

Postischemic Reperfusion Injury to Allografts – A Case for ‘Innate Immunity’?

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Key Words

Ischemia-reperfusion · Injury of allografts · Innate immunity · Reactive oxygen species · Induction of allotolerance

Introduction

There is accumulating clinical evidence suggesting that it is the degree of injury to an allograft rather than the degree of foreignness that determines or at least contributes to its short-term and long-term outcome. Reactive oxygen species (ROS)-mediated postischemic reperfusion injury – amongst different kinds of damages – appears to play a major role in injury-induced regulation of allograft outcome. Early evidence for this assumption arose from a prospective clinical trial performed at our institution in 1994 in which we could show that the free radical scavenger superoxide dismutase (SOD) – given during surgery – leads to a statistically significant reduction in incidences of acute and chronic rejection events in CsA-treated, kidney-transplanted patients [1]. This clinical observation of a beneficial effect of SOD on early and late allograft outcome provided an indirect proof for the assumption that the generation of superoxide radicals during allograft reperfusion had a significant impact on allograft survival.

In view of lack of any experimental and clinical data on the basis of which we could explain these early clinical findings, we – in close collaboration with Konrad Messmer and his group – raised a working hypothesis by discussing the possibility that it is the ROS-mediated reperfusion injury that leads to clinical events of alloimmunity (such as incidences of acute rejection episodes and acute irreversible rejection) and that, in regard to the underlying basic mechanism, it is this primary unspecific injury in terms of a key event that results in an initiation of T-cell alloactivation via activation of antigen-presenting cells [1, 2]. Subsequently, our hypothetical interpretation of the clinical findings was extended by discussing the possibility that the reperfusion injury-associated, ROS-induced inflammatory milieu of the allograft, associated with an upregulated cytokine and chemokine response, initiates activation of sentinel dendritic cells leading to upregulation of (a) MHC-molecules, (b) costimulatory molecules and (c) adhesion molecules, enabling these cells to interact with T-cells, and by this inducing an efficient T-cell alloactivation [3–5].

In regard to recent reports on new exciting findings from research in the field of innate immunity – which is precedent to adaptive immunity – it appears now possible to refine and extend our theory again.

In fact and surprisingly enough, many of those novel data obtained in the field of innate immunity – now defined as a host defence system against microbial patho-

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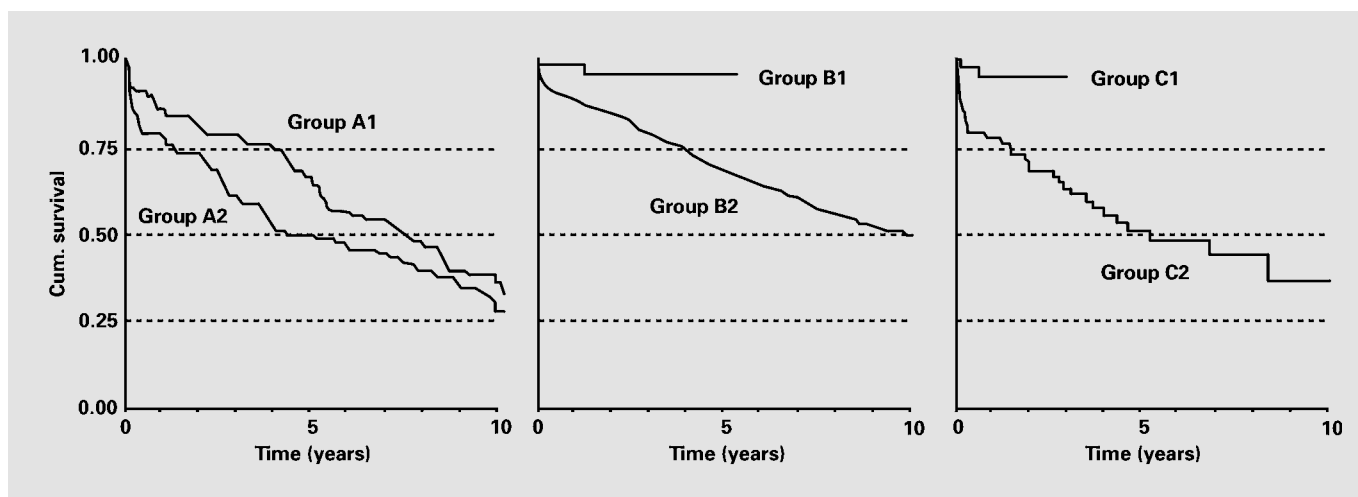


Fig. 1. Three clinical examples of injury-determined allograft outcome: Group A1/Group A2: SOD trial: 5-year results in SOD-treated patients (A1) compared to placebo-treated patients (A2). Group B1/Group B2: 5-year allograft survival in recipients of non-HLA-matched kidneys from unrelated living donors (B1) compared to historical controls (B2) of post-mortem removed kidney transplants

allocated on the basis of a good HLA match via Eurotransplant. Group C1/Group C2: 'Senior-Kidney transplant programme': 5-year results after allogeneic renal transplantation in elderly recipients of organs from elderly cadaveric donors, compared to data from the CTS study.

gens – fit very nicely in our concept that the ROS-mediated reperfusion injury to allografts creates a milieu of inflammation that operates as a danger signal and, in analogy to the infectious-agents-induced inflammation, may, thus, initiate a state of innate immunity.

In essence, the establishment of an alloimmune response to an allograft is primarily due to the inability of the innate immune system to discriminate between an inflammatory milieu of host tissue caused by pathogens on one hand, and an inflammatory state in a transplanted foreign organ induced by free-radical-mediated reperfusion injury to the allograft on the other hand. The situation is even more complicated by the fact that in the allografting setting, the innate immune response of both the donor and the recipient may become involved by flowing – as a link – in a subsequent adaptive immune response of the recipient.

By adding the new findings from research in the field of innate immunity to our reperfusion injury theory, the whole concept becomes more valid and may provide a rational approach to new strategies of immunosuppression and tolerance induction by tackling primarily the events occurring during 'innate alloimmunity' than during adaptive alloimmunity – as we are doing now.

In the following chapters, I am going to summarize some of those new findings from the literature and to try to put them in a logical and plausible way into the puzzle

posed by our early clinical observation that treatment of a primary unspecific injury to an allograft results in a remarkable reduction of subsequent specific alloimmune events.

The Degree of the ROS-Mediated Injury to an Allograft Rather than the Degree of Its Foreignness Contributes to Early and Late Graft Outcome

Clinical Examples Observed at Our Institution

Apart from the data obtained from our SOD trial [1], there are two more clinical situations that provide additional evidence suggesting that it is the degree of ROS-mediated injury to an renal allograft rather than its degree of foreignness that determines its short-term and long-term outcome: the superior results observed (a) in unrelated living donor kidney transplantation – a situation in which the degree of postischemic reperfusion injury is believed to be minimal; and (b) in kidney transplantation in elderly recipients of an elderly cadaveric renal allograft by using a treatment protocol that minimized ROS-mediated allograft injury.

Therefore, data from these two other clinical trials in this respect performed at our institution are briefly mentioned in this context as well (fig. 1). In the SOD trial

short-term and long-term results could be improved by mitigation of the reperfusion injury to the allograft with a free radical scavenger whereas a multivariate analysis of the data from this study showed that the degree of MHC/HLA incompatibilities had no significant influence on graft survival [6].

In a series of 63 unrelated living donor kidney transplantations in HLA-mismatched recipients we observed a 5-year graft survival rate of 80.8% [7]. When censoring for patient's death and technical failure, this rate was 96% compared to 70% in a series of 1,136 historical controls who had received well-matched cadaveric renal allografts allocated on the basis of a good HLA match, however, associated with long cold ischemia times.

It is well known that transplantation of kidneys from cadaveric elderly donors into elderly recipients is associated with poor short-term and long-term results. We suggested that an increased susceptibility of old organs to the attack of ROS may be responsible for these inferior results (a theory which could recently be confirmed experimentally [8]). Thus, we tried to keep the ROS-mediated injury to this category of allograft as small as possible by trying (1) to avoid application of Ciclosporin, known experimentally to contribute to the generation of reactive oxygen species [9]; (2) accepting kidneys with short cold ischemia times only in order to provide no increased postischemic reperfusion injury; and (3) by adding to the immunosuppressive induction regimen (MMF, steroids, ATG) a course of antioxidative treatment (Vitamin C, Vitamin E, acetyl cystein). As already published [10], the 3-year allograft survival rate in this prospective pilot trial was excellent and superior compared to the corresponding data from the literature.

Generation of Reactive Oxygen Species during Postischemic Reperfusion Injury of Human Cadaveric Renal Allografts

The generation of reactive oxygen species during postischemic reperfusion injury to allografts has been demonstrated in a large variety of experimental studies and in clinical studies as well [3]. We were able to measure the generation of ROS during reperfusion of human cadaveric renal allografts indirectly using malondialdehyde (MDA) as a marker. In fact, we found an increase of MDA-TBA adducts in the venous blood taken from the vein of reperfused renal allografts [11]. Because the MDA-TBA method is known to be associated with some methodologic weakness, we tried to apply methods for

direct demonstration of ROS during allograft reperfusion.

Thus recently in collaboration with Messmer and co-workers, Institute of Surgical Research, we were able to measure ROS directly during reperfusion of human cadaveric renal allografts. Using the EPR spectroscopy technique, hydroxyl radicals could be measured in the transplant vein appearing already 1 min after reperfusion and showing a peak in concentration at 5 min and 15 min (fig. 2a, b).

This very early demonstration of hydroxyl radicals immediately after onset of reperfusion – indicating the presence of superoxide and hydrogen peroxide as well – obviously represent the primary danger signal by inducing a secondarily-running cascade of events associated with any kind of oxidative stress (see below).

The Innate Immune System, Toll-Like Receptors and Their Ligands

Alloimmunity has been interpreted for a long period of time until recently in terms of a primary adaptive immunity exclusively – the basic paradigm of an adaptive immune response, clonal selection, defined in terms of a specific immune response of the recipient against the foreign donor MHC-antigens of the allograft.

In contrast, innate immunity has been considered over a long period of time as a separate entity from the adaptive immune response and has been regarded to be of secondary relevance in the hierarchy of immune functions. Recently, however, new insights in mechanisms of the first line of defence against infection (and thus, against an infection-associated inflammatory milieu) have been achieved and have again focussed on the biological phenomenon of innate immunity. In principle, modern assumptions in immunology say that the functioning of the immune system is based on two distinct recognition systems: the innate and the adaptive. The innate immunity interacts with and may lead to the second line of defence represented by the adaptive immunity. Similarities and differences in these two types of host response to infection have been reviewed recently [12, 13].

Innate immunity provides a host defence against infection and, thus, has to possess the ability to recognize pathogens, in other words to react with recognition molecules on pathogens = 'pathogen-associated molecular pattern' = PAMPs [12, 13].

The question, however, is whether or not – apart from those PAMPs on microbes – putative endogenous ligands,

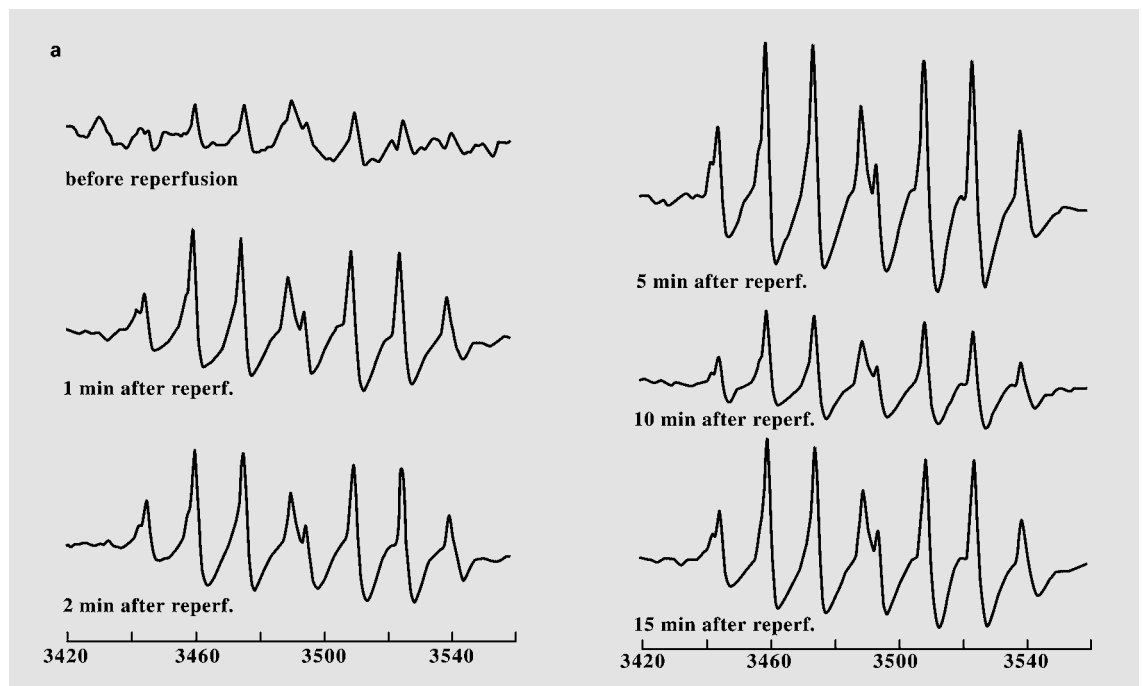
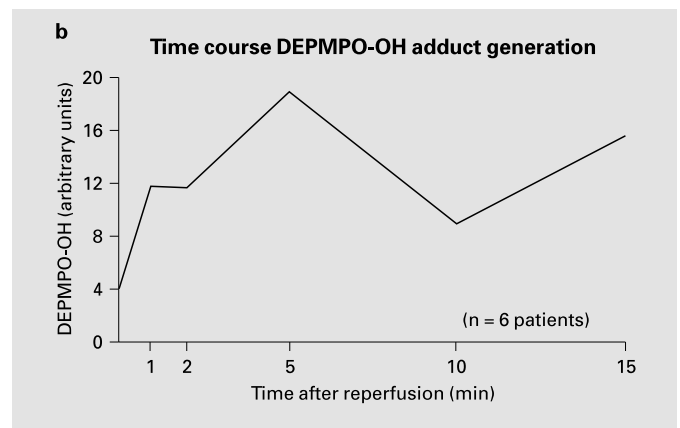


Fig. 2. a Electron paramagnetic resonance spectroscopy measurement of hydroxyl radicals in the renal allograft vein during post-ischemic reperfusion. As spin trap DEPMPO-OH was used. The typical signals of 1:2:2.1:2:2.1 characteristic for hydroxyl radicals are shown. **b** Electron paramagnetic resonance spectroscopy measurement of hydroxyl radicals following reperfusion of human cadaveric renal allografts: two peaks of hydroxyl radical generation, at 5 min and at 15 min, were observed (from J. Fertmann et al., in preparation).



e.g. on damaged host cells, may play a role in this scenario – similar to the *Drosophila* endogenous ligand ‘Spätzle’ that activates the receptors in cells of the fly in response to infection [14].

Indeed, recent studies have suggested that chaperones like autologous heat shock protein 60 (hsp60) operate as such an endogenous ligand and may serve as a danger signal to the innate immune system. Mouse or human macrophages, as well human vascular cells and PBMC, through CD14 signaling and p38 mitogen-activated protein kinase, were found to elicit a proinflammatory response when incubated with recombinant human hsp60 [15–17]. The response included the up-regulation of adhe-

sion molecule expression and the release of inflammatory mediators such as IL-6 and TNF- α . In addition, human hsp60 induced gene expression of IL-12 and IL-15.

Moreover and interestingly, microbial hsp60/65 also induces a proinflammatory response in innate immune cells [18, 19].

On the other hand, host organisms have developed a set of non-clonal receptors which can recognize PAMPs and, therefore, the presence of pathogens. These receptors have a broad specificity because they can recognize a number of different ligands, as long as the ligands share a common molecular pattern = ‘pattern recognition receptors’ = PRRs [12, 13]). These receptors which belong to

the IL-1 receptor family are known as 'mammalian Toll-like receptors' (TLR) according to the homologous receptor of the *Drosophila* Toll protein. So far, more than twelve TLRs have been published, about twenty appear to exist, TLR 4 and TLR 2 seem to play a major role in the defence against infection.

Toll-like receptors are strategically expressed on cells that are the first to encounter pathogens during infection, such as surface epithelia, and also on all types of effector cells of the innate immune system, such as dendritic cells as antigen-presenting cells and macrophages [20, 21]. The mechanism of Toll-like receptor activation is not quite clear: as already mentioned above, the question is whether or not there is a direct interaction between Toll-like receptors and their putative ligands on microbes, or there are endogenous ligands on autologous damaged cells that activate the receptors in response, e.g. to infection.

In regards to the reperfusion injury hypothesis, it is of interest that a recent study could show that the human heat shock protein 60 (hsp60) chaperokine operates as an endogenous TLR4 complex ligand suggesting that it acts as a danger antigen to the innate immune response [22].

In addition, it could be further shown that activation by LPS of Toll-like receptors on human immature dendritic cells plays an important role in activation and maturation of this category of professional antigen-presenting cells linking innate immunity to adaptive immunity [23].

Again of interest in the light of the reperfusion injury hypothesis are recent studies suggesting that Toll-like receptors share common downstream signalling molecules of the Rel/NF- κ B pathway. In fact, activation of NF- κ B occurs downstream of a number of receptors including TNF receptors, CD40, the IL-1 receptor and Toll-like receptors [20, 24, 25]. In addition, there is further evidence suggesting that TLR 4 activates not only NF- κ B but also AP-1 and JNK [20, 26] = transcription factors involved in oxidative stress situations.

In addition, in mammals, engagement of TLR4 and the IL-1 receptor leads to the sequential activation of the adapter protein My88, the IL-1 receptor-associated kinase (IRAK), TNF receptor-associated factor-6 (ZTRAF-6) and eventually the I κ B kinase complex [27].

Of further interest in this context are recent data on signal transduction processes by which injury-induced cell necrosis may lead to activation of genes involved in inflammation.

Principally, any tissue damage induced by injury or infection can result in necrosis, a mode of cell death characterized by induction of an inflammatory response. It has been shown [28] that necrotic cells, but not apoptotic

cells, activate NF- κ B and induce expression of genes involved in inflammatory and tissue-repair responses, including neutrophil-specific chemokine genes KC and macrophage inflammatory protein-2, in viable fibroblasts and macrophages. Interestingly, NF- κ B activation by necrotic cells was dependent on Toll-like receptor 2, the signaling pathway that induces inflammation in response to microbial agents. These results have identified a novel mechanism by which cell necrosis, but not apoptosis, can induce expression of genes involved in inflammation and tissue repair responses. Furthermore, these results also demonstrate that the NF- κ B/Toll-like receptor 2 pathway can be activated by exogenous microbial agents and endogenous inflammatory stimuli.

Such observations are of extreme importance for the assumption that ROS-mediated postischemic reperfusion injury, which is known to induce an inflammatory milieu, may act as an endogenous stimulus to activate Toll-like receptor-bearing cells such as dendritic cells as will be discussed here.

Possible Scenario of a 'Double' Innate Immune Response ('Innate Alloimmune Response') Initiated by Postischemic Reperfusion Injury to an Allograft Leading to an Adaptive Alloimmune Response

Recent findings from research in the field of innate immunity in terms of a host defence against infection may let suggest an involvement of the innate immune system as the first step in the initiation of the adaptive alloimmune response of the recipient against an allograft: an activation of (donor-derived) cells of the donor's innate immunity within the allograft inflamed during postischemic reperfusion injury – as well as activation of recipient-derived cells of the recipient's innate immune system entering the allograft – in terms of a transplant recipient's defence against the introduced alloantigen of the donor organ. The underlying principle would be that the innate immune system – regardless in this situation whether it is of donor origin or of recipient origin – is not able to differentiate between an inflammation of a tissue mediated by pathogens and an inflammation of a donor organ mediated by reactive oxygen species. The scenario which may start from initial unspecific reperfusion injury to an allograft and result in an adaptive alloimmune response is illustrated in figure 3: The scenario is unique as we are dealing here with the existence and activation of two innate immune systems resulting in one adaptive immune

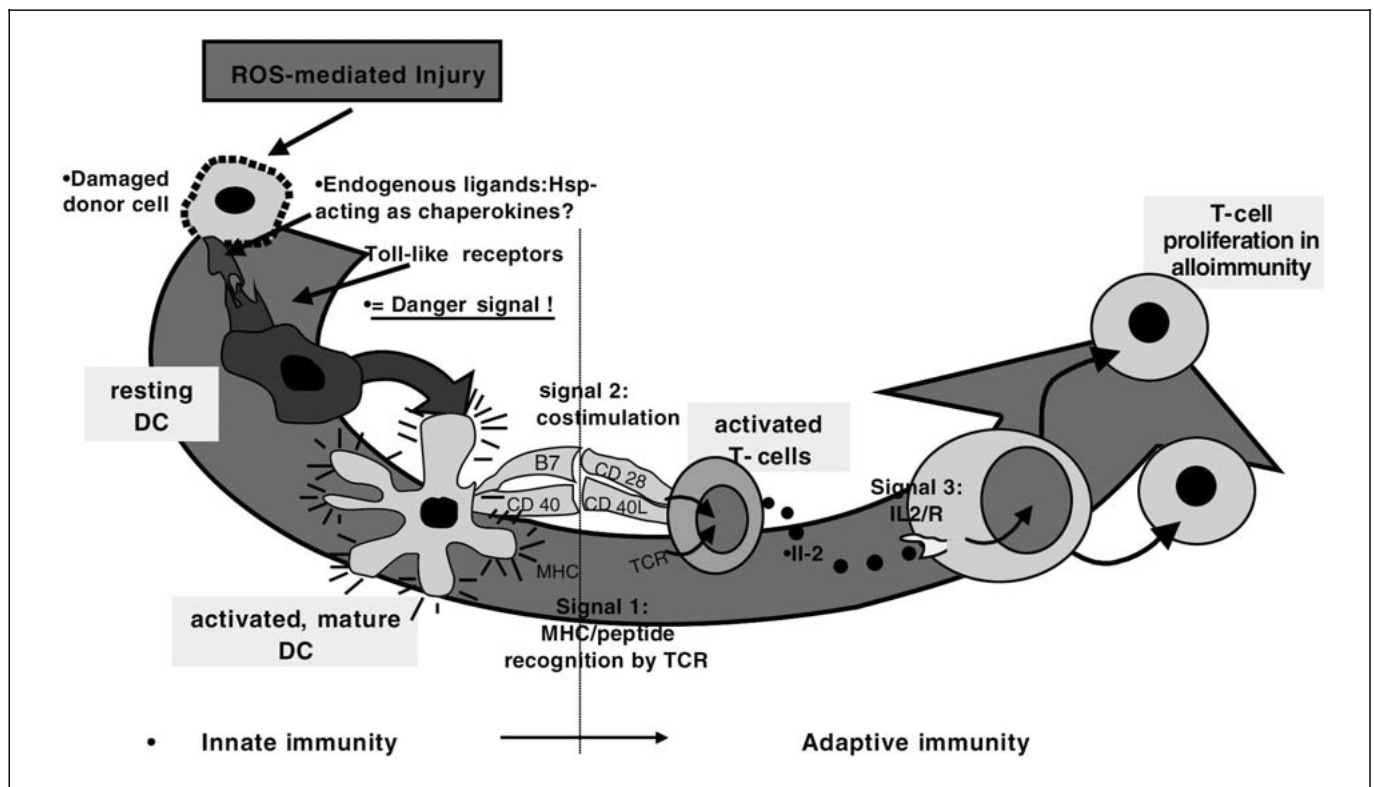


Fig. 3. Working hypotheses: initiation of adaptive alloimmunity by reperfusion injury to allografts via initiation of innate (allo)immunity (see text).

response: the innate immunity of the organ donor – represented by the existence of donor-derived dendritic cells – leading to the direct pathway of alloactivation by interacting with recipient's T-cells; and the innate immunity of the recipient – represented by graft-invading, recipient-derived dendritic cells – leading to the indirect pathway of alloactivation.

We may discuss the possibility that the scenario starts with the generation of reactive oxygen species e.g. in endothelial cells of donor organ vessel walls which has been shown to occur within the first minutes after reperfusion of an allograft (fig. 2). This early event is followed by secondary activation of monocytes and macrophages which, in turn, are stimulated to generate ROS (well-known phenomenon of a vicious circle of ROS generation). As a consequence, the generation of ROS leads to damage of the donor organ.

The next question is whether or not this injury (oxidative stress) does lead to induction of endogenous ligands on damaged allogeneic cells that may interact with and activate Toll-like receptors in response to inflamed tissue

due to this free-radical-mediated injury. Studies on endogenous ligands for Toll-like receptors have only started to emerge. As mentioned already, a recent study implicated the hsp60 chaperone as an endogenous TLR 4 ligand. In fact, hsp60 is normally sequestered in the cell interior, but is rapidly translocated to the membrane in response to any cell stress.

Indeed, numerous experiments in various models have shown that oxidative stress is able to induce heat shock proteins [29–34]. The ability of oxidative stress to damage proteins could account for its ability to induce some of those stress-induced proteins. In terms of an analogous interpretation, we would assume that these proteins (acting and functioning as chaperones) may be induced during oxidative stress in the course of reperfusion of an allograft.

ROS-induced heat shock proteins on damaged cells (or other molecules arising during injury) in terms of putative endogenous ligands may activate intragraft donor-derived and recipient-derived dendritic cells via activation of Toll-like receptors on their cell surface. This assumption can be tentatively raised under the aspect that signalling pathways

known to occur after TLR-activation (NF- κ B/Toll-like receptor pathway), leading to events of inflammation [20, 28], are induced by ROS as well. Indeed, signalling pathways downstream Toll-like receptors have remarkable similarities with ROS-induced signalling pathways.

Thus, considerable evidence has accumulated supporting a role for ROS as activators of signalling pathways and transcription factors such as NF κ B [34–36] and AP-1 [37] known to regulate and control acute inflammatory and immune events via activation of early response genes. Recent knowledge suggests that extracellular ROS may affect specific redox-sensitive signalling proteins at or near the cell membrane which will then initiate signalling pathways, whereas intracellular ROS may operate and act as second messengers for a variety of other signalling processes of the same type [38, 39]. Once donor-derived and recipient-derived antigen-presenting dendritic cells are activated and mature, they are supposed to interact with recipient's T-cells and – as a linking bridge – to induce, initiate and propagate the recipient's adaptive alloimmune response via subsequent events of alloactivation.

As already discussed by us [4, 5], postischemic reperfusion injury to an allograft has been shown to upregulate the expression of molecules on all three molecular levels of the dendritic cell surface involved in the interaction with a T-cell that leads to its activation: MHC class I and II molecules, costimulatory molecules, and adhesion molecules.

Altogether, the primary unspecific injury to the foreign donor organ induces events of the two immunological defence systems: (1) a more broadly-directed innate immune host defence (evolutionary normally directed against infection) resulting in (2) a definite adaptive immune host defence that leads ultimately to a specific injury to the allograft.

Avoidance of Reperfusion Injury to an Allograft: An Absolute Requirement for Successful Induction of Allotolerance?

We speculated already earlier that successful induction of allotolerance is impossible as long as allografts are injured during the first minutes of reperfusion after implantation in the recipient. These assumptions were based on early suggestions that professional antigen-presenting cells that react with naive T-cells only by signal 1 and not by signal 2 or costimulatory signal (elicited by a danger signal) induce and maintain an unresponsive state of tolerance [40]. Postischemic reperfusion injury to an allo-

graft, however, via establishment of an inflammatory milieu, contributes to activation and maturation of intra-graft dendritic cells and, thus, to upregulation of costimulatory molecule expression providing signal 2; the result is robust T-cell alloactivation, clonal expansion and alloimmunity [5].

Avoidance of postischemic reperfusion injury would imply a priori absence of an inflammatory state of the allograft and, thus, uptake and presentation of allogeneic antigens would be provided by immature and non-activated intragraft dendritic cells (e.g. by absence of an interaction between endogenous ligands on damaged cells with Toll-like receptors of DCs). These APCs, then, fail to support robust T-cell activation, proliferation, and survival of naive T-cells, and instead tend to induce clonal anergy and deletion of T-cells.

In addition, recent data suggest a role of apoptotic processes not only in the induction but also in the maintenance of tolerance by favouring the development of an active immunoregulatory state [41].

Anergic and apoptotic T cells generated during the process of tolerance induction actively oppose and inhibit further APCs maturation/activation, which reinforces the tolerant state. Therefore, the induction of apoptotic T-cell deletion and anergy by immature dendritic cells may represent a negative feedback loop, perpetuating a tolerant state by opposing the maturation of new dendritic cell precursors [41–49]. This scenario is illustrated in figure 4a, b.

Outlook

The concept of 'innate alloimmunity' has to be followed and investigated further, not only on behalf of the reperfused allograft in the recipient but also on behalf of the brain-dead donor. Research should include a search for the generation of reactive oxygen species, expression of heat shock proteins on donor cells and of Toll-like receptors on dendritic cells.

In case of confirmation of the concept, which may continue to gain credence, future targets of immunosuppression should then include methods to prevent injury to the donor organ and its consequences, e.g. by using antioxidants. In addition, such strategies to suppress events during innate immunity rather than events during adaptive immunity should include attempts to develop monoclonal antibodies against Toll-like receptors and/or heat shock proteins, etc.

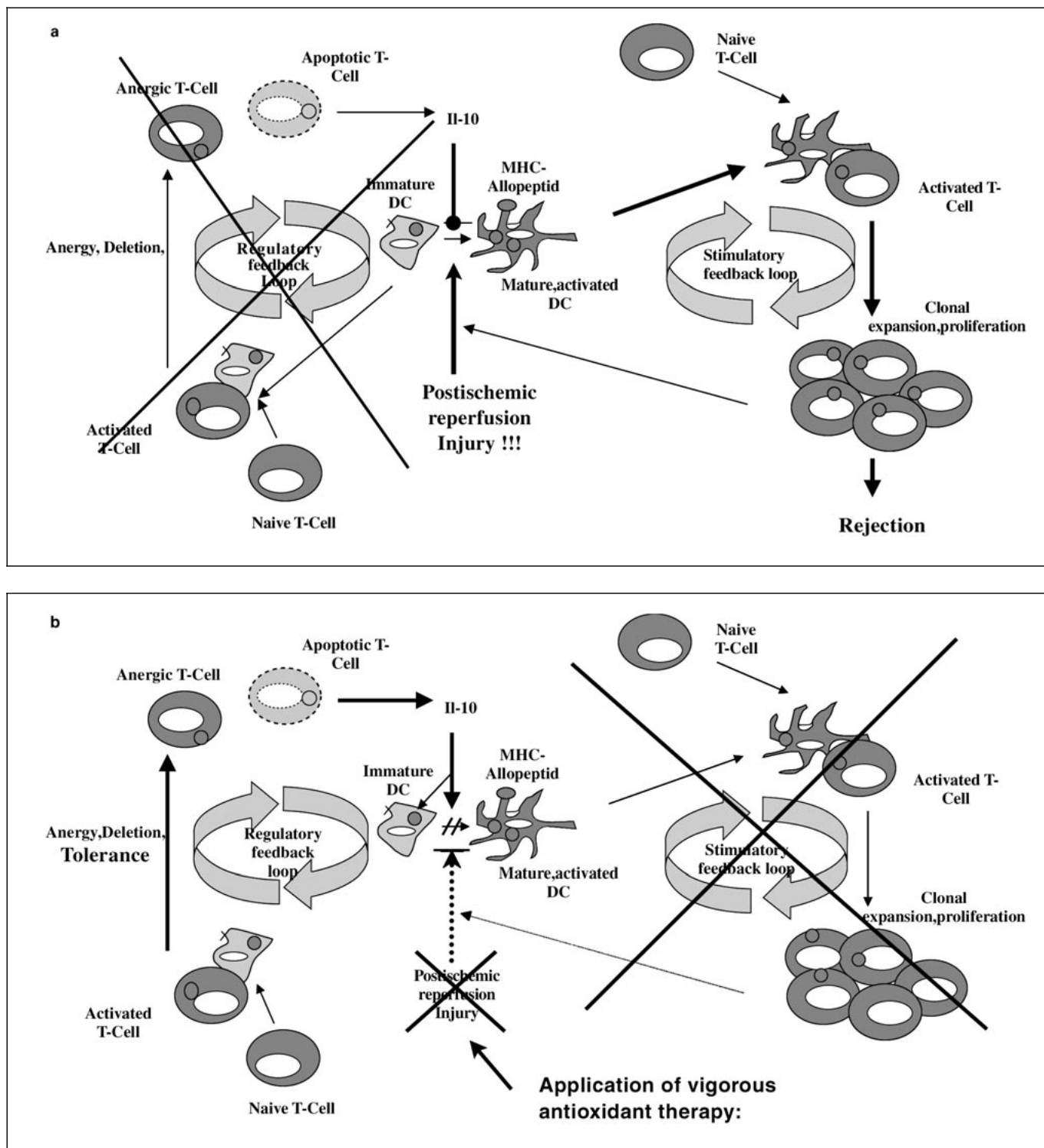


Fig. 4. a Postischemic reperfusion injury and modulation of dendritic cell maturation and activation: the role of apoptotic T-cells (figure modified according to ref. 41): Presence of reperfusion injury leads to activation of dendritic cells resulting – via a stimulatory loop – in rejection. **b** Postischemic reperfusion injury and modulation of dendritic cell maturation and activation: the role of apoptotic T-cells (figure modified according to ref. 41). Absence of reperfusion injury does not lead to activation of dendritic cells resulting – via the regulatory feed-back loop – in T-cell apoptosis and tolerance induction.

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