a lack of graft infiltrating cells, absence of arterial inflammation, and an abundance of viable nucleated myocytes (Figure 1(b)).

In contrast, GITR stimulation induced graft rejection in both WT+anti-CD40L and CD40−/− recipients. Interestingly, the rate of rejection was not consistent between the two recipient groups. One hundred percent of the CD40−/− recipients receiving anti-GITR rejected their allografts by day 15 after transplant (Figure 1(a)). Graft pathology in these recipients was characterized by the presence of interstitial hemorrhage, myocyte necrosis, and significant perivascular cellular infiltrate (Figure 1(b)). Anti-GITR mAb stimulated rejection in WT+anti-CD40L recipients was less severe, as 66% of mice rejected their grafts before day 35 after transplant (Figure 1(a); median survival 18 days). Analysis of graft pathology revealed moderate perivascular mononuclear infiltrate but no significant myocyte necrosis (Figure 1(b)).

Variations in Th cell responses specific to GITR stimulated WT+CD40L or CD40−/− recipients might be responsible for the different rates of rejection. To test this, we utilized ELISPOT assays to quantify graft reactive Th1 (IFNγ) and Th2 (IL-4) cells from recipient mice (Figure 1(c)). In both GITR-stimulated WT+anti-CD40L and CD40−/− recipients, graft rejection was characterized by a small but significant increase in IFNγ versus recipients treated with control rat IgG. It should be noted that the magnitude of the Th1 response in both GITR-stimulated WT+anti-CD40L and CD40−/− recipients was markedly reduced compared to the Th1 response in WT unmodified recipients, reflecting the ability of CD40-CD40L perturbation to reduce the Th1 response even in the presence of GITR stimulation. The magnitude of Th2 response as measured by IL-4 secretion was unremarkable in both treatment groups. Together, these results suggest that stimulation through GITR is capable of overriding CD40-CD40L perturbation and inducing graft rejection, most likely via activation of donor reactive Th1.

3.2. GITR Stimulation Does Not Induce Rejection of Established Allografts. We further investigated the ability of GITR stimulation to induce transplant rejection in established allografts. We have previously demonstrated that OX40 stimulation overrides the protective effects of CD40-CD40L blockade when given at the time of transplant. However, delayed OX40 stimulation does not induce acute rejection, instead, signs of chronic rejection are observed [9]. Therefore, we investigated the effects of delayed GITR stimulation on established allografts in WT+anti-CD40L and CD40−/− recipients. Mice received cardiac transplants and were stimulated with 1 mg of anti-GITR mAb on days 29 and 30 after transplant. As demonstrated in Figure 1(a), stimulation of GITR in recipients with established grafts did not result in graft rejection. No inflammatory infiltrate, vascular changes, or obvious differences in collagen deposition were observed between grafts isolated from WT+anti-CD40L and CD40−/− recipients that received late GITR stimulation (data not shown).
Figure 1: GITR stimulation induces graft rejection in WT+anti-CD40L and CD40−/− allograft recipients. (a) Mann-Whitney survival plot of grafts transplanted into WT (circles), WT+anti-CD40L (triangles), or CD40−/− recipients (squares). All recipients were treated with 1 mg of IgG isotype control (open symbols, dotted line), anti-GITR mAb (closed symbols, solid line) on days −2 and −1 relative to transplantation, or anti-GITR mAb that was administered at days 29 and 30 after transplantation (open symbols, solid lines), and mice were observed until 50 days after transplant. Significance was determined via logrank analysis *P < 0.001. (b) Hematoxylin and eosin (H&E; ×200) staining of cardiac allografts recovered from recipients at the day of rejection or at 35 days after transplant (controls). Arrows indicate mononuclear cellular infiltrate. (c) Recipient splenocytes were harvested and processed at the time of rejection or at 35 days after transplant for ELISPOT assays, and primed, donor-reactive IFN-γ and IL-4 producing cells were quantified as the number of spots/10⁶ splenocytes. Significance was determined by a Student's t-test with Welch's correction.
3.3. GITR Stimulation Modifies Alloantigen-Specific T Cell Proliferation and T<sub>reg</sub> Differentiation. Since GITR is expressed on both T<sub>eff</sub> and T<sub>reg</sub> [11], we next investigated whether the effects of GITR stimulation in our CD40-CD40L blockade models were due to proliferation of T<sub>eff</sub> and/or inhibition of T<sub>reg</sub> development. We characterized the proliferative effect of GITR stimulation on alloreactive splenocytes by utilizing mixed lymphocyte [³H] thymidine incorporation assays. Freshly isolated WT responder cells were cocultured with irradiated BALB/c splenocytes only, with anti-CD40L, and irradiated BALB/c splenocytes, and CD40<sup>−/−</sup> responder cells were cocultured with irradiated CD40<sup>−/−</sup> BALB/c splenocytes. As depicted in Figure 2(a), stimulation via GITR significantly increased proliferation in all treatment groups relative to isotype-treated control. Clearly, GITR stimulation resulted in an increased T<sub>eff</sub> response, even under the cover of CD40-CD40L blockade.

We next assessed the ability of GITR stimulation to inhibit T<sub>reg</sub> differentiation in vitro by utilizing lymphocytes
isolated from transgenic mice expressing the Foxp3-GFP fusion protein [19]. Freshly isolated Foxp3-GFP splenocytes were stimulated with anti-CD3 mAb and were cocultured in the presence of TGFβ and IL-2 to promote differentiation of naïve splenocytes into Treg [23, 24]. Up to 30% of naïve splenocytes were induced to differentiate into CD25+Foxp3+ Treg under these culture conditions (Figure 2(b)). Stimulation through GITR, however, significantly reduced the percentage of CD25+Foxp3+ cells, indicating inhibition of Treg differentiation in vitro. Together, these results suggest that rejection triggered by stimulation through GITR may result from a combination of Treg proliferation together with reduced differentiation of Treg.

3.4. CD4+ T Cells Are Sufficient to Mediate Rejection in Anti-GITR-Treated CD40−/− Recipients. CD4+ and CD8+ T cells have been shown to be differentially sensitive to various immunomodulatory agents in allograft rejection [25]. Therefore, we determined the requirements for CD4+ and CD8+ T cell subsets in GITR-mediated rejection induced in CD40−/− or WT+anti-CD40L recipients. Depletion of CD4+ T cells in both WT+anti-CD40L and CD40−/− recipients prevented graft rejection induced by GITR stimulation (Figure 3(a)). Depletion of CD4+ T cells resulted in interstitial mononuclear cell infiltrate in grafts; however, cardiac monocytes remained viable (Figure 3(b)). CD8+ T cell depletion in WT+anti-CD40L recipients also prevented GITR-stimulated allograft rejection (Figure 3(a)). Functional grafts recovered from these recipients showed negligible signs of inflammation around myocytes and arterioles (Figure 3(b)). In contrast, allograft rejection occurred within 15 days after transplant following depletion of CD8+ T cells in CD40−/− GITR-stimulated recipients (Figure 3(a)). Grafts isolated from these mice exhibited significant eosinophilic infiltrate extending from the periarteriolar regions of the graft into the surrounding myocytes (Figure 3(b), inset). Extensive loss of viable myocytes, interstitial hemorrhage, and arteriolar occlusion were also evident. The presence of eosinophils within the graft infiltrate suggested induction of a Th2 response [26, 27]. Confirmation of this was achieved via ELISPOT analyses, where significant IL-4, but minimal IFNγ production, was observed in GITR-stimulated CD8-depleted CD40−/− splenocytes (Figure 3(c)). Hence, CD4+ T cells alone were capable of rejecting allografts in GITR-stimulated CD40−/− recipients, but both CD4+ and CD8+ T cells are required for GITR stimulation to override the protective effects of anti-CD40L mAb treatment.

4. Discussion

This study demonstrates that stimulation through GITR can initiate graft rejection under the cover of CD40-CD40L blockade, though the magnitude of the response depends on the mode of CD40-CD40L blockade and the T cell subset present. Allograft rejection triggered by GITR stimulation in mice with whole T cell populations was characterized by inflammatory cell infiltration and Th1 cytokine secretion (Figure 1). In addition, stimulation through GITR expressed on naïve T cells resulted in enhanced proliferation of WT, WT+anti-CD40L, and CD40−/− responder cells in mixed lymphocyte reactions (Figure 2(a)), demonstrating the ability of signaling through GITR to expand alloantigen-reactive T eff populations. These results thereby reaffirm the costimulatory function of GITR-GITR ligand interactions in the development of an immune response [13, 28]. Thus, despite the protective effects of CD40-CD40L blockade, GITR stimulation and expansion of graft-reactive T eff may simply overwhelm Treg-mediated suppression. At the same time, stimulation through GITR may negatively modulate Treg function, further preventing the development of graft acceptance [29, 30]. Indeed, we also noted that stimulation through GITR significantly reduced the development of Foxp3+CD25+ Treg in vitro (Figure 2(b)).

Importantly, we observed significant differences in the magnitude of rejection responses induced by GITR stimulation in CD40−/− recipients versus WT+anti-CD40L recipients. CD40 deficiency combined with GITR stimulation resulted in a more robust rejection response. Previous evidence has demonstrated that anti-CD40L treatment may have effects beyond simple CD40-CD40L blockade. One possibility is the capacity of anti-CD40L mAb to bind to CD40L expressed on activated T cells, leading to their removal via complement or FcγR1-mediated mechanisms [31, 32]. Indeed, the effector cells generated in our experiments exhibit a polarized Th1 phenotype (Figure 1(c)), and CD40L expression is enhanced and prolonged on Th1 cells [33]. Therefore, the stimulation of T cells via GITR results in the upregulation of CD40L, and these activated cells might be targeted for clearance by anti-CD40L mAb binding.

More evidence of increased susceptibility of CD40−/− recipients to GITR stimulation was provided by our finding that CD4+ T cells alone could reject allografts in this setting. In the absence of modifying Th1 cytokines produced by CD8+ T cells, stimulation of CD4+ T cells through GITR resulted in a Th2 response characterized by significant IL-4 production and eosinophilic infiltrate of the graft (Figures 3(b) and 3(c)). This pathogenic Th2 response has previously been associated with CD8+ T cell depletion, IFNγ deficiency, or IL-12 antagonism [26, 27, 34, 35]. Thus GITR stimulation can compensate for the lack of CD40 signaling through CD40L on CD4+ T cells. In contrast, anti-CD40L mAb binding to CD40L expressed by activated CD4+ T cells in WT recipients likely results in clearance of these cells via Fc-mediated mechanisms [31, 32]. Hence, the population of cells most responsive to GITR stimulation is absent, and rejection by CD4+ T cells alone cannot occur under the cover of anti-CD40L mAb treatment.

Importantly, responsiveness to anti-GITR treatment in both WT+anti-CD40L and CD40−/− recipients does not appear to persist for a long term, as GITR stimulation 30 days after transplant did not induce rejection in either recipient strain (Figure 1(a)). This finding is intriguing especially in regards to the WT+anti-CD40L recipients, as these recipients have been shown to retain quiescent donor-reactive T cells in their spleens [1, 36], and these cells could presumably be stimulated by anti-GITR treatment. GITR stimulation likely acts in concert with the inflammatory environment present.
Figure 3: CD4+ T cells mediate GITR-stimulated graft rejection in CD40−/− recipients. (a) Mann-Whitney survival plot of grafts transplanted into WT + anti-CD40L (closed symbols) or CD40−/− (open symbols) recipients depleted of either CD4+ (squares) or CD8+ (circles) T cells and treated with anti-GITR mAb on days −1 and −2 prior to transplant. Significance was determined via logrank analysis. (b) H&E staining (×200) of transplants recovered either at the day of rejection or at the termination of the experiment at day 35 after transplantation. Black arrows indicate mononuclear cellular infiltrate, and yellow arrows indicate eosinophils. The inset represents 1000x magnification of infiltrate observed in CD8-depleted CD40−/− recipients. (c) Recipient splenocytes were harvested and processed at the time of rejection or at 35 days after transplant for ELISPOT assays, and primed, donor-reactive IFN-γ and IL-4 producing cells were quantified as the number of spots/10⁶ total splenocytes. Significance was determined by a Student’s t-test with Welch’s correction.

during early graft-reactive T cell activation. GITR may be upregulated in response to CD28 perturbation in CD4+ T cells [11]; therefore, stimulation through GITR at the time of transplantation likely targets effector T cells that have become activated in response to the graft. In addition, it has been demonstrated that stimulation through GITR results in loss of Treg function (Figure 2(b) and [13]). Therefore, early stimulation through GITR may override the protective effects of CD40-CD40L blockade by providing additional costimulatory signals to T cells and tipping the balance of Teff versus Treg. However, once acceptance has been established and allograft inflammation has subsided, GITR stimulation is not sufficient to activate T cells and induce graft rejection. Interestingly, OX40 stimulation under the cover of CD40-CD40L blockade also failed to induce graft rejection once acceptance was established [9], providing further evidence that multiple, inflammatory signaling pathways early in the anti-transplant response may contribute to rejection.

In conclusion, this study has demonstrated the ability of peritransplant GITR stimulation to reverse cardiac allograft acceptance under the cover of CD40-CD40L blockade. These findings reinforce the potential costimulatory role of GITR within the inflammatory environment found after transplant and suggest that stimulation through GITR might inhibit the
differentiation of $T_{reg}$ while simultaneously expanding the $T_{eff}$ population. We also demonstrated a marked difference between CD40 deficiency and anti-CD40L mAb treatment in the rate and pathology of graft rejection. These results support reports of the potential pleiotropic effects of anti-CD40L mAb in modulating immune responses [31, 32, 36–39] and suggest that antagonism of GITR signaling might represent a potential therapy for acute inflammatory responses in transplant.

**Abbreviations**

- GITR: Glucocorticoid-induced TRFR-related protein
- $T_{reg}$: Regulatory T cell
- $T_{eff}$: Effector T cell
- GFP: Green fluorescent protein
- i.p.: Intraperitoneally
- ELISPOT: Enzyme-linked immunospot

**Authors’ Contribution**

Kenneth T. Krill and Keri Csencsits-Smith contributed equally to this paper.

**Conflict of Interests**

None of the authors of this paper has a financial relationship with any of the commercial entities mentioned in the paper.

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**References**


Review Article

Nuclear Antigens and Auto/Alloantibody Responses: Friend or Foe in Transplant Immunology

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In addition to cellular immune responses, humoral immune responses, mediated by natural antibodies, autoantibodies, and alloantibodies, have increasingly been recognized as causes of organ transplant rejection. In our previous studies, we have demonstrated the induction of antinuclear antibodies against histone H1 and high-mobility group box 1 (HMGB1), in both experimental and clinical liver transplant tolerance. The active induction of antinuclear antibodies is usually an undesirable phenomenon, but it is often observed after liver transplantation. However, the release of nuclear antigens and its suppression by neutralizing antibodies are proposed to be important in the initiation and regulation of immune responses. In this review article, we summarize the current understanding of nuclear antigens and corresponding antinuclear regulatory antibodies (Abregs) on infection, injury, inflammation, transplant rejection, and tolerance induction and discuss the significance of nuclear antigens as diagnostic and therapeutic targets.

1. Introduction

Transplantation of cells, tissues, or organs is now widely used to cure patients with life-threatening diseases or traumatic injuries. Except for the use of self-derived grafts or grafts from an identical twin, allograft rejection can be observed acutely and/or chronically [1, 2]. In the current practice of transplantation, the administration of immunosuppressants, such as tacrolimus (FK506) and cyclosporin A, is indispensable for the prevention of allograft rejection [3]. However, the use of these immunosuppressants has limitations, including the necessity of long-term medication and serious side effects, such as nephrotoxicity [4], cardiovascular toxicity [5], and cancer [6]. Therefore, the development of safer and more effective immunosuppressants as well as useful diagnostic tools for the prediction of rejection is an important subject for further improvement of the quality of life of patients and their families after transplantation.

Since the early days of experimental and clinical liver transplantation, it has been known that this organ does not always obey the normal rules of transplant rejection (Medawar’s rule of transplantation); for example, all grafts are rejected between unrelated individuals, and the survival rate following liver transplantation is higher than that following the transplantation of other organs [7, 8]. In Dark Agouti (DA) donor livers transplanted into Piebald Virol Glaxo (PVG) recipients, allograft rejection is spontaneously overcome, resulting in a state of long-lasting and donor-specific tolerance without pharmacological immunosuppression, although PVG recipients acutely reject skin, heart, and renal grafts from DA rats [9]. Interestingly, PVG recipients bearing DA livers could accept skin, heart, and kidney transplants from the DA donor rats but rejected them from third-party strains of rats [10, 11]. The molecular and cellular basis of liver transplant tolerogenicity has not been fully elucidated, but the unique
repertoires of nonparenchymal cells including liver antigen-presenting cells (e.g., dendritic cells (DCs), Kupffer cells, and liver sinusoidal endothelial cells) and unconventional lymphoid cells (e.g., NK cells, B-1 cells, and γδ T cells), which are rarely present in the blood, may explain the immune privilege of the liver [12]. Our recent study also suggested that mast cells in the donor grafts may play important roles in the induction/maintenance of immune tolerance and liver regeneration, resulting in the replacement of hepatic cells from donor to recipient [13]. In addition, several humoral factors in the serum of a rat tolerogenic OLT model have been identified as immunosuppressive factors, including donor-soluble MHC class I molecules [14], antidonor MHC class II antibodies [15], liver suppressor factor-1 (LSF-1; 40 kDa) [16, 17], LSF-2 (87 kDa), and LSF-3 (10 kDa) [18]. However, most of these humoral factors are found only in the experimental OLT model, and it is hard to translate the findings of this animal study to clinical practice.

In the past decade, we further evaluated humoral factors, specifically IgG antibodies, which are immediately elevated and maintained at a higher level even after the recipients accept the donor liver allografts and demonstrated strong immunosuppressive activity in vitro [19, 20]. The screening of autoantigens recognized by immunosuppressive IgG antibodies in the post-OLT sera revealed the spontaneous induction of antinuclear antibodies against histone H1 and high-mobility group box 1 (HMGB1), both in the DA-PVG natural tolerance model and in a patient with operational tolerance [19–22].

In this review article, we summarize the current understanding of nuclear antigens and corresponding antinuclear regulatory antibodies (Abregs) on infection, injury, inflammation, transplant rejection, and tolerance induction and discuss the significance of nuclear antigens as diagnostic and therapeutic targets.

2. Induction of Humoral Immune Responses after Transplantation: Link to Rejection or Tolerance?

In the past, organ transplant rejection and tolerance were believed to be mediated almost exclusively by cellular immune responses. Although improvements in T-cell-directed immunosuppression have decreased the incidence of acute cellular rejection, humoral immune responses, mediated by natural antibodies, autoantibodies, and alloantibodies, have increasingly been recognized as causes of organ transplant rejection [23, 24]. The overall incidence of antibody-mediated rejection (AMR) is estimated to be 20%–30% for renal transplant recipients [25]. However, AMR is mainly discussed in ABO blood type-incompatible liver transplantation [26]. Natural antibodies against A/B carbohydrate determinants are likely to develop as a result of exposure to environmental bacteria that express similar determinants. The response of the B-cell compartment to environmental antigens/microbial products and autoantigens has been thought to be derived preferentially from the activation of CD5+ B-1 cells. Therefore, CD5+ B-1 cells have been speculated to be the major B-cell subset responding to A/B determinants in both mice and humans [27–29]. B-1 cells are present in low numbers in the lymph nodes and spleen and are instead found predominantly in the peritoneal and pleural cavity [30, 31]. Recent reports suggest that splenic CD1dhiCD5+ B cells are potent regulatory cells that produce IL-10 in models of contact hypersensitivity and experimental allergic encephalomyelitis [32, 33]. Furthermore, Moritoki et al. reported that B cells or B-cell subsets may affect the induction and function of regulatory T cells (Tregs) as suppressors of the T-cell component [34]. Chhabra et al. recently reported the prevention of autoimmune diabetes and the prolongation of islet allograft survival by the administration of naturally occurring IgM autoantibodies [35]. These findings strongly suggest that the induction of natural antibodies or autoantibodies may play an important role in immune regulation and tolerance induction after transplantation.

In our previous studies, we have demonstrated the induction of antinuclear antibodies against histone H1 and HMGB1 both in a rat tolerogenic OLT model and in a patient with operational tolerance [19–22]. In the field of liver transplantation, the induction of autoantibodies (e.g., antinuclear antibody, smooth muscle antibody, and liver-kidney microsomal antibody) has often been observed, particularly in pediatric recipients [36], while the incidence of de novo autoimmune hepatitis in children with elevated serum autoantibodies and liver function tests, hypergammaglobulinemia, and liver pathology showing necroinflammatory disease and fibrosis has been found to be just 1%–7% [37–39]. We also confirmed the significance of antinuclear antibody for protection and recovery from the concanavalin A-(Con A-) induced liver injury mimic of autoimmune hepatitis [40]. Therefore, the induction of autoantibodies in most recipients after liver transplantation may not be associated with any clinical manifestations of autoimmune disorders. A recent study also demonstrated that the long-term administration of tacrolimus to liver transplant recipients induces the production of antinuclear antibodies, whereas the autoimmune disease susceptibility of recipients treated with tacrolimus has not been elucidated [41]. The active induction of antinuclear antibodies is usually an undesirable phenomenon, but why is it often observed after liver transplantation? Is it linked to the immune privilege of the liver? The answers to these questions are still uncertain, but we speculate that the existence of antinuclear antibodies against histone H1 and HMGB1, which possess strong immunosuppressive activity in the systemic circulation, may regulate uncontrollable immune responses such as acute/chronic rejection after transplantation. In other words, the induction of antinuclear antibodies may be a “lethal weapon” to escape the breakdown of our immune system at least in transplant immunology. Our hypothesis is supported by Barnay-Verdier et al., who recently demonstrated that autoantibodies against HMGB1 are produced during sepsis and are associated with a favorable outcome in patients with septic shock [42].

3. Nuclear Antigens and Immunogenicity

Why are antinuclear antibodies against histone H1 and HMGB1 elevated in the specific condition of liver
transplantation, and do they act as Abregs? An initial mechanism for the induction of antinuclear antibodies is the release of nuclear antigens, and the primary source of nuclear antigens would be damaged hepatic cells due to peritransplant ischemia/reperfusion injury and posttransplant rejection. Specifically, hepatic cell death by necrosis, apoptosis, and autophagy during cold ischemia and warm reperfusion during the course of liver transplantation triggers liver graft dysfunction [43–45]. Indeed, the release of nuclear antigens and its suppression by neutralizing antibodies are proposed to be important in the initiation and regulation of immune responses. HMGB1 is a ubiquitous and abundant chromatin component, and it is currently well known as one of the damage-associated molecular pattern molecules (DAMPs) interacting with the receptor for advanced glycation end product (RAGE), toll-like receptor (TLR)2, TLR4, and TLR9 [46]. Wang et al. first reported the proinflammatory role of HMGB1 in endotoxin lethality in mice and in septic patients [47]. Since then, the proinflammatory roles of HMGB1 in the pathogenesis of many diseases have been reported, including acute lung inflammation [48], atherosclerosis and restenosis after vascular damage [49], severe acute pancreatitis [50], rheumatoid arthritis [51], pulmonary fibrosis [52], stroke [53], Kawasaki disease [54], cold ischemia/reperfusion-induced inflammation [55], liver fibrosis [56], systemic inflammatory response syndrome [57, 58], febrile seizures [59], hyperlipidemia [60], preeclampsia [61], and acute-on-chronic liver failure [62].

However, the roles of histones in immune responses are poorly understood in comparison with HMGB1. Histone H1 has been reported to possess various important functions including a role in transmitting apoptotic signals from the nucleus to the mitochondria, which release apoptogenic factors into the cytoplasm, following DNA double-strand breaks [63] and in normal DC differentiation, based on evidence demonstrating that the production and differentiation of DCs in histone H10-deficient mice are significantly reduced [64]. Our previous study has demonstrated that the translocation of histone H1 from the nucleus to the cytoplasm and the release of their own histone H1 are necessary for DC maturation and the T-cell activation [65]. This function is also similar to the role of HMGB1 in DC maturation [66]. In addition, recent work has clearly demonstrated the induction of inflammatory responses by extracellular histones from dying cells via TLR2 and TLR4 in acute kidney injury [67].

Taken together, these results strongly suggest the significance of nuclear antigens such as histones and HMGB1 that are released from damaged cells or actively secreted from activated immune cells such as DCs and macrophages in the initiation of immune responses during rejection as well as infection, injury, and inflammation (Figure 1). We speculate that the sensitivity to nuclear antigens (i.e., easy production of antinuclear Abregs) may be one of the key factors determining the acceptance or rejection of donor liver allografts [68]. To be exact, antinuclear antibodies include both auto- and alloantibodies due to the different sources of antigens (liver allografts: alloantigens, immune cells: autoantigens) in the case of liver transplantation. In this review article, however, we have defined the induction of antinuclear antibodies as an autoimmune response due to the homological similarity of nuclear antigens even in different species.

4. Nuclear Antigens as a Prognostic Marker for Rejection

The release of nuclear antigens into the bloodstream has been associated with the progression of several diseases. Hatada
et al. reported the elevation of plasma HMGB1 levels in patients with infectious diseases, malignancies, and traumas and suggested that HMGB1 is a potentially suitable prognostic marker of organ failure or disseminated intravascular coagulation [69]. The serum level of HMGB1 in patients with nonsmall cell lung cancer (NSCLC) was significantly higher compared to patients with chronic obstructive pulmonary disease, suggesting that HMGB1 may be a useful marker for evaluating NSCLC progression [70]. A positive association between the circulating HMGB1 level and cardiovascular mortality or traumatic brain injury has also been reported [71, 72]. As shown in Figure 2, the elevation of circulating histone H1 and HMGB1 was confirmed during the rejection phase (day 7) after OLT in a rat acute rejection combination (DA-LEW). However, mild or no elevation of circulating histone H1 and HMGB1 was confirmed in a rat tolerogenic combination (DA-PVG), suggesting the diagnostic potential for the prediction of acute rejection after transplantation. In our previous studies, we have confirmed the induction of humoral immune responses against histone H1 and HMGB1 only in the DA-PVG combination [21, 22], suggesting the blockade of the exposure of nuclear antigens by the induction of corresponding antinuclear Abregs. The induction of antinuclear Abregs could also suppress alloantibody production during the rejection phase (day 7) after OLT (Figure 3). Therefore, the balance between autoimmunity and alloimmunity is important for the prolongation of allograft survival (Figure 4).

5. Nuclear Antigens as a Therapeutic Target

To prevent the release of nuclear antigens such as histone H1 and HMGB1, resulting in the activation of innate and adaptive immune responses, several strategies have been proposed. The therapeutic potential of anti-HMGB1 antibody, soluble RAGE, and anti-RAGE neutralizing antibody has been demonstrated in experimental sepsis [73, 74], liver ischemia/reperfusion injury [75], Con-A-induced hepatic injury [76], traumatic brain injury [77], and organ transplantation [78, 79]. The therapeutic potential of antihistone H1 polyclonal antibody for overcoming rejection and liver inflammation was also confirmed by our group [19, 40]. We also confirmed the great potential of histone H1 vaccination in transplant recipients for tolerance induction [80, 81]. To further explore the roles of histone H1 and its future clinical application, we have generated antihistone H1 monoclonal antibodies (clone: 16G9, IgM), which possess immunosuppressive activity in vitro [82]. In addition, we have identified the functional epitope (SSVLYGGGPSAA) responsible for the immunosuppressive activity of 16G9 and have confirmed the diagnostic and therapeutic potential of histone H1 peptide [83]. In addition to neutralizing antibody therapy, an HMGB1 absorption column (polymyxin B-immobilized fibers) has been developed and clinically applied for the removal of circulating HMGB1 in patients with septic shock, acute respiratory distress syndrome, and idiopathic pulmonary fibrosis with acute exacerbation [84–90]. The therapeutic
Figure 3: Alloantibody response during the rejection phase after OLT. The alloantibody response was measured by flow cytometry on a single cell suspension of DA rat splenocytes. Briefly, 50 μL aliquots containing 5 × 10^5 splenocytes was incubated with 50 μL of diluted naïve or post-OLT sera (1:16, 1:64, 1:256) for 45 min at 4°C. The washed cells were reacted with 50 μL of a mixture of FITC-conjugated goat antibody specific for the Fc portion of rat IgG (×100 dilution) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBS containing 1% BSA and 0.02% NaN3. After staining, the cells were washed, fixed, and analyzed using an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). Histograms (representative of three individuals) show the percentage of DA splenocytes recognized by alloantibody (IgG) in the post-OLT sera at day 7 after OLT.

potential of HMGB1 antagonists such as HMGB1 A box peptide has also been reported [91, 92].

6. Summary and Future Directions

In this review, we have discussed the diagnostic and therapeutic potential of nuclear antigens (histone H1 and HMGB1) and the corresponding antinuclear Abregs on infection, injury, inflammation, and transplant rejection. One of the immunosuppressive mechanisms of antinuclear Abregs is the direct binding of circulating nuclear antigens, which triggers the immune response (Figure 1). In addition, our previous study strongly suggested the binding of antihistone H1 Abregs to histone H1-like molecules, which may be transiently expressed on the cell membrane of splenocytes [19]. We have also demonstrated that antihistone H1 Abregs may selectively suppress the MAPK, NF-κB, and calcineurin-NFAT signaling pathways during T-cell activation [40], coordinate the Th1/Th2 balance [81], and induce CD4+CD25+ T cells [65]. Recent evidence suggests that antihistone H1 Abregs negatively regulate the harmful T-cell response, in part through collaboration with Tregs [93]. Although further investigation is needed, the direct effects of nuclear antigens and corresponding antinuclear Abregs on immune cells may play important roles in inflammation, rejection, and tolerance induction. Interestingly, the induction of antinuclear Abregs (i.e., the autoimmune response against nuclear antigens) may suppress alloantibody production during rejection after OLT (Figure 3). A crucial issue is why cell death-associated moieties and corresponding autoantibodies, which elicit clinical autoimmunity in patients with autoimmune diseases, could be indispensable for immune regulation in other settings. In our previous study, nuclear histone H1 and Freund's complete adjuvant were injected into naïve rats and resulted in different autoantibody responses against histone H1 in tolerogenic PVG OLT recipients and rejecting LEW.
Autoimmunity
Humoral/cellular immunity
Histones HMGB1
Tolerance
Rejection
(infection, injury, inflammation and allergy)
Induction of autoimmunity
DCs, macrophages
Damaged cells
Autoimmune disorder
Alloimmunity
Autoimmunity
ALLO-immunity
Allo-immunity
Rejection
Histones
HMGB1
Humoral/cellular immunity
Tolerance
Figure 4: The balance of autoimmunity and alloimmunity (against alloantigens, pathogens, or allergens) is important for immune regulation. During the rejection phase (or when suffering from infection, injury, or allergy), alloimmunity is predominant, and nuclear antigens such as histones and HMGB1 are released from damaged cells, tissues, or organs or are actively secreted from activating immune cells such as DCs and macrophages. The induction of autoimmunity against nuclear antigens (i.e., induction of antinuclear Abregs) may regulate the balance and induce immunological tolerance. Excessive activation of autoimmunity may cause autoimmune disorders.

OLT recipients [68]. The transient induction of autoantibodies in normal mice challenged with dying cells and adjuvants (Freund's incomplete adjuvant or DCs) was also reported without clinical or histological features of autoimmunity, while clinical autoimmunity develops in autoimmune-prone mice [94, 95]. Therefore, we speculate that the response to dying cells in OLT recipients may be one of the key factors determining the clinical outcome. How to modulate the balance between autoimmunity and alloimmunity is an important issue for the extrinsic regulation of unwanted immune responses and the induction of immune tolerance (Figure 4). Our present data also reveal the diagnostic significance of nuclear antigens for the prediction of acute rejection after liver transplantation (Figure 2). The development of fast, accurate, and precise diagnostic tools by measuring the blood level of nuclear histone H1 and HMGB1 would allow clinicians to evaluate immune status and modulate the dose of immunosuppressants for rejection control. The development of absorption columns for circulating nuclear antigens (histone H1 and HMGB1) as well as neutralizing humanized monoclonal antibodies may help to establish novel immunotherapies for infection, injury, inflammation, and transplant rejection.

Conflict of Interests
The authors have declared that no competing interests exist.

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References
Clinical and Developmental Immunology


[69] T. Hatada, H. Wada, T. Nobori et al., “Plasma concentrations and importance of high mobility group box protein in the prognosis of organ failure in patients with disseminated


