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REVIEW

Immunosuppression for *in vivo* research: state-of-the-art protocols and experimental approaches

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Almost every experimental treatment strategy using non-autologous cell, tissue or organ transplantation is tested in small and large animal models before clinical translation. Because these strategies require immunosuppression in most cases, immunosuppressive protocols are a key element in transplantation experiments. However, standard immunosuppressive protocols are often applied without detailed knowledge regarding their efficacy within the particular experimental setting and in the chosen model species. Optimization of such protocols is pertinent to the translation of experimental results to human patients and thus warrants further investigation. This review summarizes current knowledge regarding immunosuppressive drug classes as well as their dosages and application regimens with consideration of species-specific drug metabolization and side effects. It also summarizes contemporary knowledge of novel immunomodulatory strategies, such as the use of mesenchymal stem cells or antibodies. Thus, this review is intended to serve as a state-of-the-art compendium for researchers to refine applied experimental immunosuppression and immunomodulation strategies to enhance the predictive value of preclinical transplantation studies.

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INTRODUCTION

Immunosuppressive treatments are routinely applied to prevent immune-borne transplant damage or rejection. These interventions continuously regain importance given the increasing need for solid organ and tissue replacement. In many countries, there has actually been a decline in the availability of appropriate transplants.[1]

In parallel, the development, optimization and implementation of emerging cell- and tissue-based regenerative therapies are underway to compensate for the paucity of transplantable organs. Regenerative therapies also provide a perspective on causal treatments for degenerative diseases that have been considered untreatable for decades.[2] Once proven effective, such regenerative therapies will most likely rely on non-autologous 'off-the-shelf' cell or tissue preparations to meet huge clinical demand. This will in turn require highly developed and effective immunosuppression or immunomodulation protocols to prevent graft rejection. Importantly, regenerative medicine approaches should be assessed in animal studies before clinical translation.

However, immunosuppressive regimens applied in preclinical research are often adopted from basic protocols that already exist in human medicine and often rely on simple body weight-adjusted dose translation. In many cases, they are even restricted to cyclosporin A (CsA) monotherapy, which is frequently reported in experimental transplantation protocols.[3] There may be some room for improvement by taking species-specific differences as well as pharmacodynamics and pharmacokinetics into full consideration, as these differences may significantly reduce the biological activity of a particular drug.[4] Neglecting these considerations not only leaves many important research questions unsolved (for example, regarding the necessity of graft survival for maximum therapeutic effect, or mitigating the effects emerging from graft decline) but may also

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severely bias the translatability of preclinical findings themselves. On the other hand, virtually all immunosuppressive strategies are accompanied by undesirable side effects and thus imperatively require a well-balanced, recipient- and species-specific design, including a combination of available protocols or even considering options currently under development.

This review provides a comprehensive, state-of-the-art overview of immunosuppressive treatments in relevant animal model species (that is, non-human primates (NHPs), rodents, dogs, pigs and sheep) and frequently investigated transplantation scenarios. Relevant physiological differences between animal species and humans as well as important differences in pharmacodynamics and pharmacokinetics are emphasized. In addition, we review promising experimental immunosuppressive protocols in terms of their potential roles in experimental cell/tissue transplantation studies. Our compendium thus aims to enable researchers to apply tailored immunosuppressive strategies with respect to the experimental question and/or the particular treatment subject under consideration, with the goal of ultimately ensuring a high predictive value of preclinical study results with respect to the clinical situation.

IMMUNOSUPPRESSIVE DRUG CLASSES

Efficient immunosuppression can be achieved via numerous mechanisms, which may be addressed synergistically. However, factors such as species physiology, age, concurrent medication, comorbidities and pharmacology can significantly affect efficacy, half-life and side effects of immunosuppressive agents.[5] The following paragraphs review relevant classes of immunosuppressive agents under current clinical or experimental use. Supplementary Table 1 summarizes the most relevant fields of application, whereas Supplementary Table 2 provides a detailed overview of side effects.

Glucocorticoids

Glucocorticoids (GCs) possess strong immunosuppressive properties and are widely used in human and veterinary medicine owing to their broad, although nonspecific, anti-inflammatory and anti-allergenic effects.[6] Common applications include the treatment of rheumatoid arthritis and asthma and concomitant administration in solid organ transplantation.[7,8] They inhibit T-lymphocytes and antigen-presenting cells (APCs) and induce a downregulation of proinflammatory cytokines.[8] The physiologically active form of GCs is cortisol (11-hydroxycortisol), whereas cortisone is a far less active 11-keto form.[9] In the blood, 80% of cortisol is inactive (bound to transcortin), whereas 20% is bound to albumin and can diffuse into tissues. The regulation of GC bioactivity is controlled hepatically by the 11β-hydroxysteroid dehydrogenase, which converts cortisone to cortisol and back (the 'cortisone-cortisol-shuttle').[9] Clinically used GCs mainly include synthetic cortisol analogs. The most common preparation is (methyl)prednisolone, but others such as dexamethasone, triamcinolone, betamethasone and paramethasone exhibit similar effects.[8,10] Synthetic GCs exclusively bind to albumin and therefore have a much higher bioavailability.[10] They also have significantly longer biological half-lives than cortisol.[10]

GCs reach the nuclei of many cell types by forming a complex after binding to a specific cytoplasmic receptor protein (Figure 1). There, they induce the synthesis of tyrosine-aminotransferase and tryptophan pyrrolase,[11] which exerts a number of tissue-specific and systemic effects as follows:

(1) reduction of chemotaxis, a pivotal process in inflammatory reactions and neutrophil activity,[12]
(2) reduction of vessel wall permeability, leading to less edema formation, exudation and migration of inflammatory cells,[10]
(3) reduction of antigen phagocytosis,[10]
(4) increase in hepatic gluconeogenesis,[10]
(5) downregulation of peripheral protein metabolism but enhanced hepatic protein synthesis,[10]
(6) alanine release from the musculature (increased plasma levels); this gluconeogenesis substrate surplus induces pancreatic glucagon secretion (from A cells) and subsequent hyperglucagonemia,[10] and
(7) fatty acid mobilization from subcutaneous storage (mainly in the extremities) and blockage of lipogenesis at these sites. In contrast, lipogenesis is increased in abdominal fat tissue.[13]

The preferred routes of administration and dosages of GCs are highly divergent across species and also depend on the condition being treated. Perioperative intravenous application of 30–500 mg/kg methylprednisolone reduced ischemia–reperfusion injuries by 24% after liver transplantation in humans without increasing susceptibility to infectious complications.[14] In the mouse, 50 mg/kg prednisolone was given intraperitoneally after allogeneic skin transplantation,[15] while rejection of a corneal xenotransplant was prevented by topical administration of 0.06 mg of dexamethasone over 8 weeks via a drug delivery system.[16] In contrast, oral application of 12.5 mg/kg prednisolone (twice a day, starting 5 days before transplantation and continuing until postoperative day (POD) 32) did not prevent graft rejection after allogeneic bone marrow transplantation in canines.[17] However, effective immunosuppression, and thereby enhanced survival of allogeneic hindlimb transplants, was achieved with a combination of CsA, mycophenolate mofetil (MMF) and GCs. This protocol was successfully transferred to a porcine model of allogeneic skin transplantation, in which 40 mg/kg per day CsA (whole blood trough levels between 100 and 300 ng/ml), 500 mg per day MMF and 2 mg/kg per day prednisolone were applied. The initial prednisolone dose, given at POD 1, could be reduced by 0.5 mg/kg per day in 3-day intervals to a final concentration of 0.1 mg/kg per day, avoiding side effects. Transplant survival was 19 to 90 days.[18,19] This protocol benefits from the perioperative administration of 500 mg of methylprednisolone, which was also confirmed for sheep in which 30 mg/kg methylprednisolone supported

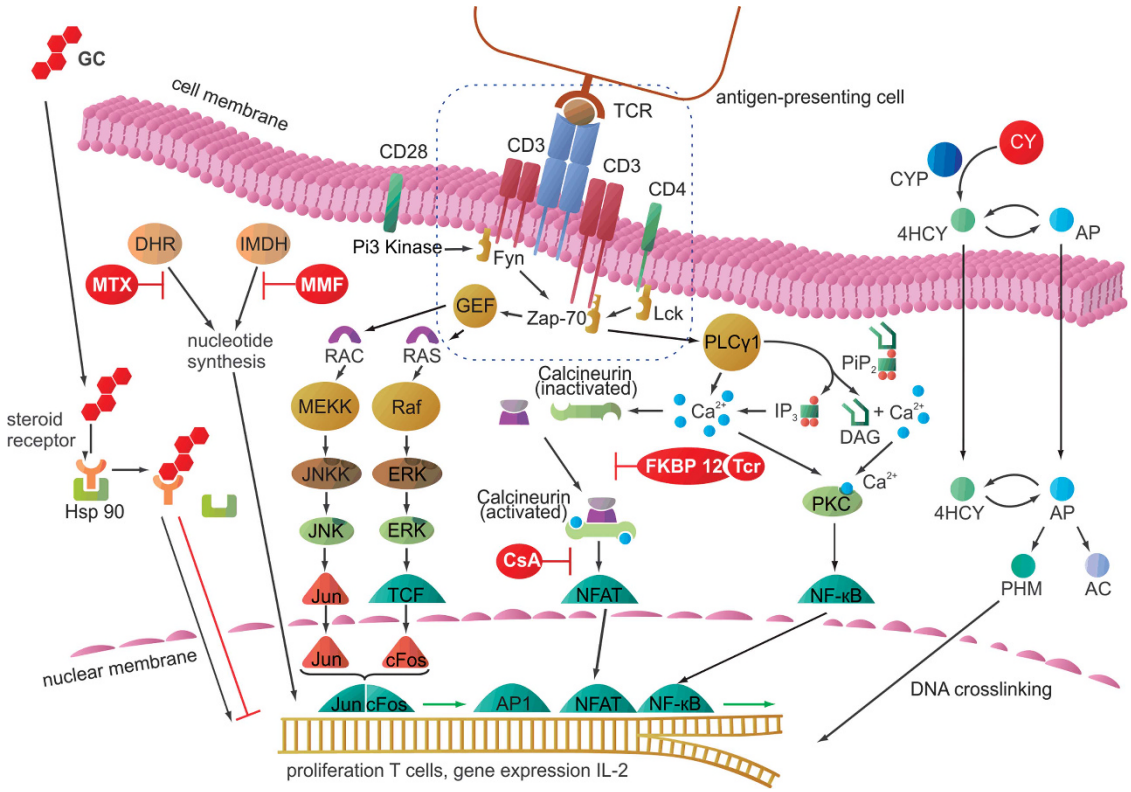

Figure 1 Cellular pathways of commonly used clinical immunosuppressive agents. GCs reach the nucleus via diffusion through the cell membrane and form a complex after binding to a steroid receptor protein following separation from Hsp 90. The complex binds to specific DNA sequences and affects the transcription of a variety of genes. MTX inhibits DHR, which is necessary for nucleotide synthesis, thereby constraining cell division. MMF blocks the IMDH, which is also required for nucleotide synthesis. CY is metabolized by CYP450 to 4HCY, which interconverts to AP. Both tautomers are able to passively diffuse into cells. Then, AP is converted to AC and PHM, which possesses DNA-crosslinking properties. CsA binds to an intracellular immunophilin and blocks calcineurin to enable NFATs, whereas tacrolimus (Tcr) binds to the intracellular FK506 binding protein (FKBP) and also inhibits NFAT activation, ultimately preventing cell proliferation. AC, acrolein; AP, aldophosphamide; CsA, cyclosporin A; CY, cyclophosphamide; CYP450, cytochrome *P*450; DAG, diacylglycerol; DHR, dihydrofolate reductase; ERK, extracellular signal-regulated kinase; Fyn, tyrosine-protein kinase; GEF, guanine-nucleotide exchanging factor; GH, glucocorticoid; 4HCY, 4-hydroxyphosphamide; Hsp 90, heat-shock protein 90; IP_3, inositol triphosphate; IMDH, inosine monophosphate dehydrogenase; JNK, c-Jun N-terminal kinase; JNKK, c-Jun N-terminal kinase kinase; RAC, guanosine triphosphate; RAS, guanosine-nucleotide-binding protein; Lck, lymphocyte-specific protein tyrosine kinase; MEK, mitogen-activated protein kinase kinase; MEKK, serine/threonine-specific protein kinase; MMF, mycophenolate mofetil; MTX, methotrexate; NF-κB, nuclear factor 'κ-light-chain enhancer' of activated B cells; NFAT, nuclear factor of activated T cell; PHM, phosphoramide mustard; Pip_2, phosphatidyl inositol bisphosphate; PKC, protein kinase C; PLCγ, phospholipase C-γ; RAF, serine/threonine-specific protein kinase; TCF, transcription factor; TCR, T-cell receptor; Zap-70, zeta-chain-associated protein kinase 70.

successful engraftment of cardiac transplants.[20] Table 1 summarizes recommendations for GC dosing in different species.

A number of GC side effects in human patients and animals have been reported since the late 1970s. These comprise musculoskeletal (myopathy, osteoporosis), gastrointestinal (ulceration), central nervous system and ophthalmic (glaucoma), cardiovascular (hypertension, peripheral edema formation), renal, metabolic (hyperlipidemia, ketoacidosis), endocrinal (reduced growth, suppression of the hypothalamic-pituitary axis) and fibroblastic (wound healing disturbance) side effects, as well as exaggerated immunosuppression.[21] Interspecies differences have also been reported in terms of fetotoxicity. GC application was shown to induce palatoschisis in rats, but no such effects have been observed in humans.[10,22] A species-specific overview of the most relevant applications and common adverse events is provided in Supplementary Tables 1 and 2.

Cytostatics

Cytostatics impair mitosis by acting as purine analogs to inhibit DNA synthesis or inosine monophosphate dehydrogenase. One relevant member of each category is described below.

Azathioprine: a purine analog. Azathioprine (Az) is a prodrug that is non-enzymatically converted in the liver into its active metabolite, 6-mercaptopurine (6-MP).[23] 6-MP is metabolized via three metabolic pathways. It can be inactivated by thiopurine methyltransferase to 6-methylmercaptopurine or by xanthine oxidase to 6-thiouric, or it can be activated to

Table 1 Glucocorticoids and their uses in experimental transplantation studies

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics bioavailability/VD*	*Application form*	*Dosage examples*
Dexamethasone (DM)					
Intracellular receptor binding Building a complex Translocation into the cell nucleus Influence transcription[201]	Human	4.7 ± 0.4,[38] 5.0[10]	Oral bioavailability of >80%[202] Protein binding: 65–70% VD: 0.8 L/kg[203]	Conjunctival, intra-articular, i.m., i.v., optic, p.o.[204]	• 16 mg per day, maintenance: <1.5 mg per day[205]
	Mouse	n.s.i.	n.s.i.	i.m., s.c.,[203] i.p.[206]	• 1 mg of DM/kg per day, q.d. tapered off towards 48 and 72 h[206]
	Rat	1.4–3.9[207]	Bioavailability of 86% CL: 0.23 L/kg/h VD: 0.78 L/kg[208] Protein binding: 84.7 ± 0.7%[209]	i.m., s.c.,[203] injection into the Tx region[210]	• 2 µl DM per animal, Tx of 4×10^6 fetal ventral mesencephalic cells[210]
	Dog	4.2 ± 0.6[211]	Biologic activity can persist for 48 h or more[212] Protein binding: 72.7 ± 1.4%[209] VD: 1.2 L/kg[213]	Conjunctival, i.m., intra-articular, i.v., p.o., s.c.,[203] i.v. dissolved in cardioplegic solution[214]	• 250 mg of BM/L cardioplegic solution[214]
	Pig	i.v.: 0.8, i.m.: 1.1[215]	Bioavailability after i.m.: 131 ± 26.05% CL after i.v.: 2.39 ± 0.57 L/kg/h VD after i.v.: 2.78 ± 0.88 L/kg[215]	i.m, intra-articular, i.v., s.c.[203]	• 0.04–0.08 mg/kg[203,213] • 1–10 mg per animal[203,216]
	Sheep	10.0 – 12.0[217]	n.s.i.	i.m, intra-articular, i.v.[203]	• 2–20 mg/kg[203]
Prednisolone (P)					
Intracellular receptor binding Building a complex Translocation into the cell nucleus Influence on transcription	Human	3.3 and more[10] 4.1 ± 0.8[218]	Protein binding: 75–90%[203] Systemic availability: 79 ± 13% CL: 0.14 ± 0.03 L/kg/h VD: 0.7 ± 0.1 L/kg[218]	i.m., intra-articular, i.v., topical,[219] ophthalmic, p.o.[204]	• 15 mg of P per day[10] • 30–500 mg of MP/kg i.v. perioperatively[14] • 4 mg of MP per day, 105.2 mg of CsA per day or 4.1 mg of Tcr per day[220]
	Mouse Rat	0.8 (at 10 mg/kg i.p. or p.o.)[221] 0.5 (at 10 mg/kg)[222]	n.s.i. CL: 2.3 ± 0.9 L/kg/h VD: 0.82 ± 0.46 L/kg (both higher in obese rats)[223]	i.p.,[15] p.o.[203] Bronchoalveolar lavage,[224] i.p.,[225] i.v.,[226] p.o., s.c.,[203] topical[16]	• 50 mg of P/kg[15] • 0.06 mg of P for 8 weeks, topical via drug delivery system[16] • 100 mg of P application by bronchoalveolar lavage[224] • single dose: 5 mg of MP/kg i.v.[226] • 10 mg of MP/kg per day, 0.2 mg of Rpm/kg per day, 20 mg of MMF/kg per day i.p. from POD – 30 to 100[225]

Table 1 (Continued)

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics bioavailability/VD*	*Application form*	*Dosage examples*
	Dog	1.3[227]	Plasma half-life not meaningful because of intermediate acting, biologic half-life 12–36 h[212] CL after p.o.: 0.43–0.58 L/kg/h[228]	i.m.,[229] i.v.,[203] p.o.[47]	• 20 mg of MMF/kg per day, 5 mg of CsA/kg per day, 0.1 mg of MP/kg per day p.o.[47] • 15 mg of CsA per day, 5 mg of Mz per day, 1 mg of P per day p.o., i.m. from POD – 5 to 20[229]
	Pig	0.73 ± 0.15[218]	Systemic availability: 27 ± 10% CL: 1.54 ± 0.13 L/kg/h VD: 1.2 ± 0.2 L/kg In general: requires 10–30 times higher i.v. or oral dose of steroids than humans[218]	i.v.,[230] p.o.[18,19]	• 40 mg of CsA/kg per day, 500 mg of MMF per day, 2 mg of P/kg per day (tapered off towards to 0.1 mg of P/kg per day)[18,19] • Initial dose: 100 mg of CsA, 50 mg of Az, 125 mg of MP, q.d. p.o.:10 mg/kg CsA, 2 mg/kg Az, 20 mg of MP[231] • 15 mg of CsA/kg per day, 2 mg of Az/kg per day, 20 mg of P per day or 1 mg of Tcr/kg per day, 2 mg of Az/kg per day, 20 mg of P per day p.o.[232] • 500 mg of MP per animal i.v. perioperatively[230]
	Sheep	0.4 ± 0.1 (at 0.5 mg/kg i.v.)[233]	CL: 0.93 ± 0.13 L/kg/h VD: 0.45 ± 0.086 L/kg[234]	i.m., i.v.[203]	• 30 mg of MP/kg perioperatively[20]

Abbreviations: Az, azathioprine; BM, betamethasone; CL, total plasma clearance; CsA, cyclosporin A; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; MMF, mycophenolate mofetil; MP, methylprednisolone; Mz, mizorbin; n.s.i., no sufficient information available; P, prednisolone; p.o., oral; q.d., once a day; Rpm, rapamycin; s.d., subcutaneous; Tcr, tacrolimus; Tx, transplantation; VD, volume of distribution.

6-thioguanine nucleotide.[24] The enzyme thiopurine *S*-methyltransferase is important for Az metabolism. Approximately 90% of human patients present a highly active form, while the enzyme is only moderately active in the remaining 10%, which explains the phenomenon of therapeutic non-responsers.[25] The active metabolite 6-thioguanine nucleotide is inserted as a base analog into nucleic acids, inhibiting DNA repair and replication. Az reaches a metabolic steady state only 4–6 months after the initiation of therapy. Hence, treatment must be initiated in a timely manner or initially supported with the use of other immunsuppressants.[26] Az causes minor, dose-independent side effects, such as nausea, diarrhea and elevated liver enzymes. Side effects can be reduced by administering 6-MP instead of Az.[27] Dose-dependent side effects include leukopenia, thrombocytopenia and hepatitis. Az was used at dosages of 2–3 mg/kg to treat acute renal allograft rejection[28] (for more details, see Supplementary Tables 1 and 2).

Cyclophosphamide and methotrexate: DNA synthesis inhibitors. DNA synthesis inhibitors comprise alkylating substances, such as cyclophosphamide (CY), and anti-metabolites, such as methotrexate (MTX).[29,30] CY, a commonly used antitumor agent, exerts cytotoxic and immunosuppressive effects and is also applied as an immunosuppressant.[29] Cytochrome *P*450 (CYP450) metabolizes CY to 4-hydroxyphosphamide, which interconverts to aldophosphamide.[31] Both tautomers are able to passively diffuse into target cells (for example, tumor cells). Then, aldophosphamide is converted to phosphoramide mustard, which exerts DNA-crosslinking properties (Figure 1).[32] The effects of cytostatic drugs are highly dependent on their impact at a particular phase of the cell cycle.[33] CY does not specifically influence a certain cell cycle phase and is thus toxic for all dividing cells, particularly rapidly proliferating cells.[33] Its primary effects involve the impairment and depletion of B-lymphocytes,[34] observed in mice, guinea-pigs and humans.[35,36] T-suppressor cells are less sensitive, whereas T-helper cells are mostly resistant to the effects of CY.[37]

There are significant interspecies differences regarding the application and dosing of cytostatic agents (Table 2; for an overview of relevant indications, see Supplementary Table 1). Immunosuppressive effects have been reported for the intramuscular administration of CY at 28.5–33.3 mg/kg twice a day in rabbits.[34] Single doses of 250–400 mg/kg were effective in mice, inducing tolerance against ovine erythrocytes.[34] In general, typical side effects of immunosuppressants, such as nephrotoxicity or hepatotoxicity, are less frequent when using cytostatic agents compared with immunophilin drugs such as CsA (Supplementary Table 2).

The side effects of CY include hemorrhagic cystitis, bone marrow suppression, alopecia and gonadal dysfunction,[38] whereas teratogenic, carcinogenic and mutagenic effects have been explained by DNA crosslinking.[29,38] In humans, single doses significantly reduce leukocyte counts to 1400/mm^3 (60 mg/kg) and 1000 per mm^3 (100 mg/kg for 5 consecutive days).[39] Reduced thrombocyte counts were observed after the application of 2000 mg of CY per m^2 body surface.[40] Gastrointestinal complications, including nausea and vomiting, have been reported in two-thirds of all human patients at doses >50 mg/kg and 6–12 h after a 1-h infusion.[41] Similarly, diarrhea and weight loss are observed in rabbits following the intramuscular application of CY.[34]

The anti-metabolite MTX also reduces purine synthesis by inhibiting dihydrofolate reductase[42] (Figure 1). Administration of the anti-metabolite MTX (for dosage regimen, see Table 2; for an overview of relevant indications, see Supplementary Table 1) may result in hepatotoxicity, pneumonitis and bone marrow suppression[30] (Supplementary Table 2).

MMF: an inosine monophosphate dehydrogenase inhibitor. MMF inhibits nucleic acid formation,[43] presents high bioavailability after oral application and diminishes transplant rejection after tissue or solid organ transplantation.[44] This agent is a potent, competitive and reversible inhibitor of inosine-5'-monophosphate dehydrogenase, inhibiting purine synthesis and thus cell proliferation[43,44] (Figure 1). *In vivo*, MMF is rapidly converted into its active metabolite, mycophenolic acid (MPA), which is excreted renally to avoid the enterohepatic circulation.[44] MMF selectively inhibits cytotoxic T cells but does not reduce proliferation of fibroblasts and endothelial cells below a concentration of 100 nM.[44] MMF was applied successfully as a stand-alone treatment after pancreatic islet transplantation in mice[45] and has been used to treat acute transplant rejection in rats (cardiac allografts), dogs (renal allografts) and humans.[46] It also prevents proliferative angiopathies (which are related to chronic rejections) after allotransplantations in rats and NHPs.[44] Daily oral application of 30 mg/kg MMF reduced antibody formation against ovine erythrocytes in rats, whereas 40 mg/kg per day effectively prevented graft rejection in mice (cardiac allografts), rats and dogs (renal allografts).[44] MMF application twice a day resulted in effective long-term plasma concentrations.[44] Moreover, the combination of MMF and CsA exerts an additive effect without known toxicity at common concentrations.[44] For example, the combination of 20 mg/kg per day MMF, 5 mg/kg per day CsA and 0.1 mg/kg per day methylprednisolone has been applied in dogs without signs of nephrotoxicity and hepatotoxicity or bone marrow suppression.[47] Immunosuppressive protocols using MMF and the reduction of CsA doses by up to 50% resulted in improved renal transplant function in human patients.[48,49] CsA dose reduction is required because CsA inhibits the active transport of MPA-glucuronide in bile, leading to reduced enterohepatic recirculation and thereby reduced MPA exposure.[50,51] Please refer to Table 3 for species- and indication-specific dosing and applications. For an overview of relevant indications, see Supplementary Table 1.

Most reported side effects of MMF are gastrointestinal complications, which have been noted in humans (2000 mg per day)[52] and dogs, but side effects have not included intestinal ulcerations.[47] Moreover, the long-term application of MMF even at doses exceeding clinically common levels does not lead to toxicity in dogs or NHPs[44] (Supplementary Table 2).

Table 2 Common cytostatic drugs and their uses in experimental transplantation studies

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics bioavailability/VD*	*Application form*	*Dosage examples*
Azathioprine (Az)					
Metabolized to 6-thioguanine Base analog in DNA or RNA Inhibits DNA repair and replication[235]	Human	Of the active metabolite 6-MP: 1.9 ± 0.6[236]	Corticosteroid-sparing effect: 4.3 mg per day[237]	p.o.[235]	• 1.5 mg/kg per day • Children: 3–5 mg/kg per day • 1 mg/kg per day • 25–100 mg per day[235]
	Rabbit	n.s.i.	n.s.i.	p.o. in the drinking water[238]	• 1.0 mg/kg dissolved in 40 ml of water in combination with CY[238]
	Dog	n.s.i.	n.s.i.	p.o.[239]	• 3 mg/kg per day for 28 or 56 day after Tx in combination with prednisolone[239]
	Pig	n.s.i.	n.s.i.	n.s.i.	• Daily up to 5 mg/kg in combination with prednisolone[240] • up to 5 mg of Az/kg daily[240]
Cyclophosphamide (CY)					
Is metabolized to 4-hydroxyphosphamide Connects to aldophosphamide Translocation into the cell Release of acrolin Transformation of aldophosphamide to phosphoramide mustard Cytotoxic effect[31]	Human	i.v.: 8.9[241] 7.7 ± 3.6[242] Children: 2.5–6.5[243]	CL: 0.04–0.18 L/kg/h VD: 0.31–1.05 L/kg[244]	i.v., p.o.[38]	• 10–15 mg of CY/kg three times per week[38] • 1–5 mg of CY/kg per day[38] • > 1.8 mg of CY/kg per day[38]
	NHP	n.s.i.	n.s.i.	i.v. as infusion[245]	• 180 mg of CY/kg[245]
	Mouse	0.2[246]	n.s.i.	i.p. or i.v.[34,247]	• Single dose: 250–400 mg of CY/kg[34,247]
	Rat	0.1[248]	n.s.i.	i.p.[249]	• 150 mg of CY/kg[249]
	Rabbit	0.4 ± 0.1[250]	n.s.i.	i.m.[34]	• 28.5–33.3 mg of CY/kg every second day[34]
	Dog	4–12 h, measurable up to 72 h after administration[212]	Pharmacokinetics not detailed for dogs, but supposedly similar to humans good oral absorption with peak levels after 1 h, distribution throughout the body, including CSF (in subtherapeutic levels); minimally protein bound; CY crosses the placenta[212]	i.v., s.c.,[251] p.o.[252]	• 2 mg of CY/kg for 15 days[252]
	Pig	n.s.i.	n.s.i.	i.p.[253]	• 50 mg of CY/kg[253]
	Sheep	n.s.i.	n.s.i.	i.v [254]	• 25 mg of CY/kg[254]
Methotrexate (MTX)					
Inhibition of dihydrofolate reductase Inhibition of purine synthesis[42]	Human	1.8–8.5 (at 50–200 mg/kg i.v.)[255]	Peak levels 4 h after oral dosing and 30 min to 2 h after i.m. injection[212]	p.o.[256]	• 7.5 mg of MTX per patient per week[256] • 7.5–12.5 mg of MTX per week[257]
	Mouse	1.42 ± 0.65[258] 0.5, up to 100 (Depo-MTX)[259]	CL after i.v.: 2.63 ± 0.76 L/kg/h VD: 2.19 ± 0.55 L/kg crosses the blood–brain barrier[258]	i.p.[260]	• 0.5–5 mg of MTX/kg[260]

Table 2 (Continued)

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics bioavailability/VD*	*Application form*	*Dosage examples*
	Rat	i.v.: 4.2[261]	Unbound fraction: 58.1% VD after i.v.: 0.27 L/kg[262]	Intrathecally and around the spinal root,[263] i.p.[264]	• 3 mg of MTX/kg per week[264] • 1 mg of MTX/kg[263]
	Dog	7.6 (intracisternal injection)[265] <10 h (2–4 h)[212]	Good gastrointestinal absorption after oral administration (<30 mg/m^2 with bioavailability ~ 60%), wide distribution in the body, active transport across cell membranes, no therapeutic levels in the CSF after oral or parenteral administration, 50% bound to plasma proteins, crosses the placenta[212]	i.m., i.v., p.o,[251] s.c.[266]	• 10 mg of MTX/kg b.i.d.[266]
	Pig	n.s.i.	n.s.i.	Into the 4. cerebral ventricle[267]	• 2 mg of MTX for 5 days[267]
	Sheep	n.s.i.	n.s.i.	i.a.[268]	• 0.25 mg of MTX/kg per day for 28 days[268]

Abbreviations: b.i.d., twice a day; CL, total plasma clearance; CSF, cerebrospinal fluid; i.a., intra-arterial; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; MP, 6-mercaptopurine; NHP, non-human primate; n.s.i., no sufficient information available; p.o., oral; s.c., subcutaneous; Tx, transplantation; VD, volume of distribution.

Calcineurin inhibitors

Calcineurin controls the transcription of interleukins (ILs) in T-lymphocytes. Binding of calcineurin inhibitors to intracellular immunophilins decreases IL-2 production and, therefore, T-cell proliferation.[53] CsA and tacrolimus are the most relevant calcineurin inhibitors.

Cyclosporin A. CsA has been very widely used both experimentally and clinically since 1978.[54] CsA is a lipophilic molecule, isolated from *Tolypocladium inflatum*,[55] that can easily penetrate the blood–brain barrier (BBB).[56,57] Major applications include stem cell and solid organ transplantations,[58] as well as the prevention and suppression of graft-versus-host-disease (GvHD).[59] Because the drug is often used nonspecifically in experimental studies, the following paragraphs provide detailed information about its mechanisms and side effects. Application regimens will also be recommended.

CsA is a calcineurin inhibitor that reversibly and selectively inhibits T-cell proliferation.[60] Its effects are mediated by the formation of intracellular complexes with immunophilin, effectively blocking both the activation of calcineurin and, subsequently, nuclear factor of activated T cells (NFATs; Figure 1).[60] Because NFAT is a key regulatory element in the transcription of proinflammatory cytokines such as IL-2, IL-3, IL-4, IL-5, IL-8, IL-13, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), application of CsA effectively inhibits proinflammatory immune reactions.[61] Reduction of IL-2 is of particular importance among the effects of CsA, as IL-2 is a potent T-cell activator both in humans and canines.[62] Because CsA is able to penetrate the BBB,[56,57] positive effects have been reported for central nervous system applications: CsA increases the proliferation and cell survival of neural precursor cells both *in vitro* and *in vivo* and is frequently used in neurological studies.[63]

CsA is metabolized hepatically via CYP450 3A.[58,64] CYP inhibitors such as ketoconazole or diltiazem can decelerate CsA metabolism,[64] whereas CYP induction by phenytoin can reduce CsA whole blood levels by up to 50%.[65] CsA pharmacokinetics depend on recipient age (younger individuals present enhanced metabolic clearance), the transplanted tissue, CYP-competitive medications and liver function, differences in CsA clearance, apparent volume of distribution and half-life with regard to age, health status and transplantation procedure have also been reported[5] (Table 4).

Important effects of individual CsA pharmacokinetics have been shown for CYP 3A5 polymorphisms: individuals presenting the CYP 3A5*3 allele require lower CsA doses to reach desired whole blood levels compared with those individuals with the CYP 3A5*1 variant.[66] An interspecies-specific comparison of CsA pharmacokinetics between humans, rats, rabbits and dogs revealed a direct proportional correlation between clearance and volume of distribution in relation to body weight. The capacity to metabolize CsA in dogs is twice as large compared with the capacities of humans, rats and rabbits.[67]

Table 3 MMF and its use in experimental transplantation studies

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics bioavailability/ volume of distribution*	*Application form*	*Dosage examples*
Mycophenolate mofetil (MMF)					
Is metabolized to glucuronide[44]	Human	16.0[269]	Oral bioavailability 94%, food reduces peak levels up to 40%[212]	p.o.[52]	• 2 g of MMF per day[52] • 20 mg of MMF/kg per day, gradually increase the dose up to 40 mg/kg per day[270]
Competitive and reversible inhibition of iosin-5'-monophosphate dehydrogenase[43]					
Inhibition of purine synthesis					
Inhibition of cell proliferation[44]					
	Mouse	n.s.i.	n.s.i.	p.o.[271]	• 40 mg of MMF/kg[271]
	Rat	4.7 ± 0.3[272]	n.s.i.	p.o.[273]	• 30 mg of MMF/kg[273] • 40 mg of MMF/kg in combination with CsA[187]
	Dog	8 (±4)[212]	Wide interpatient and interdose variability: bioavailability of 54% (10 mg/kg), 65% (15 mg/kg), 87% (20 mg/kg),[212] VD: 5.0 ± 4.5 L/kg with wide interpatient variability[212] CL after p.o. (40 mg/kg): 0.14 ± 0.12 L/kg/h[262]	p.o.[47]	• 20 mg of MMF/kg per day, 5 mg of CsA/kg per day, 0.1 mg of MP/kg per day[47] • Dosing in 8-h intervals recommended for optimal immunosuppression[212]
	Pig	n.s.i.	n.s.i.	i.v., p.o.[274]	• 25–100 mg of MMF/kg b.i.d., Tcr[274] • 1.5 g of MMF per animal b.i.d., low-dose Tcr[275] • 20 mg of MMF/kg i.v. before Tx, Tcr[276]
	Sheep	n.s.i.	n.s.i.	p.o.[277]	• 1.5 g of MMF per day, Tcr, MP[277]

Abbreviations: b.i.d., twice a day; CL, total plasma clearance; CsA, cyclosporin A; i.v., intravenous; MP, methylprednisolone; n.s.i., no sufficient information available; p.o., oral; Tcr, tacrolimus; Tx, transplantation; VD, volume of distribution.

Table 4 Differences in CsA clearance, volume of distribution and half-life[a]

	Kidney Tx		*Heart Tx*	*Healthy controls*
	Adults	*Children*	*Adults*	*Adults*
Clearance (ml/min/kg)	5.7 (0.6–23.9)	11.8 (9.8–15.5)	4.0 (2.1–5.39)	3.9 (2.8–4.2)
Volume of distribution (l/kg)	4.5 ± 3.6	4.7 ± 1.5	1.3 ± 0.2	1.2 ± 0.2
Plasma half-life (h)	10.7 (4.3–53.4)	7.3 (6.11–16.6)	6.4 (5.2–9.3)	6.3 (4.7–9.5)

Abbreviations: CsA, cyclosporin A; Tx, transplantation.
[a]In relation to age, health and transplantation procedure according to Ptachcinski *et al.*[5]

The bioavailability of CsA demonstrates relatively large intraindividual fluctuations, primarily due to reduced oral absorption in some individuals.[68] Thus, some transplantation centers have applied CsA intravenously in human patients for the first 21 days post-transplantation.[69] However, oral application is much more common. Importantly, CsA microemulsions for oral delivery reduced rejection frequencies after organ transplantation and have replaced intravenous preparations.[70] Oral application of CsA is also common for dogs[71] and pigs,[72] whereas intravenous injection is preferred in sheep models.[73] Intravenous application is also advantageous in species for which good compliance for oral application cannot be ensured and if high blood concentrations must be reached rapidly.

CsA is typically applied for GvHD prevention in humans, and different treatment regimens have been reported: CsA may be applied as a continuous infusion, once per day or twice a day with extended infusion times (2–13 h per day). Applied dosages vary between 1 and 20 mg/kg per day and are generally adopted to reach target whole blood levels, which are usually reported between 100 and 1000 ng/ml.[69] Application of CsA requires a careful balance between intended treatment and adverse side effects. Bacigalupo *et al.*[74] compared daily doses of 1 and 5 mg/kg following bone marrow transplantation to treat leukemia. Patients receiving 1 mg/kg per day presented adverse effects less frequently but had a significantly enhanced risk of GvHD.[74] In turn, long-term CsA treatments can clearly reduce the overall risk of developing GvHD.[75] Prevention of rejection was achieved with whole blood trough levels of 400–800 ng/ml CsA after experimental kidney transplantation in pigs (neonatal: 4 mg/kg per day oral; juvenile: 30 mg/kg per day).[72] Rose *et al.*[73] recommended 2–3 mg/kg CsA in combination with 10 mg/kg ketoconazole (twice a day each) as the optimal CsA dosage in a sheep model of xenologous skin transplantation[73] (for an overview of relevant indications, see Supplementary Table 1). CsA whole blood levels ranged between 1600 and 2500 ng/ml CsA in this model, and the steady state was reached after 17 days.[73] On the other hand, application of 3 mg/kg per day twice a day revealed considerable intraindividual differences and fluctuations in CsA whole blood levels (Diehl *et al.*, 2016, unpublished data). Thus, moderate dose escalation may be required to reach appropriate CsA whole blood levels. This can be considered safe because adverse effects have not been observed after the administration of relatively high doses, including 12 mg/kg per day CsA for 5 days in sheep.[76] Inhibition of calcineurin positively correlates with CsA whole blood levels and is therefore the most important indicator of CsA efficacy.[60] However, CsA whole blood levels and bioavailability may differ significantly between individuals receiving the same CsA doses[58] or even intraindividually.[68] The latter finding was corroborated by sheep studies, which strongly pointed to the necessity of thorough CsA whole blood level monitoring in CsA-based immunosuppressive paradigms (Diehl *et al.*, 2016, unpublished data). Commonly used dosages in different species are shown in Table 5.

The combination of CsA with adjuvant drugs permits dose reduction, leading to less frequent or mitigated adverse effects without compromised treatment efficacy.[77,78] Optimal combination regimens have been investigated in numerous clinical trials. It was shown that the combination of CsA (or tacrolimus (Tcr)) with MTX is highly efficient for preventing GvHD.[79] Such combination treatments do not only impact the frequency of acute GvHD but also improve long-term survival in human patients following bone marrow transplantation and therefore have become a standard approach.[69] The more recently studied combination of CsA with MMF accelerates bone marrow engraftment while reducing side effects and the frequency of mucositis compared with the CsA/MTX combination.[80] The combination of CsA with ketoconazole, a CYP inhibitor, reduces CsA clearance and thus the risk for opportunistic fungal infections.[77] This allows the reduction of CsA doses by up to 77% in humans when increasing ketoconazole to 200 mg per day.[77]

A transient increase in ovine serum bilirubin from 1 to 29 ± 11 μM has been described during CsA treatment at 2–3 mg/kg (twice a day in combination with 10 mg/kg ketoconazole) for 9 weeks[73] (Supplementary Table 2). Two weeks after the termination of CsA treatment, serum bilirubin levels returned to 2 ± 0.3 μM.[73] Moreover, albumin decreased from 32 to 22 g/l during the study.[73] Other studies reported a slight reduction in serum potassium from 4.3 to 4.0 mmol/l when 12 mg/kg per day CsA was applied.[76] CsA and prednisolone can further affect hemodynamic parameters such as blood pressure and heart rate (tachycardia), decrease the heart index and both decrease and enhance peripheral resistance in human patients.[81] In sheep, a heart rate increase from 58 to 75 beats per min and a concomitant decrease in the cardiac stroke volume from 86 to 68 ml per beat have been observed.[76] Mean arterial blood pressure increased from 73 to 90 mm Hg and

Table 5 CsA and tacrolimus and their uses in experimental transplantation studies

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics bioavailability/VD*	*Application form*	*Dosage examples*
Ciclosporine A (CsA)					
Direct inhibition of calcineurin Selective inhibition of T-cell proliferation[60]	Human	24.0–93.0[278]	Bioavailability: 61.9% CL: 29.6 L/h VD: 605 L[279]	i.v., p.o.[69]	• Initial dose: p.o. 2–12.5 (10.0) mg of P/kg per day, i.v. 1–20 (3.0) mg of CsA/kg per day q.d. or b.i.d. for 2–13 h, after 7–40 (21) days change to p.o. administration for 150–365 days, i.v. 15 mg of MTX/m^2 on POD 1 and 10 mg of MTX/m^2 on POD 3, 6 and 11[69]
	Mouse	4.1 ± 0.6[280]	At 2.6 mg/kg i.v.: CL: 0.015 L/kg/h VD: 0.07 L/kg[280]	c.p.[281]	• 35 mg of CsA/kg per day[281]
	Rat	6.0–10.0[282] 19.49[67]	CL: 0.20 ± 0.04 L/kg/h MRT: 23.20 ± 5.46 h VD: 4.54 ± 0.68 L/kg[67]	Implantation of CsA collagen matrix around the homograft,[283] s.c.[284,285]	• 10 mg of CsA/kg per day[284] • 1 mg of CsA/kg per day bound in collagen matrix[283]
	Dog	8.5[67]	Poor oral absorption and bioavailability, preparations such as Neoral, Atopica and Sanimmune are not bioequivalent, Neoral achieves much higher blood levels than Atopica (veterinary-labeled oral product): rapid absorption, bioavailability of 23–45%, filled gastrointestinal tract reduces bioavailability by ~ 20%, high distribution in the liver, fat and lymphocytes[212] CL: 6.96 L/h VD: 4.3 L/kg[67]	conjunctival, p.o., topical,[203] i.m., inhalation, i.v.[251]	• 10 mg of CsA/kg/12 h, 2–3 mg of Az/kg/48 h, 0.5 mg of P/kg/12 h (then tapered off)[286] • 10–25 mg of CsA/kg/12 h[212] • Neoral: 5–10 mg of CsA/kg/12 h[212] • Target trough levels: 100–500 ng/ml[212]
	Pig	7.7 ± 2.6[287] 8.1 ± 1.5[218]	Systemic availability: 18 ± 6% CL: 0.87 ± 0.11 L/kg/h VD: 6.5 ± 1.7 L/kg In general: requires 2–4 times higher i.v. or oral dose of CsA than humans[218]	i.m., i.v.,[288,289] p.o.[72]	• 20 mg of CsA/kg per day[288,289] • 10 mg of CsA/kg per day[288,289] • Neonatal: 4 mg of CsA/kg per day, juvenile: 30 mg of CsA/kg per day[72]
	Sheep	12.1 ± 3.1[290]	Abomasal bioavailability (6.4 mg/kg): 0.26 ± 0.09 CL: 0.45 ± 0.05 L/kg/h MRT: 9.6 ± 4.1 h MAT (6.4 mg/kg): 4.7 ± 11.1 h VD: 4.4 ± 2.0 L/kg[290]	i.v.,[73] p.o. [290]	• 2–3 mg of CsA/kg per day b.i.d., 10 mg of Kc/kg b.i.d.[73]
Tacrolimus (Tcr)					
Indirect inhibition of calcineurin Binds to intracellular FK-binding protein Selective inhibition of T cell proliferation[98,99]	Human	In liver-transplanted patients: 12.1 ± 4.7,[291] 12.0,[292] 5.5–16.6[293]	Accumulation index during chronic therapy: 1.3 CL after p.o.: 0.21 ± 0.08 L/kg/h VD after p.o.: 2.4 ± 0.8 L/kg[294]	p.o.[295]	• 0.05 mg of Tcr/kg per day b.i.d.[200]
	NHP	Baboon: 9.6 ± 2.0 (at 10 mg/kg p.o.)[293]	10 mg/kg p.o.: peak plasma concentration of 8.1 ± 1.0 ng/ml reached after 3.8 ± 1.4 h[293]	i.v.,[296] p.o.[104,297]	• 4 mg of Tcr/kg per day[104,297] • 1 mg of Tcr/kg per day, 2 or 5 mg of ASKP1240/kg on POD 0, 3, 7, 11 and 14 and on POD 28–168 0.51 mg of Tcr/kg per day, 1 or 2.5 mg of ASKP1240/kg two times per week[296]
	Rat	2.4 ± 0.3 (at 1 mg), 2.5 ± 0.3 (at 5 mg)[298]	100 times more potent than CsA at 1 mg/kg: CL: 0.42 L/kg/h VD: 4.62 ± 0.03 L/kg[298]	i.m.,[293] i.v.,[298] p.o.[299]	• 0.1 mg of Tcr/kg per day or 1 mg of Tcr/kg per day[299]

Table 5 (Continued)

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics bioavailability/VD*	*Application form*	*Dosage examples*
	Dog	9.0–13.2 (at 0.2 mg/kg i.v.), 5.6–7.9 (at 1 mg/kg p.o.)[293]	Bioavailability after 1 mg/kg p.o.: 5–12% Peak plasma concentration (1.9–4.9 ng/ml) after 1–2 h at 0.2 mg kg i.v.: CL: 0.12–0.19 L/h i.m.: slow absorption, steady-state after 4.0–12.0 (continued up to 24.0)[293]	i.m., i.v.,[293] p.o.[300]	• 0.5 mg of Tcr/kg per day, 5 mg of FTY720/kg per day[300]
	Pig	n.s.i.	n.s.i.	i.v. as continuous infusion,[301] i.v.[302]	• 0.1–0.2 mg of Tcr/kg for 12 days[301] • 0.04 mg of Tcr/kg per day[207]
	Sheep	n.s.i.	n.s.i.	i.v., p.o[277]	• 0.15 mg of Tcr/kg per day for 12 days[103] • 2 days prior Tx: 0.02–0.15 mg of Tcr/kg per day p.o., 1.5 g of MMF per day p.o., perioperatively: 50 mg of ATG i.v. (DDI), 500 mg of MP i.v. 40 mg of MP per day p.o. on POD 1–15 (tapered off towards to 16 mg of MP per day) p.o.[277]

Abbreviations: ATG, antithymocyte globulin; Az, azathioprine; b.i.d., twice a day; CL, total plasma clearance; DDI, duration drip infusion; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; Kc, ketoconazole; MAT, mean absorption time after oral dosing; MP, methylprednisolone; MRT, mean residence time; MTX, methotrexate; NHP, non-human primate; n.s.i., no sufficient information available; P, prednisolone; p.o., oral; POD, postoperative day; q.d., once a day; s.c., subcutaneous; Tx, transplantation; VD, volume of distribution.

peripheral resistance from 16 to 21 mm Hg, respectively, whereas renal function remained unaltered.[76] Those results have been confirmed by a number of studies in human patients.[82,83] Notably, the blood pressure effects in humans are the opposite of those observed in rats at 20 mg/kg but are not altered when 10 mg/kg is given.[84] When investigating the cause of the increase in peripheral resistance, Whitworth *et al*.[76] hypothesized that CsA may directly stimulate the sympathetic nervous system. This assumption was based on the observation that kidney function was normal in the sheep studies, providing no evidence for a renal cause underlying the increase in blood pressure.[76] However, more recent work confirms activation of the renin–angiotensin system in sheep.[85] A number of urogenital CsA complications have been observed in both humans and other animals. In particular, nephrotoxicity is frequently observed in human patients at 5 mg/kg per day CsA given orally.[74] Nephrotoxicity is not restricted to kidney transplantation but is also observed with the transplantation of other solid organs.[85] Frequencies of renal dysfunction at CsA trough levels between 150 and >250 ng/ml after bone marrow transplantation range between 73 and 100% in humans.[86] Consequences include fluid retention[87] and anuria.[88] The situation is similar in rats, in which reduced renal inulin clearance (at dosages of 25 and 50 mg/kg CsA as a single dose) and reduced filtration have been observed.[89] Again, this stands in opposition to the situation in sheep models, in which these effects have not been observed. Despite a distinct reduction in potassium and sodium excretion, effective plasma clearance increased, and no alteration of the glomerular filtration rate or aldosterone concentration have been observed.[73,76] This led to the assumption that CsA may exert direct effects on the renal tubular system and its epithelia,[76] and, in fact, chronic tubular necrosis has been described histologically in sheep.[73] Other studies have indicated that high-dose CsA application may decrease TNF-α levels,[90] whereas gastrointestinal side effects were reported at 5 mg/kg per day in dogs.[3] Similar results have not been reported for sheep during dermal grafting.[73]

Monitoring of the CsA concentration in the peripheral blood was clinically implemented in the early 1980s to reduce the risk of inappropriate individual dosing and since then has been considered decisive.[91] The gold standard for monitoring CsA levels is high-performance liquid chromatography because alternative methods have proven fault-prone, particularly at higher doses.[73]

A number of studies have investigated appropriate methods to monitor the biological effects of applied CsA doses in clinical settings. For example, a positive correlation between calcineurin inhibition and the maximum CsA blood concentration has been described.[92] The assessment of CsA trough levels as the indicative parameter represents a relatively old but reliable approach.[1] Thus, measuring trough levels is the current gold standard in clinics, but there is some evidence that the correlation between CsA trough levels and adverse events or effects is not very strong.[91] A more precise method to monitor CsA levels is the area under the concentration time curve

(AUC) approach. However, this is relatively time-consuming and requires that a high number of blood samples be obtained and is thus less practical in the clinical setting.[58] Some studies have shown that CsA trough levels are inferior to AUC_{0-4h} for predicting clinical manifestations and side effects.[93] A more practical approach may be the measurement of CsA levels 2 h after oral application as this parameter correlates well with the $AUC_{0-4\,h.}$[93]

Tacrolimus. Tcr (or FK506) is a potent macrolide antibiotic with similar pharmacokinetic properties to CsA but with a 10– 100-fold therapeutic potency.[94–96] Tcr forms a complex with the intracellular FK-binding protein, which in turn binds to calcineurin, preventing the NFAT activation cascade at a step that is more upstream compared with CsA (Figure 1).[97–99] Similar to CsA, Tcr is primarily metabolized by CYP450.[97]

Tcr is commonly used following kidney transplantation in humans and is widely applied in experimental transplantation studies.[97] It replaced CsA in experimental kidney transplantation in cynomolgus monkeys, and complication-free survival (>90 days) was reported at 2 mg/kg per day.[100] Dose escalation to 10 mg/kg per day oral administration was shown to significantly improve the health of experimental subjects without affecting relevant biochemical parameters, such as potassium, calcium, urea and creatinine, and without causing histopathological alterations in the liver, kidney and brain.[101] In turn, reduction of the Tcr dose to 0.5 or 1 mg/kg per day ensured mean survival of the grafts until POD 11 or 21 after kidney transplantation, respectively.[100] Target blood levels of Tcr in baboon xenotransplantation models (50% successful engraftment) were 30–40 ng/ml and were reached with dosages of 0.15–0.3 mg/kg per day.[102] In pigs, the target blood level for successful immunosuppression after renal allograft transplantation was 35 ng/ml, reached using a dosage of 0.15 mg/kg per day[103] (Table 6; for an overview of relevant indications, see Supplementary Table 1).

A common and severe adverse effect of Tcr is nephrotoxicity.[97] Lower doses of Tcr (1.0 mg/kg per day orally) could be achieved by concomitant application of the synergistically acting rapamycin (Rpm; 0.5 mg/kg per day orally) in rats and vervet monkeys, significantly reducing the frequency of nephrotoxicity.[104] Specific side effects in different species are shown in Supplementary Table 2.

mTOR inhibitors

Rpm and everolimus: an example for mTOR inhibitors. Rpm is a lipophilic substance that binds to FK-binding protein (FKBP12).[105–107] The resulting complex inhibits a protein named mTOR (*T*arget *o*f *R*apamycin), which leads to decreased protein synthesis.[105–107] In particular, Rpm arrests mitosis in the G1 phase, while binding of transcription factors to the proliferating cell nuclear antigen is also blocked, together inhibiting cell proliferation.[108] Rpm also impedes B-cell functionality via decreased levels of B-cell-activating factor.[109] The drug further inhibits T-cell activation by APCs by inhibiting antigen endocytosis into dendritic cells.[110] Moreover, Rpm increases growth factor expression (TGF-β_1), which enhances fibrogenesis and leads to long-term allograft function.[111] The agent is metabolized by CYP3A in the liver and gut.[112]

Rpm can be applied to prevent and to counter the rejection of transplanted tissue, while various studies have reported antitumorigenic effects and reduced rates of infection with post-transplantation immune suppression[110,111] (Supplementary Table 1). Monotherapy in a rodent renal allograft transplantation model (1 mg/kg per day for 24 weeks) has been described,[113] but the combination of Rpm and CsA is frequently reported for canine and swine transplantation models.[114,115] Detailed dosage regimens in different species are shown in Table 6. Side effects of Rpm application include elevated liver enzymes, thrombocytopenia and mild diarrhea.[116–118] These side effects positively correlate with the applied dose[117] (Supplementary Table 2).

Everolimus (Ev) is a semisynthetic macrolide from the family of mTOR inhibitors.[119] It is structurally similar to Rpm with similar immunosuppressive properties[120] but has better bioavailability.[121] Ev is more hydrophilic, has a shorter elimination half-life (~30 h, half of Rpm) and requires less time (4 days vs 6 for Rpm) to reach a steady state.[119] Similar to the calcineurin inhibitors and Rpm, Ev is biotransformed by CYP3A.[122] In clinical trials, Ev was used at two doses (1.5 and 3.0 mg per day) in combination with CsA and steroids in *de novo* renal transplant recipients.[119] The adverse events of Ev are, for the most part, manageable. One common side effect is hyperlipidemia, with increased serum cholesterol and triglyceride levels (in 30–50% of patients).[123]

Unfortunately, the high frequency of adverse Rpm effects calls for alternative approaches. Recently, the coapplication of Rpm with regulatory T cells (T_{regs}) has been investigated. This combination enables donor-specific tolerance after transplantation while reducing the side effects of RPM.[124] In this combination, Rpm stimulates T_{regs} and selectively blocks effector T cells (T_{effs}), efficiently preventing graft rejection.[124] Synergistic effects of Rpm and T_{regs} were obtained via the depletion of a negative regulator of the mTOR pathway through $T_{regs.}$[125] Given the exceptional benefits and the favorable therapeutic profile of this combination, some authors even expect the treatment regimen to ultimately achieve long-term, donor-specific tolerance after transplantation,[124] one of the 'holy grails' of clinical immunology.[126]

EXPERIMENTAL APPROACHES

The arsenal of 'classic' immunosuppressants, including CsA, Tcr and Rpm (including adjuvants), has been increasingly amended by novel, sometimes experimental approaches, including the use of T_{regs} and anti-CD4 antibodies, which will be reviewed in the following paragraphs.

Regulatory T cells

In vitro studies have identified a sub-population of $CD4^+$ $CD25^+$ T_{regs} that selectively inhibits the proliferative responses of both T_{effs} and naive T cells[127] (see Figure 2). These T_{regs} have

Table 6 Rapamycin and Ev and their uses in experimental transplantation studies

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics bioavailability/VD*	*Application form*	*Dosage examples*
Rapamycin (Rpm)					
Binds to intracellular protein FK506 Resulting complex inhibits mTOR Downregulation of protein synthesis and cell proliferation[108]	Human	62.0,[303] 43.8–86.5[304]	Large inter- and intrasubject variability in oral dose clearance Low systemic availability: 14% rapid absorption: 1–2 h VD: 1.7 L/kg[305]	p.o.[117]	• 24–48 h after Tx 6 mg of Rpm per day, followed by 2 mg of Rpm per day, CsA (trough level: 200–400 ng/ml), 250 mg of MP per day (tapered off to 5–10 mg of MP per day)[117]
	Mouse	2.1–4.8 (at 10–100 mg/kg)[306]	At 15 mg/kg: serum levels higher for s.c. than for p.o.[307]	i.p.,[308] p.o., s.c.[307]	• 50 000 IU per day recombinant human IL-2 starting 6 days prior Tx, 0.25 mg of anti-CD25 antibody per mouse on POD 0, 0.5 mg of anti-CD8 antibody per day on POD 0, 2, 4, 7, 1 mg of Rpm/kg p.o. starting on POD 0[308]
	Rat	25.0[121]	Poor oral bioavailability: $<4\%$[309]	Inhalation,[309] p.o.[307]	• 1 mg of Rpm/kg per day for 24 weeks[113]
	Dog	>60.0[310] 99.5 ± 89.5 h (at 0.1 mg/kg p.o.)[311]	At 0.1 mg of p.o.: $AUC_{0-48\ h}$: 140 ± 23.9 ng/h/ml Maximum plasma concentration: 8.39 ± 1.73 ng/ml[311]	p.o., s.c.[115]	• 15 mg of CsA/kg b.i.d., 0.05 mg of Rpm/kg per day[115]
	Pig	7.3 ± 0.6[312]	n.s.i.	p.o.[114]	• 1.5 mg of Rpm/kg, 15 mg of CsA/kg[114]
Everolimus (Ev)					
Similar to Rpm[313]	Human	28.1 ± 8.4[314]	CL: 15.3 ± 11.6 L/h VD: 250 ± 103 L/m^2 [314]	p.o. [313]	• 0.75 mg of b.i.d.[313]
	Mouse	3.0–6.0[315] 9.8 (at 0.9 mg/kg)[316]	At 0.9 mg/kg i.v.: CL: 0.05 L/kg/h VD: 0.42 L/kg[316]	i.v.,[316] p.o.[315]	• 1.5 mg/kg b.i.d.[315]
	Rat	14.3[316]	At 1 mg/kg i.v.: CL: 1.26 L/kg/h VD: 53 L/kg[316]	i.v., p.o.[316]	• 1.5–15 mg/kg per day p.o., 1–10 mg/kg per day i.v.[316]
	Pig	n.s.i.	Mean 24-h trough concentration: 16.3 ± 6.6 ng/ml[317]	p.o.[317]	• 1.5 mg/kg per day[317]
	Sheep	n.s.i.	n.s.i.	p.o.[317]	• 1.5 mg/kg per day; starting 4 days preoperatively[317]

Abbreviations: BID, twice a day; CL, total plasma clearance; CsA, cyclosporin A; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; IL-2, interleukin-2; IU, international units; MMF, mycophenolate mofetil; MP, methylprednisolone; mTOR, target of Rpm; NHP, non-human primate; n.s.i., no sufficient information available; p.o., oral; POD, postoperative day; s.c., subcutaneous; Tcr, tacrolimus; Tx, transplantation; VD, volume of distribution.

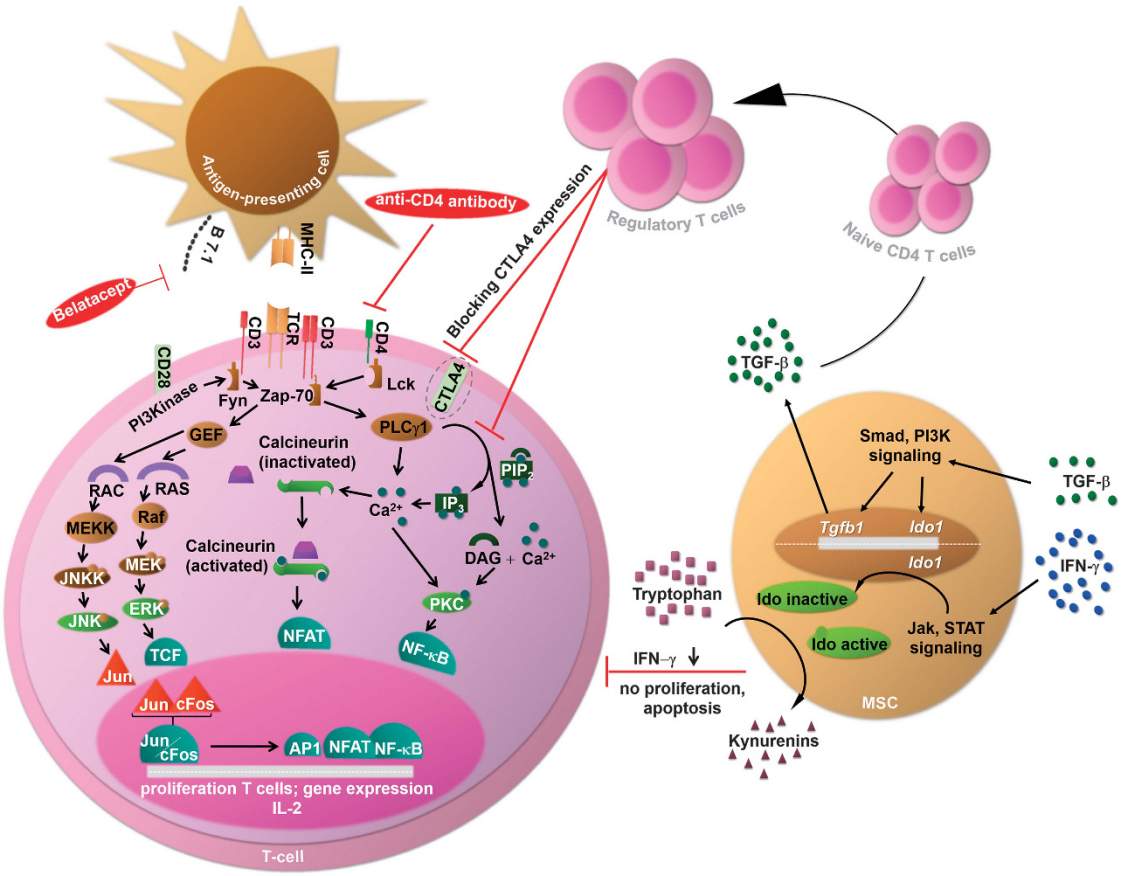

Figure 2 Mechanisms of action of the experimental approaches. Experimental approaches for immunosuppression comprise MSCs, T_{regs}, anti-CD4 antibodies and substances blocking costimulatory pathways. MSCs act on $CD4^+$ T cells via IFN-γ and TGF-β. First, the IFN-γ concentration is reduced by MSCs, inhibiting proliferation and inducing the apoptosis of T cells. Second, the TGF-β concentration is increased by MSCs, driving the differentiation of naive $CD4^+$ T cells into T_{regs}. In turn, T_{regs} directly inhibit $CD4^+$ T-cell proliferation via the suppression of Ca^{2+}-dependent pathways and indirectly act by downregulating costimulatory molecules such as CTLA4. Finally, anti-CD4 antibodies and substances blocking costimulatory pathways (belatacept) impair T-cell activation. CTLA4, cytotoxic T-lymphocyte-associated protein 4; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; Fyn, tyrosine-protein kinase; Ido, indolamine-2,3-dioxygenase; GEF, guanine-nucleotide exchanging factor; IFN-γ, interferon-γ; IP_3, inositol triphosphate; Jak, Janus kinase; JNK, c-Jun N-terminal kinase; JNKK, c-Jun N-terminal kinase kinase; Lck, lymphocyte-specific protein tyrosine kinase; MEK, mitogen-activated protein kinase kinase; MEKK, serine/threonine-specific protein kinase; MSC, mesenchymal stem cell; NF-κB, nuclear factor 'κ light-chain enhancer' of activated B cells; Pip_2, phosphatidyl inositol bisphosphate; PI3K/Pi3 kinase, phosphoinositide 3-kinase; PLCγ, phospholipase; PKC, protein kinase C; RAC, guanosine triphosphate; RAF, serine/threonine-specific protein kinase; RAS, guanosine-nucleotide-binding protein; STAT, signal transducer and activator of transcription; TCF, transcription factor; TCR, T-cell receptor; TGF-β, tumor growth factor-β; T_{regs}, regulatory T cells; Zap-70, zeta-chain-associated protein kinase 70.

also been observed *in vivo*, accounting for ~1–10% of all T cells, but are unable to completely prevent graft rejection on their own.[128] Nevertheless, some studies have shown a positive correlation between the number of circulating T_{regs} and transplant survival.[129]

The immunological impact of T_{regs} can be tremendous. Recently, syngeneic i.v. transplantation of 2×10^6 bone marrow-derived hematopoietic stem cells with 1×10^6 syngeneic T_{regs} resulted in a 100% survival rate among transplanted animals and full donor chimerism[130] (Table 7; for an overview of relevant indications, see Supplementary Table 1). Other studies demonstrated the successful introduction of T_{regs} in several animal models, which may lead to a scientific breakthrough in transplantation medicine and the treatment of autoimmune diseases.[131–133] Adverse effects after the application of T_{regs} have been reported in patients, mice and dogs, ranging from alterations in liver function to cytotoxicity and tumor induction[134,135] (Supplementary Table 2).

Mesenchymal stem cells

Mesenchymal stem cell (MSCs) are pluripotent stromal cells that can differentiate into mesenchymal tissues such as bone, cartilage or adipose tissue.[136] MSCs can easily be derived from numerous sources and expanded *in vitro*.[136,137] The cells are primarily obtained from bone marrow specimens, in which their frequency ranges between 0.001 and 0.01%.[138]

In addition to their role in regenerative medicine as a therapeutic cell population that has been experimentally tested

Table 7 Cell-based strategies for immunosuppression and immunomodulation

Mechanism of action	*Species*	*Application form*	*Dosage examples*
Regulatory T cells (T_{regs})			
Inhibit proliferative response of T_{effs} and naive T cells[127]	Mouse	i.v.[130]	• 2×10^6 BMSCs, 1×10^6 T_{regs}[130]
More effective in combination with Rpm[125]			
	Rat	i.v.[318]	• 0.5 ml (~11.7 μg) T_{regs} exosomes[318]
	Dog	i.v. as infusion[135]	• $0.6–13.7\times10^8$/kg donor peripheral blood lymphocytes[135]
	Pig	i.v.[319]	• Transfusion of 15×10^6 irradiated donor-specific peripheral blood leukocytes/kg, 10 mg of CTLA4IgG4/kg after reperfusion, 10–13 mg of CsA/kg per day for 12 days[319]
Mesenchymal stem cells (MSCs)			
Avoid host immunoreactions by not expressing MHC-II[139]	Human	i.v. as infusion[150]	• 1.4×10^6/kg MSCs ($0.4–9\times10^6$)[141]
Immunomodulation via secretion of various mediators[143]			• 0.6×10^6/kg MSCs[150]
Regulation of T- and B-cell proliferation[142,145]			
	Mouse	i.v. (Fricke, 2014, unpubl. data)	• 5×10^4 MSCs (Fricke, 2014, unpubl. data)
	Rat	i.a.[320]	• 1×10^4 MSCs 24 h after stroke (occlusion and reperfusion)[320]
	Dog	i.v. as infusion[321]	• $1–30\times10^6$ MSCs/kg per day 2–5 times per week[321]
	Pig	i.m. around the graft[322]	• 3×10^6 MSCs[322]
	Sheep	i.v.[323]	• 2×10^6 MSCs/kg[323]

Abbreviations: BMSCs, bone marrow stem cells; CL, total plasma clearance; CsA, cyclosporin A; CTLA4, cytotoxic T-lymphocyte-associated antigen 4; i.a., intra-arterial; Ig, immunoglobulin; i.m., intramuscular; i.v., intravenous; MHC, major histocompatibility complex; Rpm, rapamycin; T_{eff}, effector T cell; Tx, transplantation; VD, volume of distribution.

for a wide range of indications, MSCs also possess considerable immunomodulatory properties. Because MSCs do not express major histocompatibility complex class II (MHC-II) and many costimulatory molecules, including CD80, CD86 and CD40, they can passively avoid host immunoreactions.[139] Moreover, they can actively suppress or modulate immune responses, including complex interactions with T- and B-lymphocytes, dendritic and NK cells.[140] T-cell inhibition by MSCs is not solely MHC-mediated and thus MSCs can suppress both allogeneic and autologous T-lymphocytes.[141,142]

Their immunosuppressive effects are primarily based on soluble mediators but also direct cell interactions via vascular cell adhesion molecule, intercellular adhesion molecule-1, activated leukocyte cell adhesion molecule, lymphocyte function associated and integrins.[143] MSCs can further induce T_{regs}[144] and inhibit B-lymphocyte proliferation by IFN-γ[145] (see Figure 2). MSCs can also reduce antigen presentation on dendritic cells[146] and lead to the increased expression of a number of hematopoietic factors, such as stem cell factor, Flt3-ligand, thrombopoietin, leukemia inhibitory factor, IL-6 and IL-11.[147] MSCs are used both experimentally and clinically to support the engraftment of hematopoietic stem cells.[148]

Significantly enhanced survival rates were observed after the administration of 1.4 ($0.4–9.0\times10^6$) MSCs/kg in a phase II clinical trial enrolling patients with severe GvHD.[149] Interestingly, the results were independent of the MSC donor, indicating a reproducible and strong effect that could be used even in patients with steroid-resistant chronic excessive GvHD, resulting in partial or complete remission in 14 of 19 patients[150] (Table 7).

MSCs have been experimentally applied in models of multiple sclerosis, myocardial infarction and stroke, solid organ transplantation, liver cirrhosis and chronic inflammatory bowel diseases[151] (for an overview of relevant indications, see Supplementary Table 1). Owing to their distinct immunomodulatory properties, MSCs can be applied relatively safely in non-autologous approaches. However, the large size of MSCs can lead to complications after intravascular injection.[152] Clotting of pulmonary capillaries has been described after intravenous application,[153] whereas small cerebral infarcts were observed after intra-arterial (common carotid artery) injection[154] (Supplementary Table 2).

Antibodies

In addition to conventional immunosuppression, poly- and monoclonal antibodies can lead to severe immunosuppression in recipient animals. Anti-lymphocyte globulin (ALG) and anti-thymocyte globulin (ATG) are used to prevent graft rejection[155] and GvHD.[156] These anti-human T-cell xeno-antisera target lymphocyte sub-populations from peripheral blood, lymph nodes, thymus and some T-cell tumors.[155] Animals (rabbit, sheep, horses, donkeys and calves) can be

immunized to produce ATG and ALG. ATG and ALG are immunosuppressive drugs that exert immunomodulatory effects such as T- and B-cell depletion, modulation of leukocyte and endothelium interactions and the induction of T_{regs} and natural killer cells.[157] Furthermore, some authors have observed dysfunctions in antibody production and macrophage activity, as well as defective antibody opsonization.[155] ALG and ATG are commonly used in transplantation animal models, with dosage and application regimens shown in Table 8 (for an overview of relevant indications, see Supplementary Table 1). Side effects such as anaphylaxis, leukopenia, thrombocytopenia and granulocytopenia may occur[158] (Supplementary Table 2). When establishing a new antibody therapy, it should be ensured that the antibody only binds to the desired antigen. If the therapeutic antibodies also recognize other targets, this can lead to serious side effects such as the induction of cytokine release syndrome, a life-threatening systemic release of cytokines.[159] Nonspecific antibody binding can be avoided by *ex vivo* treatment of the graft or by performing prior testing in a humanized mouse model.[160]

Rituximab is a human immunoglobulin G1 (IgG_1) kappa monoclonal antibody.[155] Its variable regions were isolated from a murine anti-CD20 antibody (IDEC-2B8).[155,161] Rituximab binds to malignant and normal B cells expressing the CD20 molecule with high affinity[162] but not to other normal cell types.[155,163] The antibody binds to human complement, lyses lymphoid B cells and other $CD20^+$ cells through antibody-dependent cellular cytotoxicity, induces apoptosis in human lymphoma cells[164] and inhibits cell proliferation via the induction of tyrosine phosphorylation.[155,162] This antibody was tested in preclinical studies using cynomolgus monkeys (Supplementary Table 1). One model used Rituximab administration at 0.4–6.4 mg/kg[163] (Table 8). Cells that bound the rituximab molecule disappeared completely, and full B-cell reconstitution was observed within 40 days.[163] High dose studies performed with rituximab at 16.8 mg/kg lead to the depletion of all B cells in the lymph nodes in cynomolgus monkeys.[163] Specific toxicities were not observed in recipient animals[163] (Supplementary Table 2). The first *ex vivo* studies also showed no effects (binding/depletion) on canine B cells, and Rituximab failed in canine lymphoma treatment.[165] In human patients, a weekly Rituximab dose of 375 mg/m^2 is safe and shows significant clinical activity in many lymphoma patients.[155,162]

Furthermore, it is experimentally possible to reduce GvHD severity without conventional immunosuppression (one of the most important requirements for transplantation immunology[126]) via *ex vivo* treatment of the graft with anti-human CD4 antibodies; notably, the antitumor effects of the graft are not minimized[166,167] (one of the most important requirements for allogeneic hematopoietic stem cell transplantation[168]).

Blocking costimulatory pathways

Costimulatory signals have an important role in T-cell activation, proliferation and differentiation.[169] The CD28/B7 costimulatory pathway is one of the best characterized pathways. CD28 is constitutively expressed on all T-cell subsets in mice and on 95% of $CD4^+$ T cells as well as on 50% of $CD8^+$ T cells in humans.[170] B7 comprises in two subforms, B7.1 (CD80) and B7.2 (CD86), and is constitutively expressed on the surface of APCs.[171] It is also found in T cells.[172] The following three signals are required for complete T-cell activation: (i) interaction of the bound antigen with a T-cell receptor (TCR), (ii) binding of CD80 and CD86 molecules on an APC to the CD28 receptor on T cells and (iii) binding of CD28 and B7 in the presence of TCR stimulation, leading to IL-2 expression,[173–175] cytokine transcription[176–179] and T-cell proliferation.[180–182] Failure of costimulation leads to T-cell anergy, that is, reduced proliferation, differentiation and cytokine production.[183] Inhibition of the costimulatory CD28/B7 pathway is one approach to prevent graft rejection, for example, by administering belatacept.[184,185] Belatacept binds to B7.1 and B7.2 on APCs, prevents CD28-mediated costimulation and thus impairs T-cell activation[185] (see Figure 2). Typical dosing ranges between 5 and 10 mg/kg, and belatacept serum concentrations between 136 and 238 μg/ml have been described after the first application. The median elimination half-life is 8–9 days.[185] Studies using other costimulatory inhibitors are currently ongoing but thus far have revealed mixed results and some safety concerns.[186]

RECOMMENDATIONS FOR IMMUNOSUPPRESSIVE TREATMENTS AFTER EXPERIMENTAL TISSUE AND SOLID ORGAN TRANSPLANTATION

Sufficient immunosuppression is required for the successful realization of experimental transplantation in animals. In principal, long-term survival after transplantation can be ensured in the following two different ways: by the administration of immunosuppressive medication or by the induction of immunological tolerance against the donor tissue.

The classical immunosuppressive protocols have been established for years and usually include a mono- or combination therapy of immunosuppressive drugs targeting immune cells. Based on synergistic effects between different immunosuppressants,[187] a combined approach using conventional agents such as CsA, MMF and prednisolone is recommended, especially for long-term immunosuppression.[18,19] This also allows for the dose reduction of single immunosuppressants, ideally leading to less frequent and less severe side effects.[77]

The induction of immunological tolerance can be initiated by newly developed cellular therapy approaches.[131–133] One decisive factor for long-term tolerance is hematopoietic chimerism after transplantation.[188] This can be achieved, for example, by the infusion of donor lymphocytes and concurrent high-dose CY treatment after transplantation.[189] However, valid data on cell-based therapies are missing for several animal models, despite very intensive research in recent years. For this reason, successfully established immunosuppressive and immunomodulatory protocols are listed with respect to model and species (Tables 9 and 10).

Table 8 Antibodies for immunosuppression and immunomodulation

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics Bioavailability/VD*	*Application form*	*Dosage example*
Anti-lymphocyte globulin (ALG)					
Immunomodulatory effects on T and B cells, leucocytes and endothelium cells T and B cell depletion Induction of T_{regs}[157]	Human	25.5 days[324]	VD: 2.0 l CL: 0.017 l/h [324]	i.v.[325]	• 3 mg of CsA/kg on POD 1–180, 10 mg of MTX per m^2 on POD 1,3,6, total dose: 30 (20–90) mg of ALG/kg on POD 1–3[326] • 15 and 30 mg of ALG per kg per day [325]
	NHP	n.s.i.	n.s.i.	s.c.[327]	• 40 mg of ALG/kg on POD −1,1[327]
	Mouse	n.s.i.	n.s.i.	i.p.[328]	• 0.1 mg of ALG per mouse per day on POD 0,1[328]
	Rat	n.s.i.	n.s.i.	i.p.[329]	• 1 ml ALG-serum per rat, 2×10^7 donor splenocytes as intrathymic injection[329]
	Dog	n.s.i.	n.s.i.	p.o., s.c.[330]	• 2 ml ALG-Serumper kg per day on POD −5 to −1, 20 mg of CsAper kg per day on POD 2–120[330]
Anti-thymocyte globulin (ATG)					
	Human	14.3–45 days[331] 36 h–18 days[332]	VD: 0.07–0.17 l/kg [331] Maximum serum levels reached 1–3 days after final dose[332]	n.s.i.[333]	• 1 mg of ATG/kg on POD 1 and 0.5 mg of ATG/kg on POD 2–4[333]
	NHP	n.s.i.	n.s.i.	i.v.[102]	• Splenectomy and TBI on POD −7, 4 mg of LoCD2b/kg on POD −5, −4, i.v. 20 mg of ATG-serum/kg on POD −3, TI with 700 cGy on POD −2, i.v. 20 ng PC/kg/h On POD −2 to 4, 0.15–0.3 mg of Tcrper kg per day on POD −6 to 28[102]
	Mouse	n.s.i.	n.s.i.	i.p.[334]	• 0.1 mg of ATGper mouse twice per week, 0.5 mg of CTLA4-Igper mouse on POD 0, 0.25 mg of CTLA4-Igper mouse on POD 2,4,6,8,10[334]
	Rat	n.s.i.	n.s.i.	i.v.[335]	• Single dose of 10 mg of ATG/kg at the time of engraftment[335]
	Dog	n.s.i.	n.s.i.	p.o., s.c.[336]	• 5 mg of CsA/kg BID from 1 day prior to 12 weeks after infection, 7.5 mg of MMF/kg BID starting on day of infection for 4 weeks, 1 mg of ATGper kg per day. Starting 2 days prior to 2 days after infection[336]
	Pig	n.s.i.	n.s.i.	n.s.i.[337]	• CsA (target blood level: > 200 ng/ml), 50 mg of Az per animal BID, 50 mg of P per animal per day tapered, 115 mg of ATG per animal BID[337]
	Sheep	n.s.i.	n.s.i.	i.v., p.o.[277]	• 0.02–0.15 mg of Tcrper kg per day on POD −2, 1.5 g of MMF per day, 40 mg of MP per day on POD 1–15 (tapered off towards to 16 mg of MP per day), perioperatively: 50 mg of ATG (DDI), 500 mg of MP[277]

Table 8 (Continued)

Mechanism of action	*Species*	*Plasma half-life (h)*	*Pharmacokinetics Bioavailability/VD*	*Application form*	*Dosage example*
Rituximab (RTB)					
Anti-CD20-antibody which binds human complement lysis of lymphoid B Cells[289]	Human	88.0 (at 20–30 mg)[338] 445.0 ± 361.0 (at 250–375 mg per m^2)[339]	CL: 0.044 ± 0.064 l/h MRT: 516.7 ± 247.7 h VD: 11.16 ± 3.20 L[327]	n.s.i.[162]	• 375 mg of RTB per m^2 weekly[162]
	NHP	Cynomolgus monkey: ~ 168.0 (at 20 mg/kg s.c.)[340]	At 20 mg/kg s.c.: maximum plasma concentration of 300 µg/ml at day 2 At 10 mg/kg i.v.: Maximum plasma concentration of 328.5 ± 34.4 µg/ml 1 h after administration[328]	i.v.[163], s.c.[341]	• 0.4–6.4 mg of RTB/kg, for 40 days[163] • 1.5 mg of ATG/kg, 19 mg of RTB/kg weekly starting on POD 7, Tcr at dosages to reach blood levels of 20–30 ng/ml, splenectomy on POD 7[341]
	Mouse	192.0 and 264.0 (at 1 and 40 mg/kg i.v.)[342]	At 1 mg/kg i.v: CL: 8.3 ml/kg/h VD: 0.12 l/kg [330]	i.v., s.c.[342]	• 1–40 mg of RTB/kg [342]
CD28/B7					
Plays an important role in T cell activation, proliferation, and differentiation[331]	Human	196.6 ± 57.2 [331]	CL: 0.51 ± 0.14 ml/kg/hVD: 0.12 ± 0.03 l/kg [331]	i.v.[343]	• 5 mg/kg in a 4 or 8 week interval[343]

Abbreviations: Az, azathioprine; BID, twice a day; cGy, centigray; CL, total plasma clearance; CsA, cyclosporin A; DDI, duration drip infusion; GvHD, graft-versus-host-disease; h, hour; i.a., intraarterial; i.v., intravenous; LoCD2b, rat-anti-primate CD2 IgG2b; MMF, mycophenolate mofetil; MP, methylprednisolone; MRT, mean residence time; MTX, methotrexate; NHP, non-human primate; n.s.i., no sufficient information available; P, prednisolone; p.o., oral; PC, prostacyclin; POD, postoperative day; s.c., subcutaneous; TBI, total body irradiation; Tcr, tacrolimus; TI, thymic irradiation; T_{regs}, regulatory T cells; Tx, transplantation; VD, volume of distribution.

Table 9 Model-based recommendations for immunosuppressive and/or immunomodulatory protocols after cell or tissue transplantation

Species	*Indication*	*Strategy*	*Survival*	*Immunosuppressive/immunomodulatory protocol*	*Target serum levels*
NHP	Bone marrow xenoTx[102]	Immunopharmacotherapy	>187 days	Splenectomy and TBI on POD −7, 4 mg of LoCD2b/kg on POD −5, −4, i.v. 20 mg of ATG-serum/kg on POD −3, TI with 700 cGy on POD −2, i.v. 20 ng PC/kg/h on POD −2 to 4, 0.15–0.3 mg of Tcr/kg per day on POD −6 to 28[102]	• 30–40 ng of Tcr/ml[102]
	Facial alloTx[344]	Pharmacological monotherapy	>177 days	0.5–1 mg of Tcr/kg per day i.v. as continuous infusion on POD −1 to 26, following i.m Tcr. QD[344]	• On POD −1 to 26: 30–50 ng of Tcr/ml following: 10–20 ng of Tcr/ml[344]
	Islet xenoTx[345]	Immunopharmacotherapy	>158 days	Intraportal Tx of wild-type porcine islets, anti-CD25 mAb, FTY720, Ev or Tcr, anti-CD154 mAb, leflunomide[345]	• 10.8 ± 8.2 ng of FTY720/ml, 22.1 ± 13.7 ng of Ev/ml or 6.7 ± 4.1 ng of Tcr/ml, $1\,000 \pm 280$ µl of anti-CD154 mAb/ml, 12–40 µg of leflunomide/ml[345]
Mouse	Bone marrow xenoTx[346]	Immunotherapy	>233 days	i.p. on POD −6, −1: 0.1 ml of rat anti-mouse CD4, 0.1 ml of rat anti-mouse CD8, 500 µg of rat anti-mouse Thy-1.2, 400 µg of murine anti-NK1.1 mAbs, TBI (3 Gy) and TI (7 Gy) on POD 0, 60×10^6 rat BMCs[346]	• n.s.i.[346]
	Skin alloTx after successful liver alloTx[347]	Strain combination	>100 days (45–100% survival)	No immunosuppression necessary using various strain combinations, for example, liver donor: B10.AKM, recipient: B10.BR, skin donor: B10.AKM or liver donor: B10, recipient: C3H, skin donor: B10; skin alloTx was performed 3 months after successful liver alloTx[347]	
	Skin alloTx[348]	Immunopharmacotherapy and cell-based therapy	>120 days	C3H/HeN mice were primed with i.v. injection of 5×10^7 viable AKR/J spleen cells on POD −9, 150 mg of CP/kg i.p. on POD −7, skin allograft Tx from AKR/J donors on POD 0[348]	• n.s.i.[348]
	Bone marrow/splenocyte alloTx[166]	*Ex vivo* immunotherapy and cell-based GvHD prevention	>30 days	Single incubation and washing of 1.4×10^8 donor bone marrow cells and 1.4×10^8 splenocytes from human $CD4^{+/+}$, murine CD4 knockout, HLA-$DR3^{+/+}$—(TTG)-C57Bl/6 mice with 800 µg of anti-human CD4 antibodies (MAX.16H5 IgG_1) in 15 ml of Dulbecco's modified Eagle's medium for 2 h at room temperature in the dark. TBI of Balb/c^{wt} recipient mice with 8 Gy (X-ray)[166]	• n.s.i.[166]
	Islet alloTx in combination with autologous MSCs Tx[349]	Cell-based therapy	>100 days	5×10^5 recipient-derived MSCs were administered intragraft (local) with BALB/c islets into C57BL/6 kidney Tx[349]	• n.s.i.[349]
Rat	Stem cell xenoTx after MCAo[350]	Pharmacological monotherapy	Robust survival 30 days after Tx	Tx of 3×10^5 hCNS stem cells 7 days post-dMCAo, 10 mg of CsA/kg per day i.p. on POD −1 to 28[350]	• n.s.i.[350]
	Skin alloTx[351]	Pharmacological monotherapy	>120 days	3.2 mg of Tcr/kg i.m. 5 days per week for 2 weeks, starting on POD 0, afterwards maintenance with 0.32 mg of Tcr/kg × 2/week [351]	• n.s.i.[351]
	Neural xenoTx[187]	Pharmacological combination therapy	>84 days (100% survival)	i.m. 1 mg of Tcr/kg per day for 12 weeks, p.o. 20 mg of P/kg per day or 40 mg of MMF/kg per day for 2 weeks[187]	• n.s.i.[187]
Dog	Bone marrow Tx[352]	Pharmacological combination therapy	>730 days	TBI with a single dose (28.5 cGy/min) of 200 cGy, within 4 h after TBI 2.5×10^8 BMCs/kg, p.o. 15 mg of CsA/kg b.i.d. and 10 mg of MMF/kg b.i.d. on POD −1 to 63, then tapered off[352]	• n.s.i.[352]

Table 9 (Continued)

Species	*Indication*	*Strategy*	*Survival*	*Immunosuppressive/immunomodulatory protocol*	*Target serum levels*
	Skin alloTx in combination with donor bone marrow Tx[188]	Pharmacological combination therapy and cell-based therapy	Induced long-term tolerance	i.v. infusion of ~2×10^8 donor mononuclear BMCs within 8 h of TBI (400 cGy), 15 mg of CsA/kg b.i.d. p.o. on POD 1–63, then tapered (20–30% per month), 10 mg of MMF/kg b.i.d. p.o. on POD 0–63, then tapered (20–30% per month), donor skin Tx were performed 4 months after BMC Tx[188]	• n.s.i.[188]
	Islet alloTx[353]	Pharmacological monotherapy	>175 days graft survival	10 mg of CsA/kg per day on POD −2 to 11, following 5 mg of CsA/kg per day, on POD 0 microencapsulated islet alloTx was performed[353]	• Low-dose CsA, drug levels were below detectable limits: <30 ng of CsA/ml[353]
Pig	Bone marrow alloTx[354]	Immunopharmacotherapy	Induced long-term tolerance	TI (700 cGy) and 0.05 mg of pCD3-CRM9/kg i.v. on POD −2, 15–30 mg of CsA/kg per day p.o. on POD −1 to 30, Tx of $100–200\times10^8$ PBSCs on POD 0[354]	• 300–800 ng of CsA/ml[354]
	Tissue alloTx/skin xenoTx[18,19]	Pharmacological combination therapy	>90 days	P.o. 40 mg of CsA/kg per day, 500 mg of MMF per day, 2 mg of P/kg per day (tapered off towards to 0.1 mg of P/kg per day)[18,19]	• 100–300 ng of CsA/ml[18]
	Skin alloTx with donor bone marrow Tx[354]	Pharmacological combination therapy and cell-based therapy	>500 days	TI (700 cGy) and 0.05 mg of pCD3-CRM9/kg i.v. on POD −2, 15–30 mg of CsA/kg per day p.o. on POD −1 to 30, Tx of $100–200\times10^8$ PBSCs on POD 0, donor skin alloTx on POD 60[354]	• 300–800 ng of CsA/ml[354]
	Islet alloTx[337]	Immunopharmacotherapy	Up to 41 days	CsA, 50 mg of Az per animal b.i.d., 50 mg of P per animal per day tapered, 100 mg of DSG per animalper day, 115 mg of ATG per animal b.i.d. (animals weighted 48–80 kg)[337]	• >200 ng of CsA/ml[337]
Sheep	HSC xenoTx in early gestational age fetus[355]	Cell-based therapy	Long-term chimerism for several years	XenoTx of human HSCs in 50–60-day-old sheep fetus[355]	
	MPCs alloTx for posterolateral lumbar spine fusion[356]		>270 days	Hydroxyapatite: tricalcium phosphate porous ceramic graft with $25–225\times10^6$ MPCs[356]	
	Skin xenoTx[73]	Pharmacological monotherapy	>60 days	i.v. 2–3 mg of CsA/kg per day b.i.d., 10 mg of Kc/kg b.i.d.[73]	• Maintenance around 1500 ng of CsA/ml[73]

Abbreviations: ATG, antithymocyte globulin;Az, azathioprine; b.i.d., twice a day; BMCs, bone marrow cells; cGy, centiGray; CsA, cyclosporin A; dMCAo, distal middle cerebral artery occlusion; DSG, 15-deoxyspergualin; Ev, everolimus; hCNS, human central nervous system; HSC, hematopoietic stem cell; i.m., intramuscular; i.v., intravenous; Kc, ketoconazole; LoCD2b, rat anti-primate CD2 IgG2b; mAbs, monoclonal antibodies; MMF, mycophenolate mofetil; MPCs, mesenchymal progenitor cells; MTX, methotrexate; NHP, non-human primate; n.s.i., no sufficient information available; P, prednisolone; p.o., oral; PBSCs, peripheral blood stem cells; PC, prostacyclin; POD, postoperative day; q.d., once a day; Rpm, rapamycin; TBI, total body irradiation; Tcr, tacrolimus; TI, thymic irradiation; TTG, triple transgenic; Tx, transplantation.

Table 10 Model-based recommendations for immunosuppressive and/or immunomodulatory protocols after solid organ transplantation

Species	*Indication*	*Strategy*	*Survival*	*Immunosuppressive/immunomodulatory protocol*	*Target serum levels*
NHP	Heterotopic cardiac xenoTx[357]	Immunopharmacotherapy	>365 days	50 mg of anti-CD40 antibody/kg per week, 19 mg of anti-CD20 antibody on POD −14, −7, 0 and 7, 5 mg of ATG/kg on POD −2 and −1, 20 mg of MMF/kg b.i.d., 2 mg of steroids/kg tapered off in 4–6 weeks[357]	• n.s.i.[357]
	Liver xenoTx[358]	Immunopharmacotherapy	9 days	Induction therapy: Three doses of thymoglobulin on POD −3, LoCD2bn, CVF, 25 mg/kg anti-CD154 on POD −1, 0 and 5, Az on POD −1 and 0, Maintenance therapy: Started on POD −1 with Tcr and 10 mg of MP/kg on POD 0 then tapered off[358]	• 10–25 ng of Tcr/ml[358]
	Renal xenoTx[359]	Immunopharmacotherapy	>125 days	Single dose of 50 mg of anti-CD4/kg i.v. between POD −3 and −1, 50 mg of anti-CD8/kg i.v. on POD 0, 20 mg of anti-CD154/kg i.v. on POD 0, 7 and 14 and then biweekly, MMF (applied dosage in previous studies:[360] 15 mg/kg s.c. b.i.d. on POD 0–14, then q.d.) and steroids[359] (applied dosage in previous studies:[360] 20 mg of MP on POD 0, 16 mg of MP on POD 1, 12 mg of MP on POD 2, 8 mg of MP on POD 3, 4 mg of MP on POD 4, 3 mg of MP on POD 5–14, then 1 mg per day)	• n.s.i.[359]
	Renal xenoTx[361]	Immunopharmacotherapy	136 days	10 mg of ATG/kg on POD −3, 10 mg of RTB/kg on POD −2 and 50 mg of RTB/kg on POD −1, 0, 4, 7 and 14 and weekly, 100 U CVF on POD −1 and 0, 0.01 mg of Rpm/kg b.i.d. from POD −3, 5 mg of MP/kg per day tapering to 0.25/kg per day, 10 mg of tocilizumab/kg on POD −1, 7, 14 and every 2 weeks, 0.5 mg of etanercept/kg on POD 0, 3, 7, 28 and 40[361]	• 8–12 ng of Rpm/ml[361]
	Renal alloTx[362]	Immunopharmacotherapy	>3478 days	Splenectomy, TBI (1.5 Gy), TI (7 Gy) on POD −1, 15 mg of CsA/kg per day until POD 28, i.v. 0.3–3.5×10^8/kg donor bone marrow cells on POD 0[362]	• >300 ng of CsA/ml[362]
Mouse	Cardiac alloTx and pretreated donor spleen cells[363]	Cell-based therapy	35±14.4 days	Pretreatment of donor spleen cells with mitomycin C, on POD −8 transfusion of donor spleen cells, on POD −7 to −1 1.5 mg of Rpm/kg per day p.o.[363]	• n.s.i.[363]
	Cardiac alloTx in combination with liver alloTx[347]	Strain combination	>100 days (45–100% survival)	No immunosuppression necessary using various strain combinations, for example, B10 with C3H, A.TH with A.TL, B10.BR with C3H; simultaneous heterotopic cardiac alloTx of donor origin in recipients of liver allografts on the same day[347]	
	Liver alloTx[347]	Strain combination	>100 days up to >375 days	No immunosuppression necessary using various strain combinations, for example, B10 with C3H, DBA2 with B10.D2, B10.BR with C3H, B10.D2 with B10JHTG, A.SW with A.TH, B10.BR with B10[347]	
	Renal alloTx in combination with DCs and CHO cells[364]	Cell-based therapy	>80 days (60% survival)	i.p. injection of 10×10^6 CHO cells (transfected with a vector encoding murine OX-2, which leads to an overexpression of OX-2 on the surface), infusion of 5×10^6 DCs (1:1 mixture of DCs cytokine-transduced with either Ad-IL-10 or Ad-TGF-β) into the portal vein, 36 h later renal alloTx were performed[364]	
Rat	Cardiac alloTx[271]	Pharmacological monotherapy	>200 days	p.o. 40 mg of MMF/kg per day[271]	• n.s.i.[271]
	Liver alloTx[365]	Strain combination	>100 days	No immunosuppression necessary using various strain combinations, for example, donor: DA, DA-P or DA-L, recipient: PVG[365]	
	Renal alloTx and pretreated donor spleen cells[366]	Cell-based therapy	>200 days	i.v. infusion of 1×10^8 pretreated spleen cells on POD −7 and 1, 2 mg of AAT per rat i.p. on POD −1, pretreatment of spleen cells: incubation with 150 mg of ECDI per 3.2×10^8 spleen cells[366]	• n.s.i.[366]

Table 10 (Continued)

Species	*Indication*	*Strategy*	*Survival*	*Immunosuppressive/immunomodulatory protocol*	*Target serum levels*
Dog	Heterotopic cardiac alloTx[367]	Immunopharmacotherapy	>538 days	TLI (total cumulative dose of 1800 rad over 4 weeks), cardiac Tx 72 h after completion of radiotherapy, 0.25–2.0 mg of Az/kg per day i.m. for 90 days, 4 mg of ATG/kg i.m. on POD 0, 2, 4, 6, 8 and 10[367]	• n.s.i.[367]
	Orthotopic liver alloTx[368,369]	Pharmacological monotherapy	>241 days	20 mg of CsA/kg per day on POD 1–30, then reduced to 15 mg of CsA/kg per day until POD 90[369]	• >200 ng of CsA/ml[369]
	Renal alloTx[286,370]	Pharmacological combination therapy	Up to 1440 days	p.o. 10 mg of CsA/kg/12 h, 2–3 mg of Az/kg/48 h, 0.5 mg of P/kg/12 h (then tapered)[286,370]	• 400–500 ng of CsA/ml for the first 6 months after Tx, 350–450 ng of CsA/ml thereafter,[286,370] decrease of CsA dosage ~32.9±13.9% via usage of 5 mg of Fcz/kg per day[371]
	Renal alloTx in combination with donor bone marrow Tx[188]	Pharmacological combination therapy and cell-based therapy	>2078 days	i.v. infusion of ~2×10^8 donor mononuclear BMCs within 8 h of TBI (200 cGy), 15 mg of CsA/kg b.i.d. p.o. on POD 1–63, then tapered (20–30%/month), 10 mg of MMF/kg b.i.d. p.o. on POD 0–63, then tapered (20–30%/month), donor renal Tx was performed following TBI but before BMC Tx[188]	• n.s.i.[188]
Pig	Cardiac alloTx in combination with renal alloTx[372]	Pharmacological monotherapy	>269 days	i.v. 10–13 mg of CsA/kg per day on POD 0–11[372]	• 400–800 ng of CsA/ml[372]
	Liver alloTx[289]	Pharmacological monotherapy	>126 days	i.m. 20 mg of CsA/kg per day on POD −1 to 19[289]	• n.s.i.[289]
	Renal alloTx in combination with donor bone Tx[354]	Pharmacological combination therapy and cell-based therapy	Induced long-term tolerance	TI (700 cGy) and 0.05 mg of pCD3-CRM9/kg i.v. on POD −2, 15–30 mg of CsA/kg per day p.o. on POD −1 to 30, Tx of 100–200$\times10^8$ PBSCs on POD 0, donor-matched renal alloTx on POD 98 or 190[354]	• 300–800 ng of CsA/ml[354]
	Renal alloTx[288]	Pharmacological monotherapy	>100 days >730 days[373]	i.v. 10 mg of CsA/kg per day for 12 days[288]	• Reached serum levels: 701±40 ng of CsA/ml[288]
Sheep	Uterus alloTx	Pharmacological combination therapy	>180 days	p.o. 8 mg of CsA/kg per day, i.m. 5 mg of P/kg per day on POD −2 to 14[374]	• 150 ng of CsA/ml was reached after 5 days of CsA administration[374]
		Immunopharmacotherapy	>118 days, showed signs of estrus	p.o. 0.02–0.15 mg of Tcr/kg per day and p.o. 1.5 g of MMF per day on POD −2, maintain until the end of the trial, p.o. 40 mg of MP per day on POD 1–15 (tapered off towards to 16 mg of MP per day), perioperatively i.v.: 50 mg of ATG (DDI), 500 mg of MP[277]	• 3000–6000 ng of Tcr/ml[277]

Abbreviations: AAT, α1-antitrypsin (key serine protease inhibitor); ATG, antithymocyte globulin; Az, azathioprine; b.i.d., twice a day; cGy, centiGray; CHO, Chinese hamster ovary; CsA, cyclosporin A; CVF, cobra venom factor; DCs, dendritic cells, DDI, duration drip infusion, ECDI, 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide (induces apoptosis of the spleen cells); Fcz, fluconazole; GCSF, granulocyte colony-stimulating factor; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; LoCD2b, monoclonal mouse anti-human CD2b antibody; MMF, mycophenolate mofetil; MP, methylprednisolone; MSCs, mesenchymal stem cells; NHP, non-human primate; n.s.i., no sufficient information available; P, prednisolone; p.o., oral; POD, postoperative day; q.d., once a day; Rpm, rapamycin; RTB, Rituximab; s.c., subcutaneous; TPC, α-Gal-polyethylene; Tcr, tacrolimus; TI, thymic irradiation; TLI, total lymphoid irradiation; Tx, transplantation; U, unit.

GENERAL RECOMMENDATIONS FOR THE APPLICATION OF IMMUNOSUPPRESSIVE TREATMENTS IN EXPERIMENTAL STUDIES

A number of factors should be carefully considered when planning and carrying out immunosuppressive interventions. These comprise, but are not limited to, the following: bioavailability and metabolization of the immunosuppressant in the host species, the treated condition, including its pathophysiological profile, and the applied transplantation strategy. All of these factors might exert a direct influence on the effectivity of a particular immunosuppressive regimen. Thus, substantial inter- and even longitudinal intraindividual differences in immunosuppression efficacy have been reported, usually requiring multiple immunosuppressive medications as well as continuous monitoring and protocol adoption in human patients.

On the other hand, experimental studies often rely on fixed dosing schemes for immunosuppression and often do not consider interindividual disparities in pharmacokinetics and pharmacodynamics, as observed in both humans[58] and animals. Such differences may also emerge based on age, sex, comorbidities and concurrent medications (polypharmacy).[5] Importantly, a plethora of potential confounding factors may explain why it is so challenging to provide general and clear guidelines for immunosuppression in experimental studies. To cope with this complexity, exploratory pilot studies, including trough and peak level measurements of effective blood concentrations in individual species and transplantation models before the main experiment, are recommended. In the latter case, appropriate controls for immunosuppression safety/efficacy and, if possible, individualized dosing adjustment protocols should be considered to ensure the reproducibility, transferability and validity of results with respect to the clinical situation.

In addition to fixed dosing schemes, the application of single-drug immunosuppressive treatments, primarily targeting the adaptive immune system, is often reported in experimental protocols. The adaptive immune system is the most powerful instrument to damage and reject allo- and xenografts, but elements of the innate immune system, such as dendritic cells[190] and defensins,[191] also have the capacity to damage the graft and 'flare-up' immunological responses. Thus, key elements of the innate immune system (for example, Toll-like receptors triggered by pathogens) can also contribute to the (re-)activation of adaptive immune cells. Moreover, the innate immune system significantly differs with respect to its configuration, localization and activation. This is exemplified by the very complex, and thus far only partially understood, activation of microglia in the brain. CsA application, often performed after non-autologous intracerebral cell transplantation, effectively blocks T-cell proliferation and activation, but its influence on the microglia in the brain is minimal. Thus, thorough immunosuppression in experimental setups should ideally target both the adaptive and the innate immune systems in the targeted transplantation area. Therefore, to increase the security and applicability of cell and organ transplantations, appropriate preclinical and clinical trials should be performed to investigate combination therapies with the active pharmaceutical ingredients discussed in this review.

Another important factor is the targeted transplantation area. The existence of so-called 'immunoprivileged' domains within the adult mammalian body has been postulated, and immunosuppression is often reduced or omitted upon transplantation into these areas. It has been shown that transplantation of progenitor or mature cells into 'immunoprivileged' areas shielded by biological barriers, such as the intact BBB or the renal capsule, can prevent strong immuno reaction and graft rejection.[192,193] However, this notion may not be entirely correct as 'immunoprivileged' areas are not characterized by the complete absence of immunoreactions but rather by different mechanisms of immune system activation, locally maintained mechanisms of immunotolerance and/or limited vascular egress of immunocytes into these areas. However, graft rejection can occur after a breakdown of barrier function and the cessation of processes mediating immunotolerance, for example, following injury and inflammation. Furthermore, the death of mature cells within the transplant and subsequent processing of cell debris by local phagocytes and antigen processing can pave the way for an adaptive immune response.[190] In the case of blood vessel ingrowth into the area, recognition of the graft by the immune system can then induce delayed but strong immunoreactions.[194]

Another important consideration is the transformation of therapeutic drug doses between species of different sizes. In particular, therapeutic dose extrapolation between animals and humans via a linear, body weight-based conversion scheme (applying a fixed dose of mg/kg body weight (BW)) can lead to severe dose underestimation in smaller animals. A common example is metoprolol, which requires a 7.5-d to 25-fold human dose to induce comparable effects in rats.[195,196] In turn, unexpected but relevant side effects after BW-based dose conversion have been reported for psychomimetic[197] and immunomodulatory applications.[198] The body surface area (BSA) normalization method has been suggested for proper allometric dose translation. BSA correlates well with a number of relevant physiological parameters among mammals, including basal metabolism, blood volume and renal function.[199] However, BSA-based dose conversion does not prevent interindividual and intraspecies pharmacokinetic variability.[200] Thus, individual monitoring of effective blood concentrations and continuous dose adjustment remain highly recommended. This also permits compensation for size-independent, interspecies and interindividual differences,[195] which cannot be prevented by BSA-based dose transformation. Additionally, it should be determined whether biological effects can be achieved via the *ex vivo* treatment of grafts with immunomodulatory substances. Such a gentle and more clinically applicable method has the potential to decrease dosages and side effects by avoiding systemic applications.

Taken together, the plethora of relevant influential factors faced when planning immunosuppressive treatments or interventions requires that very precise information be available

when choosing the optimal treatment protocol for a specific scenario. To obtain this information and to ensure validity as well as translational relevance of the obtained data, we recommend the following measures:

(1) Choose an appropriate combination of immunosuppressants with respect to the pharmacokinetics and pharmacodynamics in the target species. Single-drug protocols should be limited to verified exemptions.

(2) Confirm the protocol's safety (including side effects) and achievement of appropriate target blood levels in the target species and model in exploratory pilot trials. Record potential blood level fluctuations.

(3) If necessary, verify the protocol's efficacy in a pilot experiment.

(4) Define interventional measures or protocol adjustments to optimize immunosuppression in the main trial. This includes the definition of circumstances under which an adjustment is necessary as well as those under which a particular adjustment might compromise the experimental results.

(5) Precisely define transplantation study end points as well as readout measures, including the expected effect size of primary study end points and standard deviation (derived from pilot trials). This will help to plan the main trial with appropriate statistical power.

(6) Strictly apply randomization and blinding protocols in the main trial, planning with appropriate negative and, if possible, positive controls.

(7) Perform thorough *post hoc* macro- and micropathological assessment of experimental subjects to exclude undetected side effects of immunosuppression.

(8) Publish neutral or negative results, including suboptimal immunosuppression protocols, in the scientific community.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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