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TOLERANCE AND MINIMIZATION OF IMMUNOSUPPRESSIVE THERAPY AFTER LIVER TRANSPLANTATION

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In this review of current publications, we look at the molecular mechanisms of tolerance of the liver and its allografts in terms of minimization and possibilities of withdrawing immunosuppressive therapy, mainly in the long-term period after liver transplantation. Information about clinical trials with regulatory T cells (Tregs) for the purpose of tolerance induction is presented. Data from a new consensus study on individualization of immunosuppressive therapy regimens are presented. Options for possible withdrawal of immunosuppression both in the early and in the long term after liver transplantation (LT) are considered. We suggest a way to study the lymphoproliferative potential of a liver transplant recipient to be investigated, since not only rejection determines life expectancy, but also the degree of immunosuppression effect on bone marrow depending on patient age.

Keywords: apoptosis, immune tolerance, immunosuppression, mesenchymal stem cells, regulatory T cells, liver transplant, NK cells.

The effects of immunosuppressive therapy in the long-term period after liver transplantation (LTx) are associated with a set of adverse effects limiting recipient survival. And if the survival rate in the first year after LTx seems satisfactory, the life expectancy of patients in the late period remains significantly lower than in the general population (Fig. 1). Some of the leading causes of negative outcomes are malignancy, infections, cardiovascular and nephrological problems [1]. Therefore, the search for rational ways to overcome undesirable effects remains relevant. Based on analysis of modern literature, the main approaches to modifying immunosuppression after LTx, include intraoperative and delayed induction of tolerance, individualization and rationalization of regimens to reduce frequency of side effects of drugs, and, finally, minimization of immunosuppression up to complete withdrawal.

Most researchers consider the liver to be an immune privileged organ that can be tolerant to various influences. Immune tolerance in the liver is mediated by specialized antigen-presenting cells (APCs), including dendritic cells (DCs), Kupffer cells, sinusoidal endothelial cells and hepatic stellate cells. By providing autoantigens to their own T cells, these cells promote their apoptosis, anergy or differentiation of T lymphocytes into regulatory T cells. The tolerogenic role of the liver immune system was first demonstrated back in 1969 by Calne R.Y. et al. [2], who found that porcine liver allografts that had mismatches in the major histocompatibility complex (MHC) survived without immunosuppression.

Explanation for the relative tolerogenicity of the liver is multifactorial: the large size of the organ results in a much larger endothelial surface area on which antibodies are distributed, thus their effects are weakened; the liver has a natural regenerative capacity, so liver tissue damage is potentially reversible in case of rejection. Expression of MHC class II antigens on liver cells is more variable than that observed in the kidneys and heart. Tolerance in the liver has an evolutionary basis because 75% of blood flow in the liver comes from the portal vein, which collects blood from the gastrointestinal tract enriched with microbial antigens. The immune system of the liver has evolved to tightly regulate immune responses to harmless intestinal microorganisms and to avoid unwanted inflammatory-type reactions [3].

Achieving immune tolerance after LTx would potentially eliminate the need for long-term immunosuppression. Currently, the mechanisms of tolerance of transplanted liver are still being actively studied and clarified. Mesenchymal stem cells (MSCs), regulatory T cells (Tregs), and donor NK cells are mainly involved in the formation of tolerance (Fig. 2). Tregs are recognized as central regulators of immune response, they express FOXP3, a transcription factor regulating the transcription of genes responsible for T cell differentiation and the expression of cytokines and other factors involved in suppression of immune response. An important marker of regulatory T cells is the expression of CD25, receptor of the IL-2 cytokine, on their surface.

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Although presentation of donor DC antigens is the main rejection factor, tolerogenic phenotypes of DCs have also been found in the liver [6, 7]. DCs are also capable of inhibiting T cell proliferation by providing a small number of costimulatory molecules, which along with high expression of PDL1 (a PD1 ligand, a membrane protein preventing T cell activation) cause anergy or deletion of the alloreactive T cell clone. DCs secrete IL-10 and TGF-β, which induce Treg differentiation. CTLA4 receptors on the Treg surface bind to the B7 protein on DCs with higher affinity than CD28, disrupting interactions between DCs and T cells. Tregs also promote a tolerogenic microenvironment by secreting TGF-β, IL-10, and IL-35, binding IL-2 to CD25 with higher affinity than effector T cells, and through direct cytotoxicity mediated by two known pathways via granzyme, perforin, and Fas-FasL interactions. Unlike recipient NK cells, which can mediate rejection, donor-derived NK cells transplanted as passenger cells can directly lyse alloreactive recipient immune cells through NKG2D-MIC-A and TRAIL-TRAILR interactions. There is evidence that recipient NK cells may also have tolerogenic potential. MSCs inhibit T cell proliferation and differentiation by secreting indoleamine-2,3-dioxygenase (IDO), an enzyme capable of metabolizing the amino acid tryptophan to kynurenine. T cells require this amino acid for activation, and tryptophan deficiency induces apoptosis or inhibits proliferation and differentiation during PD-L1-mediated cell-to-cell contact [4, 5]. Kupffer cells can be polarized by the M2 phenotype, producing IL-10 and TGF-β and thus also promoting tolerance. They can also release nitric oxide (NO) with IFN-γ to inhibit T cell proliferation. Liver sinusoidal endothelial cells (LSECs) act as 'amateur' APCs with typically low levels of MHC class II antigen expression. LSECs, together with liver stellate cells, induce T cell apoptosis through PDL1-PD1 interactions [3–8].

The liver retains a tolerogenic potential even when alloreactive T cells have gained access to the parenchyma and tissue damage has begun. According to some data, alloreactive T cells cause hepatic cell death either through apoptosis or lysosomal degradation of hepatocytes. This process also partly depends on Treg, which inhibits rejection reaction [9, 10].

The study of these mechanisms seems to be important for justification of the possibilities of tolerance induction after LTx.

Treg-based cell therapy is a promising alternative approach to promote allograft engraftment, potentially minimizing the use of traditional immunosuppression [11, 12]. In 2020, the Lancet journal published data from the ONE study demonstrating the safety of Treg in kidney donor recipients [13]. Other centers are nearing completion of clinical trials, which are in different phases, focusing on the study of alloantigen-specific Tregs, as they have a better suppressive function against alloreactive effector T cells than polyclonal Tregs [14]. Treg expansion in vivo represents another interesting therapeutic strategy. Tregs exhibit a higher affinity for IL-2, so the use of low concentrations of this molecule

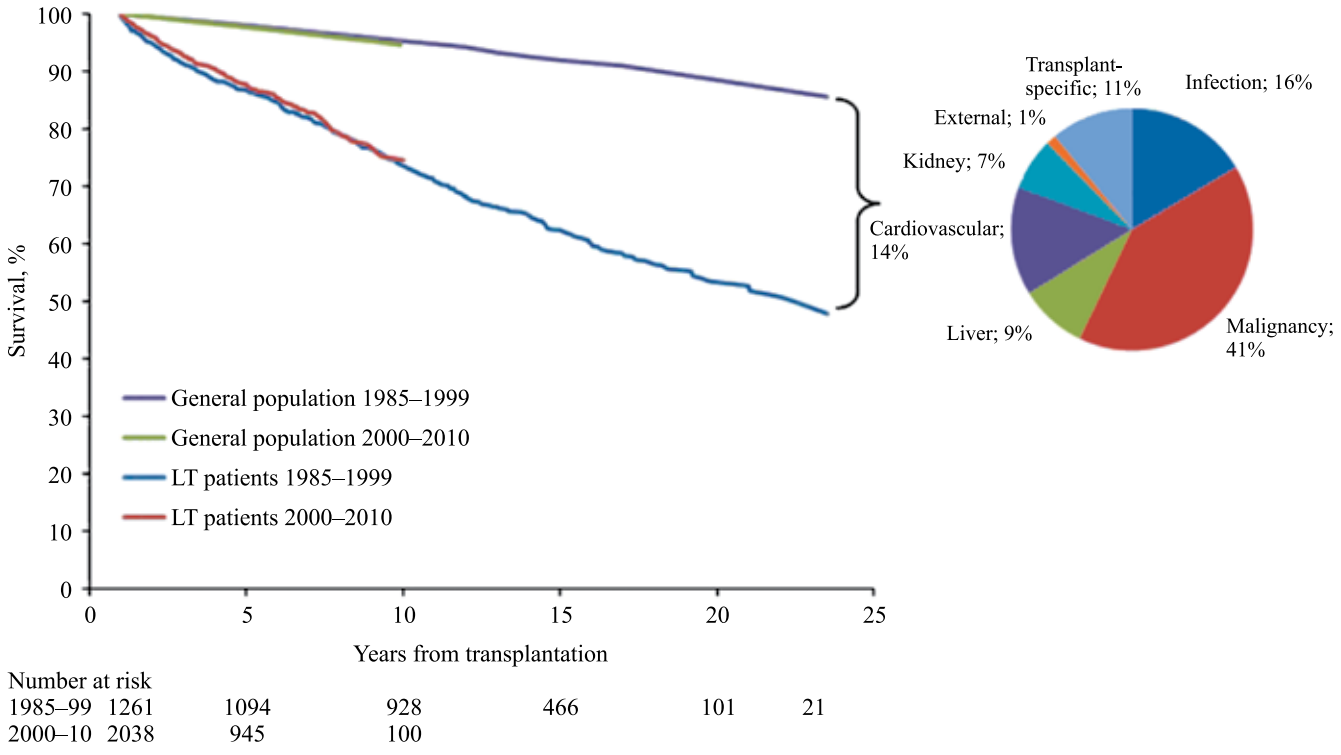


Fig. 1. Differences in long-term survival among liver transplant recipients and the general population [1]

can increase the Treg pool in vivo several times without significantly increasing the count of effector T cells [15].

Several clinical trials using a number of Treg drugs in LTx are currently underway worldwide (Table). Polyclonal regulatory T cell therapy with ex vivo cell augmentation is used in the ThRIL study at King's College Hospital (UK), the DeLTA and ARTEMIS studies at the University of California (San Francisco, USA) using donor alloantigen, reactive regulatory T cells (darTregs), and at Nanjing Medical University (China) using donor antigen-specific Tregs in patients in the early and late periods after liver transplantation.

In the case of related LTx, the cell product can be produced before the transplant surgery and infusion can be performed in the early post-transplant period. However, LTx from a deceased donor is prevalent worldwide, making it difficult to access and obtain donor tissue. In addition, production of Tregs is very resource-intensive. There are very few licensed laboratories capable of producing Tregs under GMP conditions [16]. There are still many technical problems in terms of production, scaling, and storage of the cell product. However, this direction seems to be the most inspiring for tolerance induction both in the early and late periods after LTx.

Since the donor liver is a foreign organ continually providing APCs, while tolerance induction has not become widely available, immunosuppressive therapy remains necessary for prevention and treatment of graft rejection.

In real clinical practice, the range of drugs for immunosuppression is not so large. Tacrolimus remains the mainstay. Regimens consisting of a combination of mycophenolates and calcineurin inhibitors (CNIs), or, more recently, everolimus, are commonly used as a maintenance immunosuppression regimen after LTx.

The mechanisms of suppression of T cell activation by these drugs are well studied. Nevertheless, the effects of these immunosuppressants on hepatocyte apoptosis have not been investigated. Hepatocyte apoptosis stimulates fibrogenesis in various liver injuries [17]. From this point of view, a study by Lim E.J. et al. [18] is of interest. The authors evaluated hepatocyte death based on apoptosis markers in biopsy by immunohistochemical method against immunosuppression and was compared with the state of liver cells in people without liver disease, operated on for other reasons. The level of hepatocyte apoptosis markers was significantly higher in liver transplant recipients compared to the control. Apoptotic hepatocytes are engulfed and utilized by both

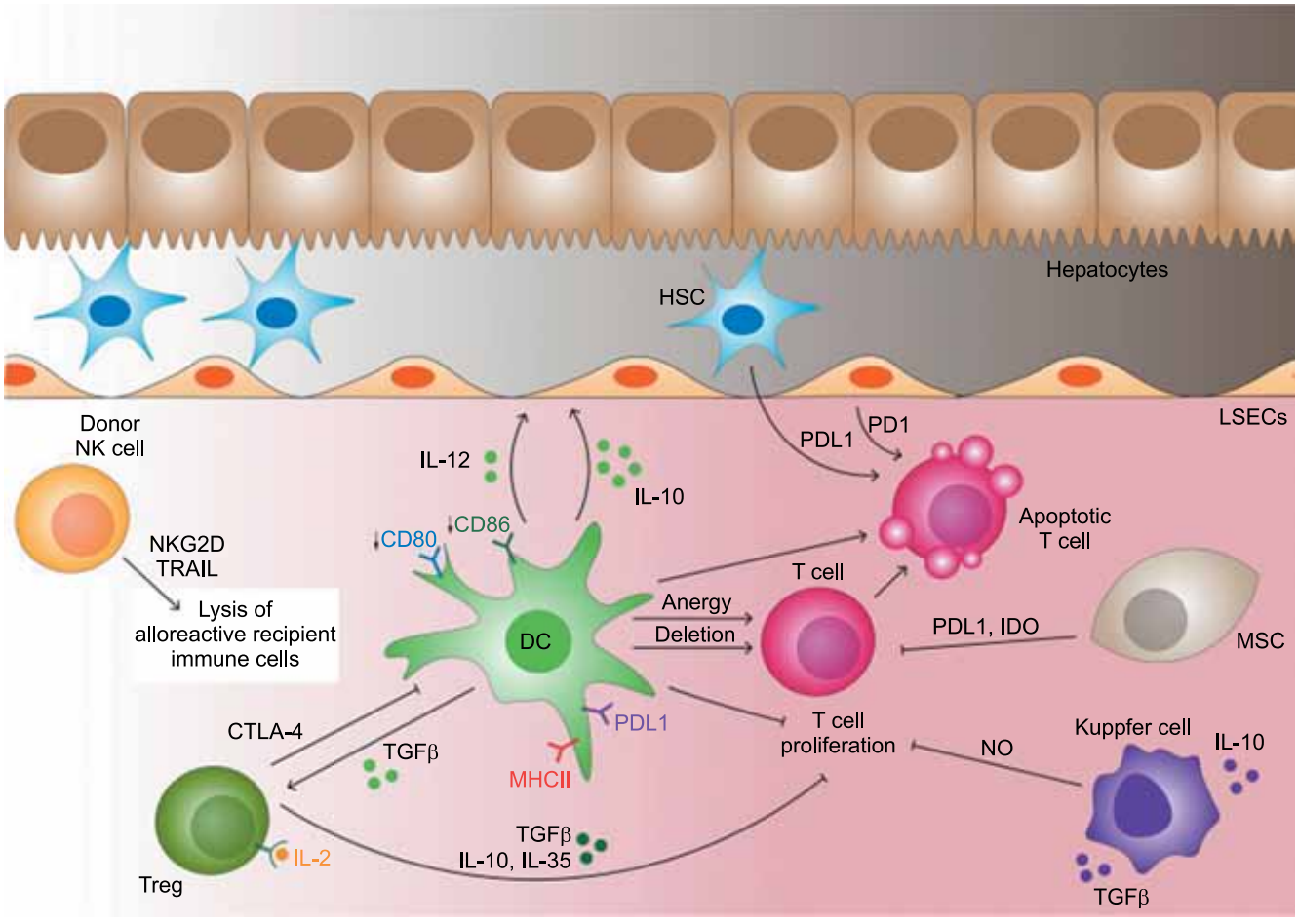


Fig. 2. Cellular mechanisms of liver allograft tolerance (description in text) [3]

Kupffer cells and stellate liver cells. Kupffer cells that have engulfed apoptotic hepatocytes secrete TGF-β, a major fibrogenesis factor [19]. On murine hepatocyte culture, it was shown that combinations of cyclosporine (CyA) and mycophenolate (MMF), tacrolimus (Tac) and MMF reduced cell viability and increased hepatocyte apoptosis, while isolated Tac and CyA did not cause such effects. Sirolimus as a monocomponent had no effect on hepatocyte viability and apoptosis, and in combination with MMF, these effects were expressed minimally [20]. Thus, it is possible to choose a rational immunosuppression regimen taking into account the effect of drugs on hepatocyte apoptosis, and, consequently, on fibrogenesis. The CNI/mTOR and mTOR/MMF regimens in the long-term period after LTx appear to be reasonable [18].

Although much experience has been accumulated in the use of immunosuppressive agents after LTx, the regimens are nevertheless heterogeneous and not ideal. Long-term recipient survival is associated not only and not so much with rejection reactions, but also with accumulation of adverse events. For their correction, two approaches can be implemented: preliminary individual selection before LTx and standard regimens, which are modified as complications develop [21]. In 2020, the Italian consensus on the use of everolimus in clinical practice after LTx was published, where algorithms for modification of immunosuppressive therapy were proposed. The choice of immunosuppressive therapy regimen should take into account a set of clinical variables, including the primary disease, patient transplant status, type of surgery, characteristics of the early postoperative period, events and expected complications associated with the long-term use of CNIs, and the risk of de novo malignancy. Strategies to prevent or limit CNI-related adverse events are worth using as early as possible after LTx. Currently, the most effective nephroprotective strategies are recognized as reducing CNIs exposure, which is possible with early administration of everolimus or mycophenolate if everolimus is not indicated. The presented algorithms allow minimizing the risks of adverse events and toxicity of immunosuppressants (Fig. 3, 4) [22]. These recommendations are also consistent with the above experimental data on rational immunosuppression regimens based on the effects of known drugs on liver fibrogenesis.

Unfortunately, results from experimental studies on tolerance mechanisms are not conclusive enough for us to establish certain factors for effective minimization and withdrawal of maintenance immunosuppressive therapy. In the absence of reliable tolerance biomarkers [23], with the complexity and inconsistency of molecular mechanisms, the only reliable way to confirm tolerance is if there is no graft rejection after deliberate cessation of immunosuppression.

From a clinical point of view, tolerance is defined as stable graft function in an immunosuppressed recipient with no clinically significant immune response.

In 2015, Chi-Xian Zhang et al. presented a review of data on minimization and possible withdrawal of immunosuppressive therapy after LTx [24], based on evidence of congenital liver tolerogenicity and careful evaluation of clinical parameters. Successful weaning from immunosuppression was possible in nearly 20% of selected liver transplant recipients.

In a later study [25], 95 recipients 1–2 years after LTx were randomly selected for withdrawal (n = 77) and continuation (n = 18) of maintenance therapy. Inclusion criteria were: monocomponent immunosuppression; ade-

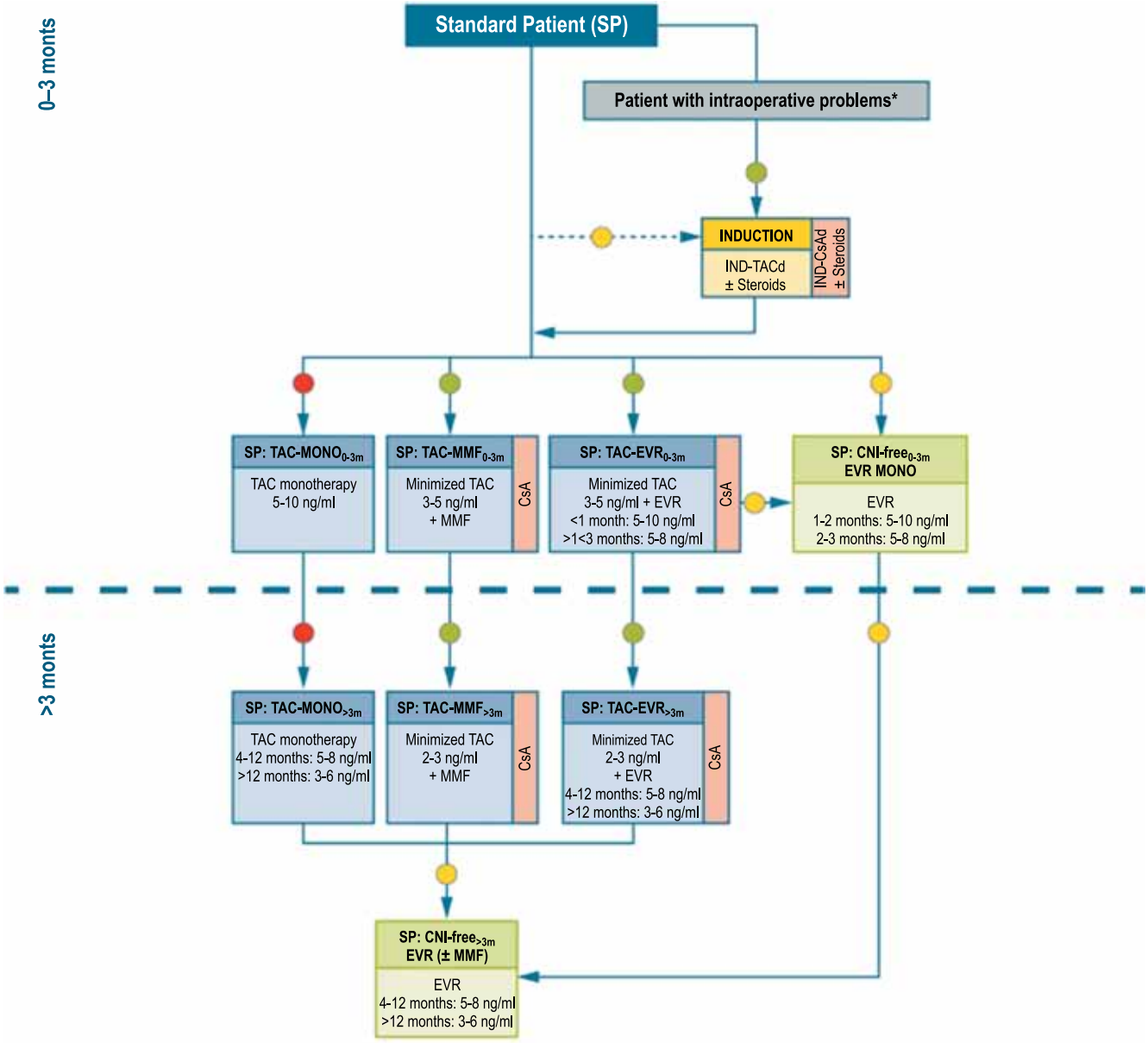
Table

Clinical trials using Treg *ex vivo* in liver transplantation [16]

Test	Liver transplant setting	Country	Study design	Age (years)	Treg dose	Repeated administration	Immunosuppression withdrawal
NCT 01624077	Chronic rejection	China	Open-labeled single group assignment	10–60	1×10^6 cells/kg donor	Yes	Yes
ARTEMIS (NCT 02474199)	Induction of tolerance in living-related LT	USA	Open-labeled single group assignment	18–70	$300–500 \times 10^6$ cells donor	No	Yes
ThRIL (NCT 02166177)	Induction of tolerance in living-related LT	UK	Open-labeled single group assignment	18–70	Autologic polyclonal Cohort 1: $0.5–1.0 \times 1 \times 10^6$ cells/kg Cohort 2: $3.0–4.5 \times 1 \times 10^6$ cells/kg Cohort 3: $5.0–6.5 \times 1 \times 10^6$ cells/kg	No	No
dELTA (NCT 02188719)	Induction of tolerance in deceased-donor LT	USA	Nonrandomized open-labeled parallel assignment	21–70	Donor alloreactive Cohort 1: 0 Cohort 2: $25–60 \times 10^6$ cells Cohort 3: $100–240 \times 10^6$ cells Cohort 4: $400–960 \times 10^6$ cells	No	Yes

quate liver and kidney function; ≤2 Ishak fibrosis stage; no rejection on biopsy. Immunosuppression was reversed according to an 8-step reduction algorithm at 8-week intervals. Fifty-two of 77 recipients (67.5%) reduced the dose to ≤50% of baseline, and 10 of 77 (13.0%) completely discontinued immunosuppression for ≥1 year. Immunosuppression was intensified in cases of rejection or veering of laboratory tests. Bolus injection of methylprednisolone was required in 5 of 32 cases of rejection. A composite end point (death or graft loss; grade 4 secondary malignancy or opportunistic infection; Ishak stage ≥3; or >25% decrease in glomerular filtration rate within 24 months of randomization) was achieved in 12 of 66 (18%) and 4 of 13 (31%) in the withdrawal and minimization groups, respectively. Thus, early minimization of immunosuppression was feasible in selected liver recipients, while complete withdrawal was successful in only a very small proportion.

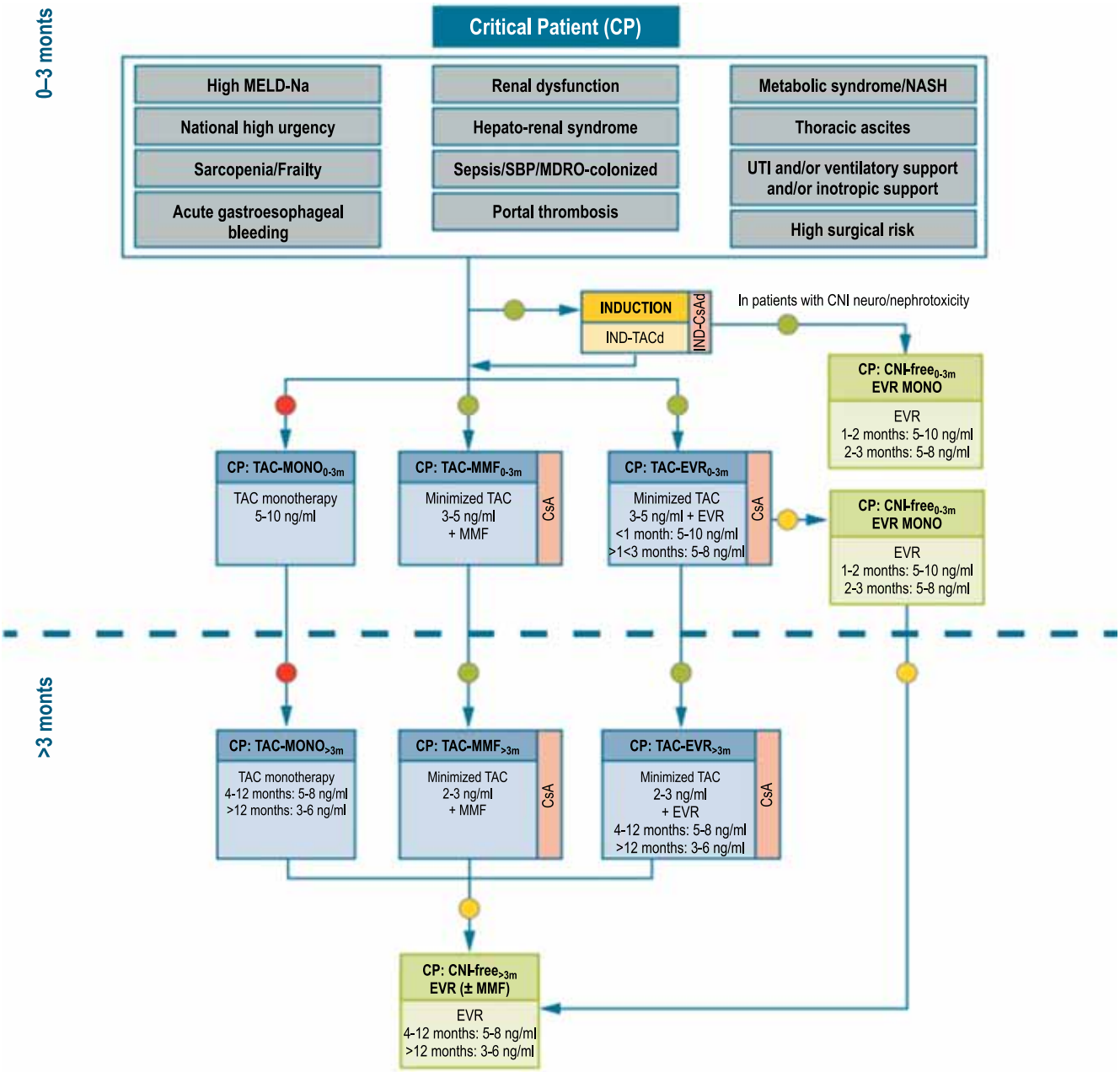
Data from another prospective randomized trial [26], suggest that over 40% of carefully selected liver recipients can have their immunosuppression reversed several years after LTx. 102 stable liver recipients 3 years or more after LTx were selected for withdrawal. Medications were gradually discontinued over several months. The primary endpoint was development of tolerance, defined as successful discontinuation of immunosup-



*Se grave evento intraoperatorio considerare switch a "Paziente critico – Alto rischio chirurgico".
CNI = Calcineurin inhibitors; CsA = Cyclosporin A; d = delayed; EVR = Everolimus; IND = Induction; MMF = Mycophenolate mofetil; TAC = Tacrolimus;

Fig. 3. Immunosuppression algorithm for standard patients. Key: green circle = recommended; yellow circle = caution advised; red circle = not recommended [22]

pressive drugs when graft function remained stable for at least 12 months and there were no histological signs of rejection. Of the 98 recipients, 57 retained and 41 successfully discontinued all immunosuppressive drugs. In tolerant patients, medications were discontinued for an average of 8.0 ± 4.6 months. Twenty-three tolerant patients had one episode of temporary allograft dysfunction unrelated to rejection. All episodes resolved spontaneously without increasing doses of immunosuppressants. Two tolerant patients died with normal graft function 16 and 26 months after complete cessation of medication because of postoperative complications of colorectal cancer resection and metastatic ovarian cancer, respectively. None of the tolerant recipients developed rejection during follow-up. In 57 intolerant patients, acute rejection was detected at 6.4 ± 4.4 months after the start of minimization therapy. All intolerant patients received immunosuppressants at the time of rejection. Liver biopsy was performed in 89% of cases and rejection was classified in most cases as borderline or mild according to Banff criteria. No cases of chronic rejection were observed. In 21 patients, rejection was cured



CNI = Calcineurin inhibitors; CsA = Cyclosporin A; d = delayed; EVR = Everolimus; IND = Induction; MDRO = Multidrug-resistant organism; MMF = mycophenolate motetil; MELD-Na = Model for End-stage Liver Disease – Sodium; NASH = Non alcoholic steatohepatitis; SBP = Spontaneous bacterial peritonitis; TAC = Tacrolimus; UTI = Urinary tract infection;

Fig. 4. Immunosuppression algorithm for critically ill patients. Key: green circle = recommended; yellow circle = caution advised; red circle = not recommended [22]

by returning to the baseline immunosuppression. In the remaining patients, restoration of baseline immunosuppression was combined with low or moderate doses of oral steroids, and only once was their bolus injection required. In 55 recipients (96.5%), all parameters were completely normalized 5.6 ± 5 months after rejection. There were no graft losses.

At the end of the study, successful reversal of immunosuppression was significantly more likely to occur in men ($p = 0.009$), in those older at the time of LTx ($p = 0.05$), and in those who lived longer after LTx ($p < 0.0001$). Time after LTx was identified as the strongest predictor of clinical tolerance. Among patients living over 10 years after LTx, 79% were successful in withdrawing immunosuppression, compared to patients living for more than 5.7 years, where 38% were successful in withdrawing all medications. It was significantly easier to wean off immunosuppression for those who lacked CNIs in the study enrollment regimen ($p = 0.005$).

Nevertheless, there are very few randomized trials of stepwise minimization and complete withdrawal of immunosuppressive therapy in the long-term period, and withdrawal still seems inappropriate at 1–2 years after LTx.

There is another not quite traditional view on the problem of long-term survival or maintenance of the functional state of a liver transplant. In some recipients, loss of a liver transplant may be due to not graft rejection but depletion of the proliferative potential of the lymphocytic growth of the bone marrow (the product of the number of stem/progenitor blood lymphocytes and mitotic activity), whose value limits the life span during natural aging [27]. Individual monitoring of lymphoproliferative potential could contribute to timely dose adjustment and modification of immunosuppressive therapy regimens. The study of the least studied functional relationship of own regulatory T lymphocytes with hematopoietic stem cell pool, seems relevant to objectivize the individual monitoring of immunosuppression in the remote period after LTx. This aspect can be considered as one of the approaches to minimize, and possibly cancel immunosuppression.

In conclusion, based on further studies on molecular mechanisms of tolerance, ways of inducing it in clinical practice may be determined, and then immunosuppressive therapy may lose its relevance. Prospective and promising is the introduction of allogeneic Tregs in order to induce immune tolerance. In the long-term post-LTx period, preservation of immunosuppression regimens, taking into account the recipient's individual profile, remains relevant. Dose minimization and immunosuppression cancellation in carefully selected recipients with stable graft function seem possible but require careful monitoring of liver graft status. Additional prospective studies are now needed to confirm the safety and efficacy of complete withdrawal of immunosuppression compared with maintenance therapy, as well as to verify tolerance biomarkers.

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The authors declare no conflict of interest.

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