Immune recognition and rejection of allogeneic skin grafts

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Abstract

The transplantation of allogeneic skin grafts is associated with a potent infammatory immune response leading to the destruction of donor cells and the rejection of the graft. Shortly after transplantation, skin dendritic cells (DCs) migrate out of the graft through lymphatic vessels and infiltrate the recipient's draining lymph nodes where they present donor antigens via two mechanisms: the direct pathway, in which T cells recognize intact donor MHC antigens on donor DCs; and the indirect pathway, involving T-cell recognition of donor peptides bound to self-MHC molecules on recipient DCs. Some recent studies have suggested that T cells can become activated via recognition of donor MHC molecules transferred on recipient antigen-presenting cells (semidirect pathway). Activation of T cells via direct or indirect allorecognition is sufficient to trigger acute rejection of allogeneic skin grafts. In addition, allospecific antibodies contribute to the rejection process either by killing allogeneic targets in a complement-dependent fashion or by opsonizing donor cells and forming immune complexes. Finally, several studies demonstrate that NK cells, activated due to missing self-MHC class I molecules on allogeneic cells, are involved in allogeneic skin graft rejection via direct killing of donor cells and through the production of proinflammatory cytokines including IFN-γ and TNF-α.

Keywords

allorecognition; B lymphocytes; NK cells; passenger leukocytes; skin transplantation; T lymphocytes; transplant rejection

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In the USA, approximately 2.4 million burn injuries are reported every year. Many patients with major burns involving at least 25% of their total body surface die. The transplantation of large patches of skin would save their lives. However, allogeneic skin grafts are invariably rejected in an acute fashion. While current immunosuppressive treatments are effective in preventing early rejection of organ transplants, such as kidney, they have little or no effect in skin transplantation. In addition, until now, many attempts to engineer artificial skin or grow skin tissue *in vitro* have been ineffective. As a result, current clinical skin transplantation is confined to autotransplantation (i.e., the grafting of small patches of an individual's own skin from one anatomical site to another). This underscores the need for

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engineering strategies designed to induce immune tolerance to skin allografts, defined as indefinite survival in the absence of chronic, widespread immunosuppression.

The placement of an allogeneic skin patch triggers a potent reaction by the host's immune system eventuating in rapid elimination of donor cells and rejection of the graft. Initial trauma and tissue injury resulting from placement of allografts is associated with an inflammatory process and the initiation of an innate immune response. During this response, polymorphonuclear cells, macrophages, dendritic cells (DCs), releasing cytokines, acute-phase proteins and angiogenic factors infiltrate the graft. Concomitantly, donor DCs migrate from the graft to the recipient's secondary lymphoid organs where they can present donor antigens, thereby eliciting an adaptive immune response. This response is initiated by recipient T lymphocytes recognizing genetically encoded polymorphisms between members of the same species, a phenomenon referred to as T-cell allorecognition. Following allorecognition, $CD4^+$ and $CD8^+$ T cells become activated, proliferate, secrete proinflammatory cytokines and differentiate into effector cells. Such activated effector T cells leave the secondary lymphoid organs and infiltrate the graft where they mediate its r ejection, presumably by killing donor cells.

Most of the knowledge pertaining to the immunological mechanisms involved in the rejection of allogeneic transplants is derived from studies on skin transplantation. Initial studies by Medawar *et al.* were performed during wartime, first in humans and then in animal models [1,2]. In the 1980s, the generation of mice displaying discrete mutations of genes of the MHC locus and of monoclonal antibodies against CD4 and CD8 accessory molecules rendered possible studies exploring the roles of MHC class I and II genes in the allorecognition by $CD4^+$ and $CD8^+$ T-cell subsets and in the rejection of skin allografts [3]. Recent engineering of genetically modified mice including transgenic mice, and mice with selected gene deficiencies, have provided new insights into the immunology of skin allograft rejection. In the present article, we describe the studies that have led to our current perspective on the cellular and molecular mechanisms underlying the allorecognition and rejection of skin allografts.

Cutaneous dendritic cells & skin transplantation

Following placement of skin allografts, donor DCs, often referred to as passenger leukocytes, migrate out of the graft through lymphatic vessels and infiltrate the recipient's draining lymph nodes (LNs) where they present donor antigens to alloreactive T cells. Therefore, skin DCs play an essential role in the initiation of adaptive alloimmunity and skin graft rejection (Figure 1).

Skin DCs initiating adaptive alloimmune responses are comprised of two distinct cell subsets: Langerhans cells (LCs) in the epidermis, and dermal DCs in the dermis. LCs, which are regularly spaced throughout the epidermis, represent 3% of epidermal cells. LCs are typically characterized by the expression of cytoplasmic organelles termed Birbeck granules, and the expression of CD1a membrane molecules in humans (not present in mice) capable of presenting microbial lipid antigens to T cells [4]. In addition, the C-type lectin langerin (CD207) has been recently identified as a key marker of LCs [5]. The hematopoietic origin of LCs is well established. However, in steady-state conditions, LCs can replenish locally as evidenced by the host origin of LCs found in mice even after 1 year of parabiosis [6]. By contrast, other DCs comprise mixed populations in LNs and spleen of parabiotic animals [7,8]. In steady-state conditions, LC half-life is estimated to be 53–78 days, while other DCs display a half-life of 7–8 days [8–12]. The proliferation rate of LCs is estimated to be approximately 1–2%, compared with 5% for lymphoid organ DCs [8–10]. Skin LC precursors coexpress langerin and CD14 (LPS receptor); and their final migration into the

suprabasal layer of the epidermis is controlled by CXCL14, produced by keratinocytes [13,14]. During inflammation, LCs repopulating the skin derive from peripheral blood precursors. For instance, after UV light exposure or cutaneous graft-versus-host disease (GVHD), which result in major LC loss, LC repopulation occurs from circulating monocyte precursors that express CCR2 and CCR6 [6,15]. Conversely, in less severe injuries that result in moderate LC loss, LCs can be repopulated locally [6,16]. Final LC differentiation, ultimately, appears to depend on the cytokine environment of the epidermis [14], with contribution from granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-15 and TGF-β.

Dermal DCs belong to a broader subset known as interstitial DCs [17]. The majority of dermal DCs are langerin-negative cells expressing high levels of the integrin CD11b and macro-phage markers such as F4/80, SIRP-α and CX3CR1 [10]. In addition, approximately 20% of the dermal DCs comprise a langerin-positive population [10,18,19]. Unlike LCs, these DCs lack expression of E-cadherin (E324 or uvomorulin), EpCAM (CD326) and CD103. Finally, dermal DCs contain a third subpopulation expressing a distinct set of surface markers, CD11c$^+$ MHC class II$^+$ langerin$^-$CD103$^-$ CD11b$^-$, which migrates to draining regional LNs in inflamed settings [20].

Initial studies of DC migration in skin transplantation relied on tracking cells labeled with fluorescent agents [21]. When the labeling agent was painted onto the skin [22], this agent was retrieved in the draining LNs, either carried by migrating cells or in free form. Kripke *et al.* observed skin-cell migration in a contact hypersensitivity model. In this study, mice were transplanted with an allogeneic skin that had been painted with fuorescein isothiocyanate. Subsequently, antigen-presenting cells, capable of transferring an allogeneic MHC-restricted contact hypersensitivity reaction against fluorescein isothiocyanate antigens could be isolated from the recipient's draining LN [23]. Similarly, Hoefakker *et al.* showed that, following placement of human skin grafts on nude mice, HLA-DR$^+$ cells migrated from the graft into the draining LN of this recipient [24]. In another study, Richters *et al.* demonstrated that 4 days after skin transplantation in rats, cells bearing a marker for the leukocyte common antigen of donor origin, and with a dendritic morphology, were present in the T-cell area of the recipient's draining LNs [25,26]. Finally, studies by Larsen *et al.* demonstrated that skin DCs (both dermal DCs and LCs) emigrate rapidly out of skin explants placed in culture for 24 h [27–29], providing evidence that this migratory behavior is an intrinsic property of these cells. Based upon these observations, it was postulated that donor DCs migrating from the skin graft (referred to as 'passenger leukocytes') to the recipient's LNs played a key role in the presentation of donor antigens to recipient T cells and the initiation of an adaptive alloimmunity [30]. It is probable that DCs migrate out of skin allografts via lymphatic vessels. Seminal studies performed in the 1970s provided indirect evidence of the key contribution of lymphatic drainage in the initiation of an alloreactive T-cell response to, and subsequent rejection of, a skin allograft [31,32]. In these studies, lymphatic drainage of the graft was prevented by raising the skin graft off the recipient bed while preserving blood circulation through a pedicle. These grafts were not rejected by the host's immune system and survived as long as the pedicle was still intact. Altogether, these studies support the view that shortly following placement of skin allografts, donor DCs migrate out of the transplant via lymphatic vessels and infiltrate the recipient's draining LNs where they present alloantigens to recipient T cells (Figure 1).

Adaptive alloimmunity by T lymphocytes

Direct allorecognition by T cells

The process by which recipient T cells located in secondary lymphoid organs (draining LNs and spleen) interact with allogeneic MHC molecules, displayed on donor bone marrow-

derived MHC class II+ passenger leukocytes, is termed 'direct allorecognition' (Figure 2). It involves CD4+ and CD8+ T cells recognizing intact donor MHC class II and I molecules, respectively. This type of antigen recognition induces a vigorous polyclonal T-cell response engaging up to 10% of the entire peripheral T-cell repertoire. The exceptional strength of this type of immune response is refected in mixed lymphocyte reactions in which naive T cells become activated *in vitro* upon exposure to allogeneic stimulators. At first glance, such recognition of a foreign MHC molecule by a T lymphocyte appears to violate the dogma of self-MHC restriction of T cells. Some evidence has been provided suggesting that cross-reactivity of T cells specific for a self-MHC molecule with an allogeneic MHC molecule can provide an explanation for this paradigm. Indeed, it is clear that alloreactive T cells do not constitute a distinct population within the overall pool of T lymphocytes. Second, T cells utilize the same T-cell receptor (TCR) to recognize both nominal antigens and allogeneic MHC proteins. Based upon these principles, it has been proposed that direct allorecognition results from the cross-reactivity by T cells specific for a self-MHC molecule 'A' + peptide 'x' with an allogeneic MHC molecule 'B' + peptide 'y' [33]. This view is supported by numerous instances in which alloreactive T-cell clones with dual recognition for a nominal antigen have been described. Recent studies using alloreactive T-cell clones, exon shuffling and single-site mutations within MHC genes and crystallography have shown that two nonmutually exclusive mechanisms contribute to the high frequency of T cells engaged in direct allorecognition:

■ The high determinant density model, in which the structure of the allogeneic MHC molecule governs T-cell recognition [34];

■ The multiple binary complex model, in which alloantigen recognition is dictated by the peptides bound to allogeneic MHC molecules [35].

In the high determinant density model [34], TCR interaction is focused on exposed amino acid polymorphisms of the foreign MHC molecule, independently of the nature of the peptides bound to it. Each individual MHC molecule displayed on an allogeneic antigen-presenting cell (APC) could serve as a ligand for TCR binding, thereby providing a very high ligand density and activating a polyclonal T-cell response. The multiple binary complex model [35] is based upon the principle that allogeneic MHC molecules are loaded with a wide variety of peptides differing from those presented by self-MHCs. Therefore, a single allogeneic MHC molecule could stimulate a multitude of alloreactive T cells, each specific for a particular peptide–MHC complex. The prevalence of either high determinant density versus multiple binary complex Model in allorecognition is likely to depend upon the degree of heterogeneity (structural and/or conformational) between recipient and donor MHC molecules.

Indirect allorecognition by T cells

Seminal studies by Lechler and Batchelor showed that allosensitization could occur in the absence of donor-derived passenger leukocytes following retransplantation of kidney grafts in rats [36,37]. Based upon the assumption that donor parenchymal cells are not capable of sensitizing recipient alloreactive T cells, an alternative pathway of allorecognition was proposed as the trigger for host T-cell sensitization. In 1992, three independent studies performed in rodents and humans demonstrated that following allotrans-plantation, recipient APCs process and present donor MHC molecules in the form of peptides, thus eliciting a T-cell response by CD4+ T cells [38,39,40]. This phenomenon was referred to as the 'indirect allorecognition' pathway (Figure 2). Unlike the classical direct alloresponse, it was observed that the indirect alloresponse was oligoclonal in that it was mediated by a limited set of T-cell clones displaying discrete TCRs for antigens. Furthermore, in skin-transplanted mice, indirect alloresponses were invariably directed to a single or a few dominant determinants on donor MHC antigen. However, while different regions of MHC were found to be

immunogenic depending upon the donor/recipient combination, these alloimmunogenic determinants were always located in polymorphic regions of donor MHC molecules [41,42]. Similar restriction of indirect alloresponses by T cells to dominant determinants on human MHC HLA molecules has been reported [43–45]. Collectively, these studies show that while direct allorecognition is characterized by its polyclonality and polyspecificity, indirect allorecognition follows the rules of immuno dominance in that it is restricted to a few dominant determinants on donor MHC molecules.

Initial ELISPOT studies in skin transplant models have suggested the prevalence of MHC versus other antigens in indirect alloreactivity. However, low, but detectable, responses following incubation of recipient T cells with APCs plus third-party sonicates (MHC-mismatched to the donor) were detected. This suggested that non-MHC-derived peptides presented by recipient APCs could activate T cells. It is note-worthy that MHC-matched skin allotransplants are often acutely rejected. In fact, it is known that a variety of polymorphic proteins, referred to as minor histocompatibility antigens [46], can serve as a source of determinants for recognition by allospecific T cells following transplantation [47–49]. It is clear that these proteins may represent a source of antigens for exogenous processing by recipient APCs and subsequent presentation in MHC class II complexes to recipient CD4$^+$ T cells (i.e., in an indirect fashion). In a recent study, we measured the frequencies of indirectly activated T cells responding to MHC or minor antigens in mice transplanted with a fully allogeneic skin graft. We found that 50–60% of the indirect response was mediated by T cells recognizing peptides derived from donor MHC molecules, while the response to minor antigens accounted for 40–50% of the overall indirect alloresponse [50]. However, it is noteworthy that this feature of the immune response to skin allografts does not reflect a general phenomenon in transplantation since 100% of T cells responding to corneal allografts are directed to minor antigens while, after cardiac transplantation, virtually all T cells are activated in response to MHC antigens [50].

Indirect allorecognition by CD8$^+$ T cells

It has been assumed that the indirect allo-response is mediated exclusively by CD4$^+$ T cells recognizing donor peptides generated from exogenous processing by recipient APCs and presentation in the groove of self-MHC class II molecules. However, it is now well established that MHC class I processing regularly leads to presentation of extracellular antigens to CD8$^+$ T cells, a phenomenon termed 'cross-priming' [51–53]. This suggests that, following allotransplantation, some recipient CD8$^+$ alloreactive cells may become activated indirectly via recognition of donor-derived pep-tides presented by self-MHC class I molecules on recipient APCs. In fact, the initial observations supporting the existence of cross-priming were published in 1977 by Matzinger in a skin transplantation model [54]. This was later supported by Ivanyi's observations that a fraction of the alloreactive cytotoxic T lymphocyte (CTL) population, directed against class I HLA alloantigens generated *in vitro* during primary mixed lymphocyte reactions, is restricted to self-HLA MHC class I molecules [55–57]. Subsequently, studies by Popov *et al.* demonstrated that indirect presentation *in vivo* of a single MHC class I peptide was sufficient to trigger the activation and differentiation of functional donor-specific CD8$^+$ CTLs and rejection of MHC class I disparate skin allografts [58]. Further evidence of the actual contribution of indirect CD8$^+$ T-cell allorecognition to the overall alloresponse and graft rejection was provided in a recent study by Heeger and Valujskikh using a murine skin transplant model [59]. In this study, IFN-γ-producing CD8$^+$ T cells, activated in an indirect fashion were detectable in allograft-primed mice at a frequency of 50–100 per million cells, compared with 3000 per million for responses through the direct pathway, and were similar in frequency to indirectly primed CD4$^+$ T cells [59]. A subsequent study from the same scientists showed that these indirectly activated CD8$^+$ CTLs contributed to the rejection of skin allografts by attacking recipient

vasculoendothelial cells upon graft revascularization [60]. Taken together, these studies show that CD8^{+} T cells primed through the indirect pathway play a key role in the indirect T-cell alloresponse triggered after placement of allogeneic skin transplants and represent an essential element of the rejection process.

The initial indirect alloresponse, induced to donor MHC antigens after placement of skin allografts, is limited to a few dominant determinant peptides [41,44,45]. However, we and others have provided evidence indicating that at later time points after transplantation, new allodeterminants begin to be presented [62–65] – a phenomenon referred to as antigen spreading. This phenomenon had been previously described in chronic inflammatory autoimmune disease models including experimental autoimmune encephalitis (EAE) and Type 1 diabetes [66–68]. Similar to that observed in autoimmune models, the immune response diversifies to formerly cryptic peptides on the target antigens. These determinants comprise poorly processed peptides, which, despite their high affinity to self-MHC molecules, do not reach the threshold of presentation required to trigger a T-cell response. It is believed that, over the course of an inflammation process, secretion of proinflammatory cytokines such as TNF-α and IFN-γ can alter antigen processing and trigger or enhance the presentation of formerly cryptic determinants, thus perpetuating the T-cell response in autoimmunity and alloimmunity. Such spreading of indirect alloreactivity to new determinants on allogeneic MHC molecules has been observed in patients displaying chronic allograft vasculopathy [64,69]. It is probable that this phenomenon contributes to the induction and/or maintenance of a chronic form of transplant rejection [63,64].

Semidirect allorecognition by T cells

Along with the classical direct and indirect allorecognition pathways in transplantation, a third mechanism has been proposed, notably by Herrera and Lechler [70], that can blur the distinction between the two classical pathways and potentially add complexity to the model. This 'semidirect' pathway involves the phenomena of MHC molecules being transferred between donor and recipient APCs, which is also known as 'cross-dressing' (Figure 2) [71]. Unlike the indirect pathway, which involves donor MHC fragments processed and presented on host MHCs [72], the semidirect pathway involves entire MHC complexes, removed or released from donor tissue and used as antigen-presenting molecules by host APCs. As a result of this process, a single host APC could both present allopeptides via self-MHC II to CD4^{+} T helper cells (the indirect pathway) and directly stimulate cytotoxic CD8^{+} T cells by an acquired donor MHC I molecule.

This dual presentation from host DCs to CD4^{+} and CD8^{+} T cells, as a result of the semi-direct pathway, could provide a 'three-cell model' of T-cell activation, where the CD8^{+} cell is fully activated by both its contact with the APC's MHC class I:antigen complex and the CD4^{+} cell concurrently stimulated by the same APC. Jiang *et al.* argue that this three-cell model overcomes the difficulty of explaining how cytotoxic cells are partially activated by the donor APCs and then, in an unconnected manner, fully activated by CD4^{+} T cells that have been activated by the indirect pathway [73]. The three-cell interaction of DCs with CD4^{+} and CD8^{+} T cells has been shown to dramatically impact CD8^{+} T-cell function [74]; but this interaction is not indispensable for mounting a rejection response. Studies show that severe combined immunodeficiency (SCID) mice, reconstituted with either CD4^{+} T cells or CD8^{+} T cells, can still reject allogeneic skin transplants [75], and CD4^{+} knockout mice can reject fully allogeneic skin transplants (although they can accept minor antigen mismatched cardiac transplants) [76]. However, there is mounting evidence that CD4^{+} T-cell help is required for the establishment of a CTL memory population [77] and strong recall response [78,79]. The latter studies present strong evidence that a DC needs to be fully activated, or 'licensed,' by interaction with a CD4^{+} T cell, including CD40/CD40L costimulation, before it can initiate the differentiation of CD8^{+} T cells to memory CTLs. The semidirect pathway

provides a mechanism for a recipient DC to play a role in both the direct and indirect stimulation of CD4^{+} and CD8^{+} T cells and the creation of a CD8^{+} alloreactive memory effector T-cell population.

Evidence is gathering for the intercellular transfer of MHC molecules that form the basis of the semi-direct pathway of allorecognition. Extensive sharing of surface molecules, including MHCs, between T and B cells and their conjugate APCs has been demonstrated in the related study of trogocytosis [80]. The semidirect pathway involves sharing between two APCs. Herrera *et al.* demonstrated that DCs can acquire MHC molecules from allogeneic DCs or endothelial cells *in vitro*, and that the recipient DCs were able to activate T cells via the transferred MHC [70], with further *in vitro* evidence from the Lechler laboratory (King's College, London, UK) [81]. Moreover, the MHC transfer occurred between DCs separated by a semipermeable membrane, blocking any intact cell passage, although less efficiently than with cell-to-cell contact, implicating exosomes (discussed later) as a means of transfer. Most importantly, this MHC transfer also occurred *in vivo* in their mouse model. Transgenic DCs, lacking both MHC class I and II molecules, were able to acquire these molecules from the host. Similarly, Brown *et al.* demonstrated bidirectional *in vivo* sharing of MHC molecules between APCs [82]. Using fow cytometry, immunohistochemistry and confocal microscopy, they found cells displaying both donor and host MHC class II molecules after kidney and cardiac allografts. They further verify that these molecules are present on the cell surface, and that the allogeneic MHC molecules never move to the interior of the recipient cell, strongly suggesting that the surface expression of these molecules is not simply a preliminary step towards the endocytosis and processing of these molecules. Their investigation was limited to MHC class II molecules, but certainly does not rule out similar results for MHC class I. These studies clearly suggest a process wherein recipient T cells can encounter allo-MHC molecules on either donor APCs (the direct pathway), or recipient APCs, displaying intact, acquired donor MHC on their surface (the semidirect pathway).

One possible method for the transfer of MHC:antigen complexes between APCs is via exosomes, which are small (generally 50–100 nm) vesicles released by a number of cell types and capable of containing MHC molecules in their membranes. The addition of exosomes as MHC donors in the semidirect pathway could expand both the scope of the pathway and give us valuable tools to manipulate it. And there is strong evidence that exosome to DC transfer of MHC molecules occurs. In one study, the stimulation of antigen-specific CD8^{+} T cells by DCs lacking MHC class I was only possible when the cells were incubated with exosomes bearing MHC class I:antigen complexes [83]. This immunogenic potential of MHC:antigen bearing exosomes is being explored *in vivo*. The mechanism for exosome interaction with APCs is not yet clear; but MHC transfer has been shown to occur and to lead to both activation of T cells and tolerance induction *in vivo* [83]. Our growing understanding of the semidirect pathway and MHC transfer in transplantation suggests more tools for promoting transplant tolerance. The presence of self-MHC on recipient APCs displaying both intact and processed alloantigens may promote the activation of regulatory mechanisms associated with transplant tolerance. It is noteworthy that the treatment of transplant recipients by immature donor DCs has been a promising but elusive method of tolerance induction. Introduction of recipient immature DCs, loaded with donor-derived exosomes, could give the same positive results, without the potential dangers of the injected donor DCs maturing *in vivo* and inducing proinflammatory antidonor immunity and rejection.

Contribution of direct & indirect T-cell allorecognition pathways to allograft rejection

It is firmly established that CD4^{+} T cells, activated via direct allorecognition, provide help for the differentiation/expansion of functional antidonor CD8^{+} CTLs [84], which can mediate tissue injury and subsequent acute rejection of skin grafts. This has been proven in

recipient mice lacking MHC class II molecules (no indirect CD4^{+} T-cell recognition) and depleted of CD8^{+} T cells [85]. These mice acutely rejected allogeneic skin grafts, thus confirming rejection mediated exclusively via allorecognition by CD4^{+} T cells. In another study, Pietra *et al.* used BALB/c *Rag1*^{−/−} and SCID mice (devoid of both CD4^{+} and CD8^{+} T cells) as recipients of allografts from C57BL/6 and C57BL/6 MHC class II knockout donors. Reconstitution of hosts with either LN T cells or purified CD4^{+} T cells ensured acute rejection of wild-type, but not MHC class II knockout transplants [86]. By contrast, T cells depleted of the CD4^{+} subset did not elicit graft rejection. Therefore, in this model, direct CD4^{+} T-cell allorecognition was necessary and sufficient to mediate the rejection. On the other hand, there is plenty of evidence showing that adoptive transfer of CD8^{+} T cells, recognizing alloantigens in a direct manner in immunodeficient RAG knockout mice, is sufficient to ensure acute rejection of skin allografts. Taken together, these studies show that either CD4^{+} or CD8^{+} T cells activated through the direct allorecognition pathway can, on their own, mediate acute rejection of allogeneic skin transplants. This implies that depletion of donor passenger leukocytes that serve as APCs in direct allorecognition should promote graft survival. In fact, this was achieved in a mouse kidney transplant model by 'parking' the allograft in a first recipient to allow the migration of donor DCs out of the graft. These kidney grafts, now devoid of DCs, were then collected and transplanted into a second recipient, genetically identical to the first [36,37]. This manipulation resulted in long-term survival of the allograft. Injection of donor DCs to the recipients of these retransplanted kidneys led to rapid rejection. Furthermore, reconstitution of the intermediate recipient with donor-derived bone marrow induced mixed hematopoietic chimerism and resulted in prompt rejection of the allogeneic kidney transplant (presumably by replenishing DCs in the graft) [36]. Similar experiments performed in our laboratories in skin graft models led to different conclusions. Fully allogeneic grafts were cultured for 24 h in Petri dishes, a process that resulted in removal of dermal DCs from the graft. These allografts failed to induce a direct alloresponse when placed on an allogeneic host (as evidenced using an ELISPOT assay), but they were rejected at the same rate as normal skin allografts. This suggests that, unlike renal allotransplants, direct allorecognition is sufficient, but not necessary, to induce skin allograft rejection. Finally, it is important to note that while direct activation of naive allospecific T cells may require donor MHC presentation by so-called professional APCs (i.e., graft passenger leukocytes), recipient memory T cells may be activated by donor endothelial and epithelial cells expressing allogeneic MHC class II molecules as a consequence of local inflammation. This process may not be critical in laboratory mice displaying very few memory T cells at the time of transplantation (<5%). By contrast, nonhuman primates and humans display high frequencies of alloreactive memory T cells recognizing donor MHC molecules directly pretransplantation (>50%). These allospecific memory T cells are thought to derive from the priming of T cells by crossreactive microbial antigens [87–89], a phenomenon referred to as heterologous immunity [90,91]. These memory T cells may become activated following antigen presentation by nonprofessional APCs and play an important role in the initiation of direct alloresponses and in its perpetuation following elimination of *bona fide* APCs of donor origin. It is probable that the presence of such alloreactive memory T cells in primates explains their resistance to transplant tolerance protocols, which are traditionally effective in laboratory mice [92].

It is now well documented that T cells, activated through the indirect allorecognition pathway, provide help for the differentiation of antidonor CD8^{+} cytotoxic T cells [85,93,94], for the production of allospecific antibodies by B cells [95–97], and for delayed type hyper-sensitivity reactions [98]. The first evidence that indirect T-cell alloreactivity can influence the rejection process was obtained by Fangmann *et al.* who reported that immunization of recipients with allogeneic MHC peptides resulted in accelerated graft rejection [38,99]. Recent studies using genetically engineered mice and T-cell clones have allowed the study of indirect alloresponse in isolation and helped evaluate its potential role in the rejection

process. Seminal studies from Auchincloss' laboratory showed that skin transplants from MHC knockout donors, whose APCs lacking MHC expression cannot trigger a direct alloresponse, are acutely rejected. This suggested that the indirect pathway of allorecognition is sufficient, on its own, to cause rejection of an allogeneic skin transplant [85,93,94]. In another series of experiments, Valujskikh *et al.* generated a Th1 T-cell line from BALB/c recipient mice (H-2^d) of B10.A (H-2^a) skin transplant that was specific for a single immunodominant, self-restricted allogeneic MHC class II peptide, I-A^k β (58–71). When transferred into BALB/c SCID mice recipients of B10.A skin allografts, this cell line-mediated skin graft rejection, characterized by the presence of Th1 cytokines, macrophage infiltration and extensive fibrosis. Analysis of the rejected skin transplant showed only the presence of the peptide-specific CD4$^+$ T cells and no detectable direct pathway alloresponse [100,101].

B cells, antibodies & skin allograft rejection

The essential role of T lymphocytes in the process of skin graft rejection stemmed primarily from studies showing that these allografts were not rejected in animals lacking T cells. In addition, there is ample evidence indicating that adoptive transfer of purified T cells is sufficient to restore the rejection of skin allografts in immunodeficient rodents. The role of B cells in skin graft rejection is somewhat more intriguing and controversial. Initial studies performed in the 1960s and 1970s favored the view that production of antidonor antibodies by allospecific B cells is an essential element of the rejection process. However, later studies demonstrated that B cell-deficient mice acutely reject skin allografts in a manner indistinguishable from wild-type mice. It has now become clear that B cells contribute to the allograft rejection process either through alloantibody production or as an APC (Figure 3).

Seminal studies by Koene and others showed that passive transfer of alloantibodies alone generally failed to cause rejection of skin allografts in nude mice or other strains [102,103]. Actually, it was reported that transfer of donor-specific antibodies can promote allograft survival in certain conditions, a phenomenon referred to as 'enhancement mechanism' [104]. On the other hand, the adoptive transfer of B lymphocytes from sensitized mice caused accelerated rejection of grafts. In fact, established skin grafts were hyperacutely rejected in nude mice if the administration of specific alloantibody was combined with efficient species of complement [102,103]. Furthermore, later studies showed that in order to cause rejection, antiserum had to be administered after the vascular connection between the host bed and skin grafts were established, which typically occurs within 4–6 days after transplantation [105]. Interestingly, skin grafts became less sensitive to antibody-mediated rejection after prolonged residence on the recipients [106], usually several weeks following transplantation, which was presumably due to replacement of graft vessels with recipient endothelium [107]. It is commonly accepted that B-cell activation and alloantibody production requires signals provided by CD4$^+$ Th cells, activated through the indirect allorecognition pathway. This conclusion was drawn from studies showing that lack of MHC class II gene expression in recipients and, presumably, absence of indirect allorecognition leads to significant impairment for alloantibody production in skin-transplanted mice [108]. In the presence of recipient MHC class II molecules, indirectly activated T cells can provide help for memory B-cell generation, antibody class switching and affinity maturation through various cytokines and costimulatory molecules that recognize receptors expressed on B cells (Figure 3). Memory B cells may be able to contribute to allograft rejection through their ability to serve as APCs and directly activate allospecific T cells. Likewise, some recent studies have revealed the essential role of B cells in the differentiation, expansion and survival of alloreactive memory T cells following skin transplantation [109]. It was observed that alloantigen presentation by B cells promotes the proliferation of CD4$^+$ central memory T cells and the survival (via BCL2) of effector CD8$^+$ memory T cells [109].

NK cells, allorecognition & skin allograft rejection

Traditionally, NK cells represent an essential element of the innate immune system as they are developmentally programmed to kill targets, either directly or via antibody-dependent cellular cytotoxicity (ADCC), in the absence of antigen sensitization (Figure 3) [110,111]. Despite the fact that NK cells do not express germline-encoded antigen receptors, they can discriminate between cells of self- and nonself-origins. In fact, NK cells express clonotypic receptors specifically for self-MHC class I molecules [111]. Following recognition of autologous MHC class I molecules, engagement of a series of inhibitory receptors, including killer inhibitory receptors, prevents the development of an autoimmune process by NK cells [111–114]. Alternatively, lack of self-MHC class I interaction by NK cells leads to NK-cell activation, cytokine release and cytotoxicity [115–117], a phenomenon referred to as 'missing self' [118,119]. Likewise, NK cells are traditionally associated with the destruction of virally infected cells and tumor cells [110,120–122]. In addition, some evidence has been provided showing that NK cells can recognize and destroy allogeneic cells and tissues lacking self-MHC class I molecule expression such as skin grafts from β2 microglobulin knockout mice and semiallogeneic F1 donors [123,124]. By contrast, it has been reported that NK cells contribute to induction and maintenance of tolerance. NK cells could favor allograft survival by eliminating donor professional APCs [125,126] and by secreting immunoregulatory IL-10 cytokines and the activation of anti-inflammatory Th2 and Treg cells [127]. In addition, a recent report indicates that NK cells can promote tolerance of allogeneic transplants by downregulating the homeostatic proliferation of CD8^{+} memory T cells [128]. Therefore, contrary to previous beliefs, NK cells appear to play an essential role in the initiation and regulation of alloimmune responses and in the rejection or tolerance of allografts. The mechanisms underlying the impact of NK cells on APC functions and on T-cell activation are poorly understood. However, it is clear that the dual nature of the 'NK-cell effect' may vary depending upon the type of NK cell subset activated and engagement of different inhibitory or stimulatory receptors on NK cells.

Conclusion

In summary, placement of allogeneic skin allografts is associated with a potent immune response involving both innate and adaptive arms of the host's immune system. T cells activated via direct, indirect and, presumably, semidirect pathways, as well as B cells and NK cells, can ensure the rejection process via direct killing of donor cells or via the production of proinflammatory cytokines. Many attempts to induce tolerance to allogeneic skin transplants (indefinite survival in the absence of chronic immunosuppression) have been unsuccessful, probably owing to the exceptional potency and diversity of the alloimmune response evoked by these transplants. So far, induction of mixed chimerism following donor bone marrow transplantation is the most reliable method for achieving tolerance to fully allogeneic skin grafts [129–132]. However, this method relies on stable mixed chimerism associated with deletion of alloreactive T cells in the host's thymus, a process that has been achieved only in l aboratory rodents.

Future perspective

Recent development of new tolerance protocols, including mixed hematopoietic chimer-ism and T-cell costimulation blockade, have proven to be effective in achieving tolerance to fully allogeneic skin allografts in laboratory mice. However, these strategies have consistently failed to significantly prolong skin allograft survival in primates. A number of studies indicate that, unlike laboratory mice, primates display large numbers of alloreactive T cells prior to transplantation. These memory T cells have been shown to prevent tolerance induction to alloantigens. Therefore, successful tolerance induction to skin allografts will

require elimination or inactivation of such alloreactive memory T cells in patients. On the other hand, there is increasing evidence suggesting that lack of vascularization of skin allografts at the time of their placement is responsible for increased immunogenicity of these grafts by favoring skin DC trafficking via lymphatic vessels and preventing some regulatory immunity associated with antigen delivery via blood vessels. Based on this, it is probable that vascularization of skin allografts at the time of transplantation, along with blockade of lymphangiogenesis, will promote tolerance induction to skin allografts using strategies that have proven to be successful for primarily vascularized solid organ transplants. Work is in progress in our, and other, laboratories to explore these new directions in skin transplantation.

Acknowledgments

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Executive summary

- Following skin transplantation, cutaneous dendritic cells, including Langerhans cells and dermal dendritic cells, migrate to the recipient's draining lymph nodes where they present donor antigens to alloreactive T cells.

- Recipient T cells recognize donor antigens via two distinct pathways: the direct pathway (intact donor MHC molecules displayed on donor antigen-presenting cells [APCs]) and the indirect pathway (donor peptides bound to self-MHC molecules on recipient APCs).

- Both direct and indirect alloresponses by T cells are sufficient, on their own, to induce donor-specific T-cell-mediated cytotoxic responses and trigger acute rejection of an allogeneic skin graft.

- The indirect allorecognition pathway is required for B-cell activation and alloantibody production and it is presumably the driving force behind chronic allograft rejection.

- There is accumulating evidence for the existence of a third allorecognition pathway, the semidirect pathway involving T-cell recognition of donor MHCs transferred on recipient APCs. The contribution of this mechanism to skin graft rejection is still unknown.

- B cells contribute to the rejection of skin allografts by presenting donor antigens to T cells, via opsonizing donor cells (through binding of alloantibodies) and via direct complement-dependent, antibody-mediated cytotoxicity.

- B cells play a critical role in skin allograft rejection by promoting the differentiation, expansion and survival of donor-specific memory T cells.

- NK cells contribute to the rejection of skin allografts by directly killing donor cells (via detection of missing self-MHC class I molecules) or indirectly through antibody-dependent cellular cytotoxicity.

- The potency and diversity of the innate and adaptive immune responses to allogeneic skin grafts is associated with resistance to tolerance induction.

- Future tolerance strategies in skin transplantation will rely on primary vascularization of skin patches (to reduce their immunogenicity) and on inactivation of alloreactive memory T cells (in primates), which prevent the development of regulatory immunity.
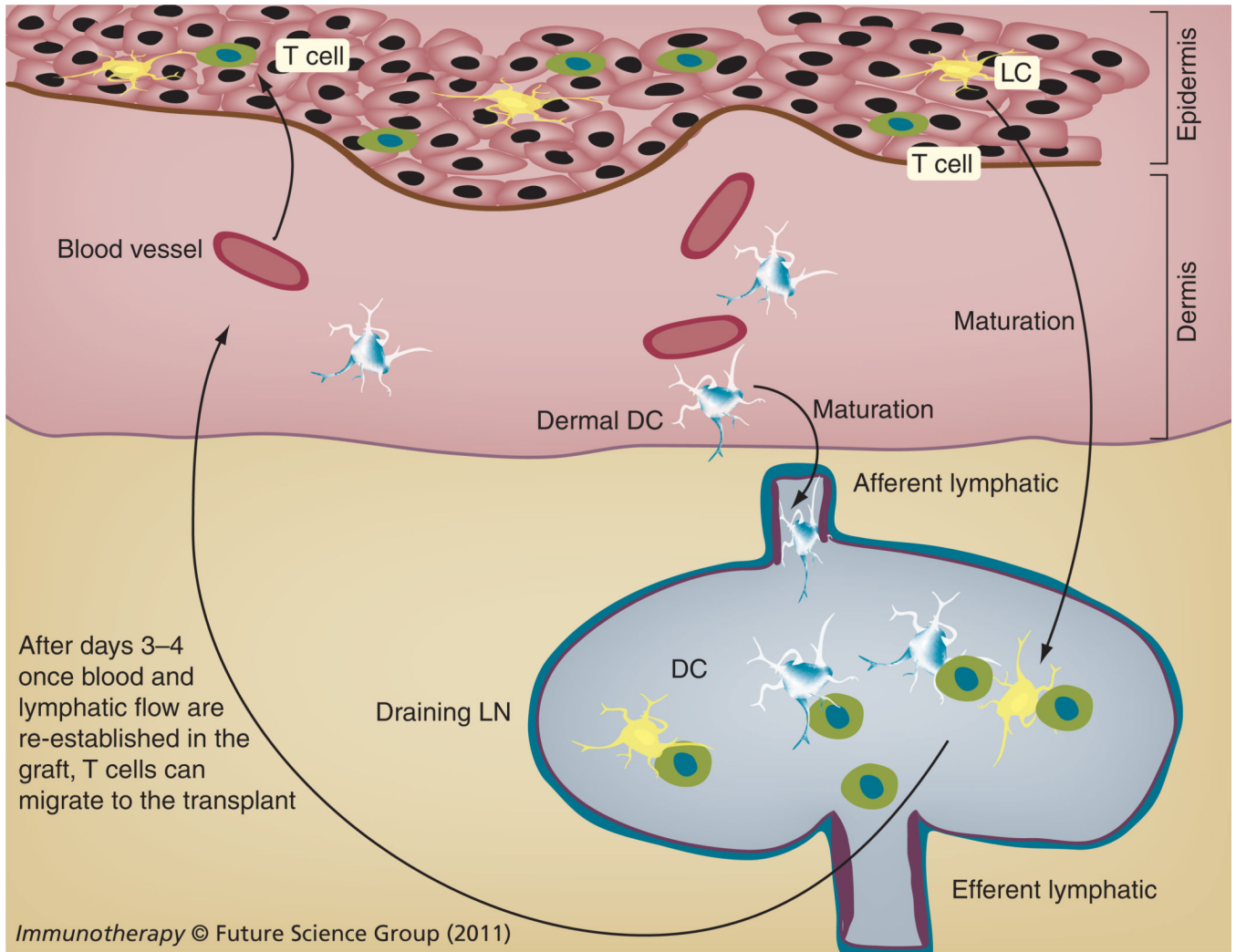
Figure 1. Antigen-presenting cell and T-cell trafficking following skin transplantation
At 3–4 days following placement of skin allografts, when the blood and lymphatic circulation is restored, graft APCs (bone marrow-derived MHC class II+ cells) migrate to the draining lymph nodes of the recipient where they can activate T cells through the direct pathway. Once activated, these T cells can now migrate to the site of the graft through the blood vasculature and participate in graft rejection.
APC: Antigen-presenting cell; DC: Dendritic cell; LC: Langerhans cell; LN: Lymph node.
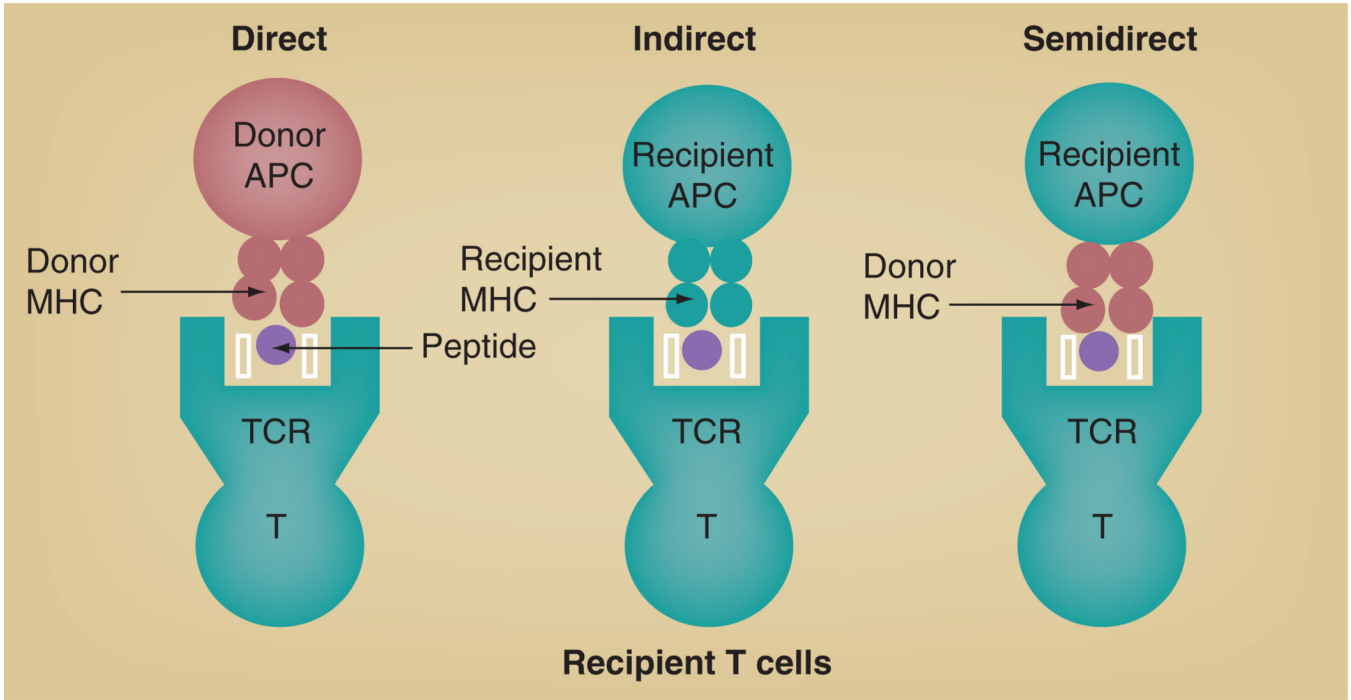
Figure 2. Pathways of T-cell allorecognition

Following skin transplantation, recipient alloreactive T cells can be activated to donor antigens presented via three distinct pathways: the direct pathway, in which T cells recognize intact allo-MHC molecules on donor APCs; the indirect pathway, in which recipient T cells recognize processed donor antigens (MHC and minor antigen peptides) presented by self-MHC molecules on host APCs; and the semidirect pathway, in which recipient T cells can be activated by acquired donor MHC molecules displayed on recipient APCs.

APC: Antigen-presenting cell; TCR: T-cell receptor.
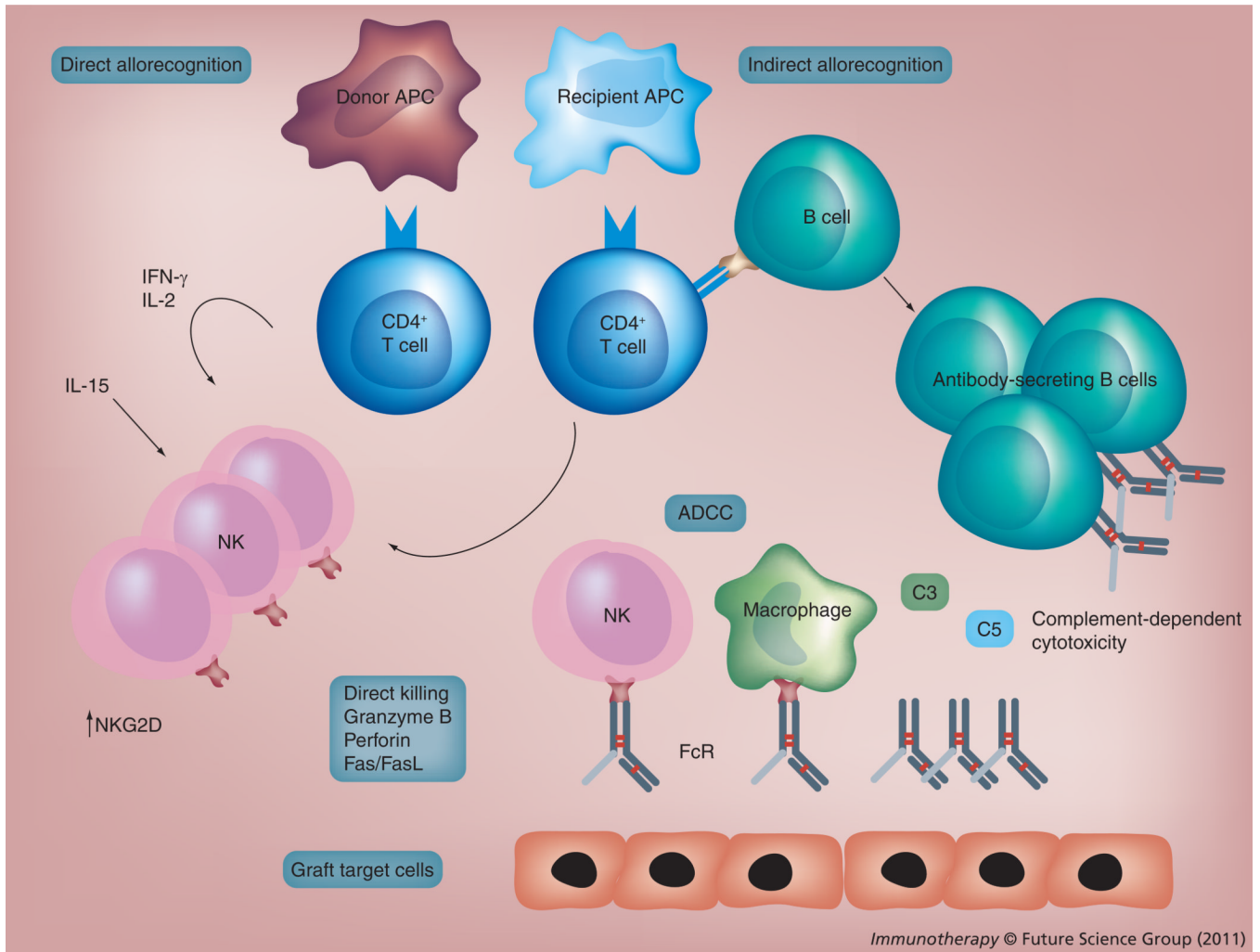
Figure 3. Alloresponses mediated by B cells and NK cells after skin transplantation
Following skin transplantation, B cells and NK cells can contribute to alloimmunity and allograft rejection via several mechanisms. Antidonor antibodies can directly kill donor cells via complement-dependent cytotoxicity or opsonize allogeneic cells (i.e., target cells for ADCC). NK cells can kill donor cells lacking expression of self-MHC class I molecules (missing self), or can contribute to DTH reactions and activation of cytolytic cells by producing proinfammatory cytokines including IFN-γ and TNF-α.
ADCC: Antibody-dependent cell-mediated cytotoxicity; APC: Antigen-presenting cell; DTH: Delayed type hypersensitivity.