CTLA4–Ig fusion proteins: promise for improved therapy of transplant rejection and autoimmune diseases

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T-cell activation is critically involved in the pathogenesis of both transplant rejection and autoimmunity, making its modulation a promising approach for immunotherapy. One attractive target for that purpose is provided by the CD28/B7 costimulatory pathway which is composed of the T-cell-associated CD28 molecule and its B7 ligands (CD80 and CD86) on antigen-presenting cells. This pathway is pivotal in complementing, in a non-antigen-specific manner, the antigen-specific signals of T-cell activation that are delivered upon triggering of the T-cell receptor. In the absence of CD28-mediated costimulation, T-cells activated through their T-cell receptor may acquire a state of antigen-specific unresponsiveness or undergo apoptotic cell death, leading to an abortive immune response. A competitive inhibitor of the CD28/B7 pathway has therefore been designed for use as a selective immunosuppressant. This reagent, called CTLA4–immunoglobulin (Ig), is a soluble fusion protein that binds B7 molecules with greater affinity than CD28. Extensive studies in animal models of allograft rejection and autoimmune diseases demonstrated that CTLA4–Ig exerts immunosuppressive effects without major toxicity. Over the past few years, the development of CTLA4–Ig and several analogs further proceeded to enter clinical trials in kidney transplantation, psoriasis and rheumatoid arthritis, with encouraging results. The present article will discuss the therapeutic potential of CTLA4–Ig molecules in the light of these new data.

The development of effective strategies for preventing acute and chronic allograft rejection and treating inflammatory and autoimmune diseases is an important goal of contemporary biomedical research [1]. Currently available immunosuppressive/anti-inflammatory drugs are less than optimal, in part due to their low immune cell selectivity, resulting in associated side-effects (Table 1). Moreover, many of these drugs act by inhibiting lymphocyte activation and/or proliferation in an indiscriminate manner and suppress protective immunity at the same time as detrimental immune responses, thereby exposing patients to increased risks of opportunistic infections and malignancies. Conventional methods of immunosuppression also do little to promote immune tolerance, or specific unresponsiveness to alloantigens and pathogenic self-antigens, which often necessitates life-long drug treatment with potential adverse consequences. Additional reagents with novel modes of action and improved safety are therefore needed.

One potential field of discovery for such reagents is T-cell activation, as this process is crucially involved in both transplant rejection and autoimmunity. Optimal activation of naive T-cells, leading to clonal expansion and acquisition of effector functions, requires at least two sets of signaling events. The first is initiated by the specific recognition through the T-cell receptor (TCR) of an antigenic peptide combined with major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs) [2]. The second set of signals is antigen nonspecific and is delivered by T-cell costimulatory receptors interacting with their ligands on APCs [2]. In the absence of costimulation, T-cell activation is impaired or aborted, which may result in an antigen-specific unresponsive state of clonal anergy [3], or in deletion by apoptotic death [4]. Hence, the blockade of T-cell costimulation has been thought to provide an approach for suppressing unwanted immune responses in an antigen-specific manner while preserving normal immune functions [5,6].

Of several T-cell costimulatory pathways identified to date [7], the most prominent and best characterized is provided by CD28, a cell surface molecule constitutively expressed on T-cells, and its counter receptors, the B7.1 (CD80) and B7.2 (CD86) molecules that are present on dendritic cells (DCs), macrophages...
This pathway has thus been regarded as a particularly attractive target for interrupting T-cell costimulatory signals. The design of a competitive inhibitor of CD28/B7 interactions, first described by Linsley and colleagues [12], took advantage of the existence of a second T-cell surface molecule homologous to CD28, CTLA4 (CD152), which is expressed at low levels late after activation, but has a 20-fold higher affinity for the B7 ligands than CD28 [10,13]. The immunosuppressive activity of this reagent, a recombinant fusion protein termed CTLA4–Ig, was demonstrated by extensive studies in preclinical models of transplantation and autoimmunity. Over the past few years, CTLA4–Ig and several analogs were further evaluated in patients with kidney transplant, psoriasis or rheumatoid arthritis (RA) and demonstrated favorable efficacy and tolerability. The present article will provide an overview of the therapeutic potential of these novel immunosuppressive agents.

### CTLA4–Ig fusion proteins
Several versions of soluble CTLA4–Ig fusion proteins, which are all dimers with a molecular weight of approximately 92–97 kDa, have been generated (Table 2). The prototypic form of CTLA4–Ig (referred to as CTLA4–Ig throughout, and developed by Bristol–Meyers Squibb as abatacept™) was engineered by fusing the extracellular domain of human CTLA4 to a fragment of the Fc portion of human IgG1 [12]. Mutational analyses of CTLA4 have identified several amino acid residues which are critical for its interaction with the B7 ligands. These include the hexapeptide motif, MYPPPY, that is present in the

<table>
<thead>
<tr>
<th>Type</th>
<th>Drug</th>
<th>Main indications</th>
<th>Limiting side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticosteroids</td>
<td>Prednisone, Prednisolone</td>
<td>Transplant rejection, CD, RA, SLE</td>
<td>Broad toxicity</td>
</tr>
<tr>
<td>DNA synthesis inhibitors</td>
<td>Thiopurines (Azathioprine, 6-mercaptopurine)</td>
<td>CD</td>
<td>Bone marrow toxicity</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>SLE</td>
<td>Bone marrow toxicity</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
<td>RA</td>
<td>Bone marrow toxicity, liver toxicity, gastrointestinal intolerance</td>
</tr>
<tr>
<td></td>
<td>Leflunomide (Arava®, Aventis)</td>
<td>RA</td>
<td>Liver and cardiac toxicity, Gastrointestinal toxicity</td>
</tr>
<tr>
<td></td>
<td>Mycophenolate mofetil</td>
<td>Transplant rejection</td>
<td></td>
</tr>
<tr>
<td>Calcineurin inhibitors</td>
<td>Cyclosporine A</td>
<td>Transplant rejection</td>
<td>Nephrotoxicity, neurotoxicity, hypertension, diabetes</td>
</tr>
<tr>
<td></td>
<td>(Sandimmune®, and Neoral®, Novartis AG; Gengraf®, Abbott)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tacrolimus (Prograf®, Fujisawa)</td>
<td></td>
<td></td>
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<tr>
<td>TOR inhibitors</td>
<td>Sirolimus (Rapamune®, Wyeth)</td>
<td>Transplant rejection</td>
<td>Lipidemia</td>
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<tr>
<td></td>
<td>Everolimus (Certican®, Novartis)</td>
<td></td>
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</tr>
<tr>
<td>Anti-CD3</td>
<td>Muromonab-CD3 (Orthoclone OKT®3, Roche)</td>
<td>Transplant rejection</td>
<td>Cytokine release syndrome, risks of infections</td>
</tr>
<tr>
<td>Anti-T-cells</td>
<td>Anti-thymocyte globulin</td>
<td>Transplant rejection</td>
<td>Risks of infections, malignancies</td>
</tr>
<tr>
<td></td>
<td>(Thymoglobulin®, Sangstat)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD25 (IL-2Rα)</td>
<td>Basiliximab (Simulect®, Novartis)</td>
<td>Transplant rejection</td>
<td>Risks of infection</td>
</tr>
<tr>
<td></td>
<td>Daclizumab (Zenapax®, Roche)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD2</td>
<td>Alefacept (Amevive™, Biogen)</td>
<td>Psoriasis</td>
<td>Risks of infection</td>
</tr>
<tr>
<td>Anti-CD11a (LFA-1)</td>
<td>Efalizumab (Raptiva®, Genentech)</td>
<td>Psoriasis</td>
<td>Risks of infection</td>
</tr>
<tr>
<td>TNF-α inhibitors</td>
<td>Infliximab (Remicade™, Centocor)</td>
<td>CD, RA</td>
<td>Risks of infection, antinuclear antibodies, demyelination</td>
</tr>
<tr>
<td></td>
<td>Adalimumab (Humira®, Abbott)</td>
<td>RA</td>
<td>Risks of infection</td>
</tr>
<tr>
<td>IL-1 inhibitor</td>
<td>Anakinra (Kineret®, Amgen)</td>
<td>RA</td>
<td>Injection site reactions, risks of infection, neutropenia</td>
</tr>
</tbody>
</table>

CD: Crohn’s disease; IL: Interleukin; LFA: Leukocyte function-associated antigen; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; TOR: Target of rapamycin; TNF: Tumor necrosis factor.
complementarity-determining region (CDR)3-like region of both CTLA4 and CD28 [14–17]. Substitution of the first tyrosine to a phenylalanine in this motif gave rise to a variant whose fusion construct with IgG1 (Y100F–Ig) retained full affinity for CD80 but was unable to bind to CD86 [18]. Although Y100F–Ig is not being developed for clinical use, it proved helpful to delineate the specific contributions of CD80 versus CD86 during immune responses [18–20]. Residues of CTLA4 that are not conserved in CD28 and are present within the CDR3-like region and the spatially adjacent CDR1-like region, also play an important role in the binding to B7 molecules [14–17]. Thus, changing Leu to Glu at position 104 within the CDR3 domain and alanine to tyrosine at position 29 within the CDR1 domain of CTLA4 yielded a variant fusion protein with IgG1, designated LEA29Y, that exhibits approximately twofold greater binding avidity for CD80 and about fourfold greater binding avidity for CD86 than CTLA4–Ig [21].

Another fusion protein consisting of the extracellular domain of human CTLA4 joined to an Fc portion of human IgG4 with a modified CH2 domain (substitution of Leu at position 235 with Glu and substitution of Gly at position 237 with Ala) was also made. This construct, called CTLA4-Cγ4, has markedly reduced complement activation ability and FcR binding activity compared with CTLA4–Ig, while displaying the same binding affinity to B7 molecules as the latter [22]. These various CTLA4–Ig proteins have been produced from transfected cells followed by purification with protein A affinity chromatography. For clinical development, large-scale production has been achieved in the milk of transgenic goats expressing the gene for CTLA4–Ig under the control of a lactation-specific promoter [23]. Different versions of murine (mu)CTLA4–Ig proteins and adenoviral vectors encoding muCTLA4–Ig have also been generated for use in murine experimental systems.

Pharmacokinetic profile

Owing to their Fc moiety, CTLA4–Ig fusion proteins were expected to possess a long circulating half-life (T1/2) after parenteral administration. Accordingly, initial studies in mice showed a serum elimination T1/2 of 143 h for muCTLA4–Ig given as a single intravenous (i.v) dose of 0.2 mg, although human CTLA4–Ig was cleared approximately fivefold faster [24]. After single or multiple dosing in mice or rats, the serum concentrations of human CTLA4–Ig increased in a dose-proportional manner with i.v treatment but not with subcutaneous (s.c) treatment [24,25]. Analysis of human CTLA4–Ig pharmacokinetics in cynomolgus monkeys after six i.v injections in doses of 1.0, 2.9 and 8.7 mg/kg revealed a dose-proportional relationship for the mean area under the curve (AUC), Cmax and Cmin values at steady state, but the mean T1/2 values remained similar in all dose groups (76–207 h) [26]. Linear pharmacokinetic parameters were also recorded, with little inter-individual variations, in psoriatic patients who received i.v infusions of CTLA4–Ig at doses ranging from 0.5–50 mg/kg [27]. Following administration of four doses, the Cmax was 17.0 ± 4.6 µg/ml at 0.5 mg/kg and 2201 ± 578 µg/ml at 50 mg/kg. The mean T1/2 was approximately 14.7 days regardless of the dose and remained constant throughout dosing, consistent with an absence of substantial antibody response to CTLA4–Ig [27].

Mechanisms of action

The basic mode of action of CTLA4–Ig proteins involves their binding to B7 molecules on APCs. This is thought to result primarily in a blockade of CD28-mediated T-cell costimulation [8,9,12,13]. However, CTLA4–Ig may also hinder the interaction of B7 molecules with CTLA4, which normally serves to downregulate T-cell responses [10]. Furthermore, over the past few years, it has been realized that the binding of CTLA4–Ig to B7 molecules may also affect the function of a subset of APCs [28].

Table 2. CTLA4–Ig fusion proteins under development.

<table>
<thead>
<tr>
<th>Name</th>
<th>Other name</th>
<th>Construct type</th>
<th>Developer</th>
<th>Developmental status</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA4–Ig</td>
<td>BMS-188667 (Abatacept)</td>
<td>CTLA4 fused to IgG1</td>
<td>Bristol–Meyers Squibb</td>
<td>Phases II/III in rheumatoid arthritis</td>
</tr>
<tr>
<td>LEA29Y</td>
<td>BMS-224818</td>
<td>Mutated CTLA4 (Glu104, Tyr29) fused to IgG1</td>
<td>Bristol–Meyers Squibb</td>
<td>Phase II in kidney transplantation</td>
</tr>
<tr>
<td>CTLA4-Cγ4</td>
<td>RG-1046</td>
<td>CTLA4 fused to IgG4</td>
<td>Repligen Corp.</td>
<td>Phase I in idiopathic thrombocytopenic purpura</td>
</tr>
</tbody>
</table>
Most experimental evidence indicated that disruption of the CD28/B7 costimulation pathway is a major mechanism accounting for the immunosuppressive effects of CTLA4–Ig. Such effects were documented in vitro as an inhibition of antigen-specific T-cell activation, resulting in markedly diminished IL-2 secretion and proliferation, especially when the concentration of stimulating antigen was limiting [29,30]. Although increasing the concentration of antigen allowed T-cells to undergo cell division in the presence of CTLA4–Ig, T-cell survival and clonal expansion were still strongly suppressed [30]. A recent study further demonstrated that CTLA4–Ig inhibits human T-cell activation in vitro at concentrations (3–100 µg/ml) encompassing those achieved in the serum of patients treated with a therapeutically effective dose of the compound (10 µg/kg) [31]. The mutated molecule, LEA29Y, was also found to inhibit T-cell responses in vitro but with an approximately tenfold higher potency than CTLA4–Ig, consistent with its greater avidity for B7 ligands. Studies in mice provided evidence that CTLA4–Ig inhibits the clonal expansion of antigen reactive T-cells in vivo [30,32]. In models of allograft transplantation, CTLA4–Ig treatment needed to be initiated at the time of graft implantation, or soon thereafter, in order to be fully effective [33], supporting the notion that CTLA4–Ig interferes with immune responses during antigen recognition by the T-cells. In agreement with a predominant role of CD28/B7 blockade in mediating the in vivo action of CTLA4–Ig, the compound prolonged cardiac allograft survival in wild-type but not CD28-deficient mice [34]. However, CTLA4–Ig was shown to be less effective at inhibiting CD8+ T-cells and memory T-cells than naïve CD4+ T-cells [34–37], as these functionally distinct T-cell subsets differ in their reliance on CD28/B7 costimulation for productive activation. While the suppression of T-cell-mediated responses is likely to play a crucial role in the beneficial activity of CTLA4–Ig in transplantation and autoimmunity, CTLA4–Ig proved also able to strongly inhibit antibody responses, as seen in the context of renal transplantation [38]. Indeed, when given at the time of immunization or immediately before antigen challenge, CTLA4–Ig inhibited primary and secondary antibody responses to T-cell-dependent antigens in mice [12] and monkeys [26,39]. Likewise, CTLA4–Ig abrogated the antibody response to repeated blood transfusion or a murine antibody in rats [40,41]. Studies in CTLA4–Ig transgenic mice indicated that CTLA4–Ig exerts such suppressive effects on antibody production, not by acting directly on B-cells but by impairing T-cell help for B-cell responses [42]. CTLA4–Ig may also inhibit T-cell-dependent B-cell maturation, as observed in a murine model of systemic lupus erythematosus (SLE) [43].

One important issue concerns the induction of antigen-specific tolerance by CTLA4–Ig treatment. As mentioned previously, one might expect CD28/B7 blockade during antigen encounter to induce T-cell anergy, a potential mechanism of peripheral immune tolerance [3]. This was observed in some experimental systems [32,44–48]. However, in many other instances, tolerance induction could not be achieved with CTLA4–Ig treatment alone [49,50], but required additional immunological manipulation, such as donor-specific transfusion [51], bone marrow (BM) transplantation [52], and blockade of other costimulatory pathways [53–57]. Lasting robust tolerance may thus involve multiple mechanisms that are not all recruited by merely disrupting the CD28/B7 pathway [6]. Furthermore, CTLA4–Ig may deplete peripheral tolerance-promoting regulatory T-cells in vivo, as CD28/B7 interactions appear essential for the development and survival of these cells [58]. In addition, CTLA4–Ig was found to impede tolerance induction in some situations [59,60], due to the fact that it disrupts CTLA4/B7 interactions that play a critical role in regulating T-cell anergy [10,61–63], even though such interactions may not be required for the maintenance of established allograft survival [63]. Therefore, the simultaneous blockade of CD28 and CTLA4 signaling by CTLA4–Ig in T-cells may exert complex effects whose tolerance-inducing potential may depend on the context of the immune response.

The finding that CTLA4–Ig proteins may also influence APCs introduced another element of complexity in its mechanism of action. Cross-linking of B7 on a subset of monocyte-derived DCs by CTLA4–Ig was shown to stimulate the release of interferon (IFN)-γ, which in turn caused the production of indoleamine-2,3-dioxygenase (IDO) [64], an enzyme that catabolizes tryptophan to its byproduct, kynurenine [65]. Such up-regulation of IDO in DCs is believed to result in reduced local concentrations of tryptophan and a concomitant increase of kynurenine and other metabolites, causing an inhibition of T-cell functions [64–66]. Remarkably, treatment with a pharmacologic agent that inhibits IDO activity abrogated the protective effect of CTLA4–Ig in a model of pancreatic islet allograft rejection in mice [64]. While murine
versions of CTLA4–Ig (muCTLA4–IgG3 and muCTLA4–IgG2a) were used in these experiments, human CTLA4–Ig was recently shown to also upregulate IDO in the presence of IFN-γ in human DCs [67]. Since ligation of B7 by CTLA4 expressed on CD4+ T-cells similarly enhanced IDO activity in DCs [67], one might consider that CTLA4–Ig mimics such a physiological interaction. At present, however, the relative contribution of this action to the overall immunosuppressive and tolerogenic effects of CTLA4–Ig in vivo remains unclear, and further studies in this area are much warranted.

Prevention of transplant rejection

Preclinical studies

The ability of CTLA4–Ig to suppress detrimental immune responses was first established in the transplant setting. A short treatment with CTLA4–Ig was shown to prolong the survival of transplanted human pancreatic islets in mice [44] and cardiac allografts in rats [49]. Many subsequent studies in rodent models corroborated and extended these observations for transplantation of diverse types of organs (reviewed in [68,69]) (Table 3). Note, however, that the efficacy of CTLA4–Ig varied depending on the model, possibly due to organ-related differences in the immune processes of allograft rejection. For instance, models such as skin or intestinal transplants, in which CD8+ T-cells are capable of mediating graft rejection, appeared less susceptible to CD28/B7 blockade than cardiac rejection that involves predominantly CD4+ T-cells [6]. Although the administration of CTLA4–Ig alone often prolonged graft survival only modestly, its combination with other immunosuppressive agents usually resulted in additive or synergistic effects. Besides the above mentioned procedures favoring tolerance induction [51–57], antagonists to cytokines [70,71] or the adhesion molecule, CD11α (LFA-1) [72], as well as the chemical immunosuppressant, sirolimus (Rapamune®, Wyeth) [73], augmented allograft protection when coadministered with CTLA4–Ig. However, variable results were obtained in the case of calcineurin inhibitors, such as cyclosporine A (CsA) and tacrolimus (Prograf®, Fujisawa) whose association with CTLA4–Ig either facilitated or impaired long-term allograft survival or transplant tolerance depending on the level of immunosuppression produced by CTLA4–Ig and the strength of the antiallograft response [74].

Table 3. Activity of CTLA4–Ig§ in preclinical models of transplantation.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Animal model</th>
<th>Effect of CTLA4–Ig treatment</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac allograft rejection</td>
<td>Rat, mouse</td>
<td>Prolonged graft survival when given alone at the time of transplantation. Combination with donor-specific cell transfusion or BM transplantation or with anti-CD40L or anti-CD4 mAb allowed for indefinite graft survival and prevented graft arteriosclerosis associated with chronic rejection.</td>
<td>[49]</td>
</tr>
<tr>
<td>Renal allograft rejection</td>
<td>Rat</td>
<td>Prolonged graft survival and prevented graft nephritis associated with chronic rejection.</td>
<td>[46]</td>
</tr>
<tr>
<td>Liver allograft rejection</td>
<td>Rat</td>
<td>Prolonged graft survival, prevented chronic rejection and induced donor-specific tolerance when given in multiple doses from the time of transplantation.</td>
<td>[78,111]</td>
</tr>
<tr>
<td>Small bowel allograft rejection</td>
<td>Rat</td>
<td>Combination with anti-CD4 mAb allowed for indefinite allograft survival and induced donor-specific tolerance.</td>
<td>[112]</td>
</tr>
<tr>
<td>Lung allograft rejection</td>
<td>Rat</td>
<td>Reduced intragraft vasculitis and hemorrhage but did not induce long-term graft acceptance in a model of aggressive rejection. Prevented acute rejection in a less stringent model.</td>
<td>[113]</td>
</tr>
<tr>
<td>Limb allograft rejection</td>
<td>Rat</td>
<td>Treatment on day 2 after transplantation prolonged graft survival.</td>
<td>[114]</td>
</tr>
<tr>
<td>Islet allograft rejection</td>
<td>Monkey</td>
<td>Prolonged graft survival in tw out of five animals.</td>
<td>[83]</td>
</tr>
<tr>
<td>Renal allograft rejection</td>
<td>Monkey</td>
<td>Delayed rejection by 20–30 days when given alone. Combination with anti-CD40L mAb resulted in more prolonged (&gt;150 days) graft survival.</td>
<td>[84]</td>
</tr>
</tbody>
</table>

§Studies were performed using human or mouse CTLA4–Ig.
BM: Bone marrow; mAb: Monoclonal antibody.
In addition to preventing acute allograft rejection, CTLA4–Ig also inhibited the chronic rejection of kidney, heart and liver allografts [20,75–78]. The progression of chronic allograft rejection was interrupted even when CTLA4–Ig treatment was initiated late post-transplantation, after initial graft injury. This is an important finding since chronic rejection is poorly suppressed by currently available immunosuppressants and, therefore, remains a major cause of late graft loss in clinical transplantation [79].

Studies in mice further demonstrated that CTLA4–Ig could attenuate the severity of graft-versus-host disease (GVHD) [80], which can be a major complication of BM transplantation [81]. Accordingly, the establishment of stable mixed hematopoietic chimerism was facilitated by CTLA4–Ig after BM transplantation in dogs [82].

To better assess the potential of CTLA4–Ig for clinical development in transplantation, it was imperative to determine its immunosuppressive effect in nonhuman primate models. CTLA4–Ig monotherapy was found to significantly delay the rejection of islet and renal allografts in rhesus monkeys, but not in all treated animals [83,84]. More consistent and pronounced prolongation of renal allografts was obtained when CTLA4–Ig treatment was carried out in conjunction with the blockade of the CD40/CD40L costimulation pathway [84,85]. A brief course of CTLA4–Ig following induction therapy with an anti-CD4 antibody similarly resulted in significant prolongation of cardiac allograft survival in monkeys [86]. The more potent CTLA4–Ig analog, LEA29Y, was also tested for efficacy in a model of islet transplantation in rhesus monkeys. Addition of LEA29Y (10–20 mg/kg) to a base regimen of rapamycin and anti-IL-2R antibody, which by itself was ineffective, significantly prolonged the survival of allogeneic islets and inhibited antidonor T-cell and antibody responses [87].

Preliminary results of a multicenter Phase II trial of LEA29Y carried out in kidney transplantation have been recently presented [89,90]. The objective of this study was to compare the safety and efficacy of LEA29Y versus CsA in renal transplant patients concomitantly receiving a triple regime of mycophenolate mofetil (CellCept®), basiliximab (Simulect®, Novartis) and corticosteroids. Patients on this regimen were randomized to be given LEA29Y (n = 148), initially at 10 mg/kg every other week, and then monthly at 5 mg/kg, or to continue standard maintenance co-treatment with CsA (n = 73). Safety data monitored over a 6-month period showed lower hypertension and hyperlipidemia and slightly but significantly better renal function in the LEA29Y-treated than in the CsA-treated cohorts. Moreover, at 6-months, the incidence of biopsy proven acute rejection (19% for LEA29Y vs 18% for CsA) and graft loss (3% for LEA29Y vs 4% for CsA) were similar in the two treatment arms, and the rate of death was lower with LEA29Y (1%) than CsA (5%). Discontinuation and adverse events, including infection and malignancies, were also similar across the treatment groups. Although definitive assessment of the effects of LEA29Y in this study must await further follow up and publication of the full set of data, the current findings suggest that LEA29Y might provide a favorable alternative to CsA-based immunosuppression [90]. A second clinical trial of LEA29Y in renal transplantation that will avoid both CsA and steroids, is planned to be conducted through the Immune Tolerance Network [201].

Therapy of autoimmune diseases

Preclinical studies

The therapeutic potential of CTLA4–Ig for the treatment of autoimmune diseases was examined using various models in mice or rats, including experimental allergic encephalomyelitis (EAE), type I diabetes in NOD mice, oophoritis, experimental autoimmune glomerulonephritis, experimental myasthenia gravis, experimental skin inflammation and colitis, collagen induced arthritis (CIA), and SLE-like disease [68,69] (Table 4). When administered before the onset of disease, CTLA4–Ig attenuated inflammation markers and pathological manifestations in these models. In NZB/NZW mice, which spontaneously develop SLE, short-term combination therapy with CTLA4–Ig and an anti-CD40L monoclonal antibody (mAb) was sufficient to induce a sustained suppression of anti-dsDNA

Clinical studies

A pilot study in human BM transplantation showed that, when co-cultured with irradiated cells from the recipient in the presence of CTLA4–Ig ex vivo, T-cells in donor BM could be rendered anergic to recipient alloantigens. Upon transfer to the recipients, such treated donor BM cells were fully capable of restoring hematopoiesis in vivo but with a reduced risk of GVHD [88]. However, no further exploration of this approach for alleviating GVHD in the clinical setting has been reported to date.
autoantibody production [91,92]. Importantly, such a treatment was effective at prolonging life even if delayed until 8 months of age, when the disease was already evident [91]. Likewise, when administered in conjunction with cyclophosphamide, CTLA4–Ig reversed proteinuria and attenuated renal damage in NZB/NZW mice with advanced lupus nephritis [93–95]. In the case of CIA, a model for human RA, CTLA4–Ig treatment was also beneficial in reducing joint damage and anticollagen antibody formation when commenced after the onset of disease [96].

Clinical studies

Psoriasis
CTLA4–Ig was first evaluated in psoriasis, a chronic inflammatory skin disorder characterized by increased proliferation and altered differentiation of keratinocytes, dermal angiogenesis and immune cell infiltration [97]. This open-label, dose-escalation study involved 43 patients with moderate-to-severe psoriasis to whom four i.v infusions of CTLA4–Ig were administered at eight doses ranging from 0.5 to 50 mg/kg over a 5-week period [27]. Overall, 46% of the treated patients achieved a 50% or greater improvement in their Physician’s Global Assessment of disease activity, compared with baseline psoriasis, versus only 4% of 23 patients in a control group. A 50% or greater improvement in global clinical parameters of psoriasis was observed in nine out of 11 patients accrued to the two highest CTLA4–Ig doses and in one out of nine patients at the two lowest doses. Sustained clinical improvement was observed in some cases for at least 147 days following administration of the final dose of CTLA4–Ig, well after the elimination of detectable CTLA4–Ig from the circulation [27]. Analyses of serial skin biopsies showed that clinical improvement was associated with reduced T-cell infiltration and cellular activation of T-cells, keratinocytes, DCs, and vascular endothelium in the psoriatic lesions [27,98]. CTLA4–Ig treatment was also associated with reduced antibody responses (especially IgG) to T-cell-dependent antigens. However, no evidence of tolerance induction to these antigens was obtained [27].

Rheumatoid arthritis
Several studies have been conducted to determine the possible efficacy of CTLA4–Ig molecules in the treatment of RA, a systemic autoimmune disease leading to chronic joint inflammation partially mediated by T-cells [99].

In a pilot, double-blind, placebo-controlled trial, CTLA4–Ig (Abatacept, Bristol–Meyers Squibb) was tested in parallel with LEA29Y in RA patients (n = 214) refractory to standard disease-modifying antirheumatic drugs (DMARDs) [100]. DMARD treatment was discontinued and patients received four i.v infusions of either

Table 4. Activity of CTLA4–Ig§ in preclinical models of autoimmune diseases.

<table>
<thead>
<tr>
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<th>Effect of CTLA4–Ig treatment</th>
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<tbody>
<tr>
<td>Collagen-induced arthritis</td>
<td>Rat</td>
<td>Treatment started 1 day before immunization with type II collagen prevented the clinical and histological manifestations of arthritis.</td>
<td>[115]</td>
</tr>
<tr>
<td>Collagen-induced arthritis</td>
<td>Mouse</td>
<td>Treatment prevented joint damage and anticollagen antibody formation if given at the time of type II collagen immunization, and still significantly reduced the pathology if given after the disease onset.</td>
<td>[95]</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>Mouse</td>
<td>Treatment suppressed anti-dsDNA antibody and prolonged life even when initiated at disease onset. Effect potentiated by co-treatment with anti-CD40L mAb. Combination with cyclophosphamide reversed proteinuria and prolonged survival in mice with advanced lupus nephritis.</td>
<td>[91,92]</td>
</tr>
<tr>
<td>Experimental allergic encephalomyelitis</td>
<td>Rat</td>
<td>Treatment suppressed clinical disease and prevented death if started before immunization. Delayed treatment was either effective or ineffective depending on the study conditions.</td>
<td>[116]</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>Mouse</td>
<td>Treatment prevented diabetes if started early, but had no effect if started at disease onset.</td>
<td>[117]</td>
</tr>
<tr>
<td>Skin and colon inflammation</td>
<td>Mouse</td>
<td>Inhibition of pro-inflammatory cytokine generation and suppression of psoriasis-like skin inflammation and colitis.</td>
<td>[118]</td>
</tr>
</tbody>
</table>

§Studies were performed using human or mouse CTLA4–Ig. mAb: Monoclonal antibody.
CTLA4–Ig or LEA29Y (both at 0.5, 2, or 10 mg/kg) or of placebo. Patients were evaluated on day 85, with a further follow-up period through day 169. Efficacy was expressed as the proportion of patients meeting the American College of Rheumatology 20% improvement criteria (ACR20). Overall, the incidence of discontinuations due to worsening of RA was found to be lower in the CTLA4–Ig or LEA29Y-treated patients than in the placebo group. Within the patient cohorts who completed the study to day 85, a dose-dependent improvement in ACR20 responses was observed after treatment with CTLA4–Ig (23% at 0.5 mg/kg, 44% at 2 mg/kg, and 53% at 10 mg/kg) or LEA29Y (34% at 0.5 mg/kg, 45% at 2 mg/kg, and 61% at 10 mg/kg), as compared with placebo treatment (31%) [100].

A second double-blind, randomized, placebo-controlled investigation (Phase IIb trial) of the effectiveness of CTLA4–Ig therapy was carried out in RA patients (n = 339) with inadequate response to methotrexate therapy [101]. All patients continued to receive a stable dose of methotrexate (1–30 mg, weekly) and, if needed, low-dose of corticosteroids and nonsteroidal anti-inflammatory drugs, but any other treatment was stopped. Patients received i.v. infusions of placebo or CTLA4–Ig at 2 mg/kg or 10 mg/kg on days 1, 15, and 30 and monthly thereafter for 6 months. More patients in the placebo group discontinued the trial because of worsening of arthritis than in the CTLA4–Ig groups. After 6 months, the ACR20, ACR50 and ACR70 responses were significantly higher in the patient group given CTLA4–Ig at 10 mg/kg (60, 37, and 16%, respectively) than in the placebo group (35, 12 and 2%, respectively). Patients treated with CTLA4–Ig at 2 mg/kg also achieved significantly higher ACR50 and ACR70 responses than with placebo [101]. Levels of several serum biomarkers of inflammation, including C-reactive protein (CRP), rheumatoid factor (RF), soluble interleukin-2 receptor (sIL-2R), IL-6 and intercellular cell-adhesion molecule (ICAM)-1 were found to decrease in relation to CTLA4–Ig dosage with the greatest reductions in the 10 mg/kg group at both 6 and 12 months, which correlated with the ACR responses [102,103]. Furthermore, patients who received CTLA4–Ig at 10 mg/kg experienced statistically significant improvements in multiple parameters assessing physical function, compared with the placebo group. This effect was observed as early as 30 days after treatment and further improved and sustained to 6 months [104]. Patients in the CTLA4–Ig 10 mg/kg group also had clinically and statistically significant improvements in parameters of health-related quality of life [105,106].

A third study evaluated CTLA4–Ig in RA patients who experienced active disease despite treatment with the tumor necrosis factor (TNF)-α inhibitor, etanercept (Enbrel™, Wyeth). In this randomized, double-blind, placebo-controlled trial (Phase IIb), all patients continued to receive etanercept (25 mg, twice weekly) in addition to once-monthly infusions of either CTLA4–Ig at 2 mg/kg (n = 85) or placebo (n = 36) for 6 months [107]. A total of 48.2% patients in the CTLA4–Ig-treated group achieved an ACR20 as compared with 27.8% in the placebo control group. Among the subjects who had an ACR20 response at 6 months, there was a decrease from baseline in the serum levels of CRP, RF, sIL-2R and IL-6 [108]. Moreover, an ACR70 was observed in 10.6% patients receiving CTLA4–Ig but in none of the patients receiving placebo [106]. CTLA4–Ig treatment also resulted in meaningful improvement in health-related quality of life of the patients as compared with placebo treatment [109].

Multicenter Phase III studies have now been initiated to further assess the efficacy and safety of CTLA4–Ig treatment (10 mg/kg) in RA patients who failed DMARD and/or anti-TNF therapy [202]. No data from these trials are available to date.

Safety & tolerability

The preclinical studies in rodents and nonhuman primates demonstrated that CTLA4–Ig proteins are well-tolerated and devoid of organ toxicity at efficacious doses. Likewise, in the clinical studies conducted thus far, treatment with CTLA4–Ig or LEA29Y was not associated with any major side effects. During the 6-month studies of CTLA4–Ig therapy combined with methotrexate or etanercept in RA, the most frequently reported adverse events were headache, upper respiratory tract infection, musculoskeletal pain, nausea and vomiting, but these events occurred at comparable rates in the CTLA4–Ig and placebo groups. There were no notable renal, hepatic, or hematologic adverse events. Moreover, trials in normal volunteers and in patients with idiopathic thrombocytopenic purpura showed that CTLA4-Cp4 was well tolerated. It is not clear, however, whether the latter would offer any safety advantage over the other CTLA4–Ig variants. Although CTLA4–Ig
may mediate complement- and antibody-dependent cellular cytotoxicity via its IgG1 Fc portion, unlike CTLA4-CF4 \[22\], treatment with CTLA4–Ig in psoriasis or RA patients did not produce any detectable cellular depletion of B7-bearing cell populations or alterations in lymphocyte subset distribution in the blood \[27,101\]. Therefore, the overall safety profile of CTLA4–Ig proteins appears encouraging. Nevertheless, as with all immunosuppressive agents, one major concern is whether CTLA4–Ig proteins may cause global immunosuppression leading to increased risks of opportunistic infections or malignancies. This did not seem to be the case in the animal studies, nor in the human studies \[27,101\]. Furthermore, CTLA4–Ig did not decrease total serum Ig levels after four doses in psoriasis patients \[27\]. Longer term studies will be needed, however, before the possibility that CTLA4–Ig proteins may induce adverse sequels of general immunosuppression can be ruled out. In any case, it is worth noting that repeated injections of CTLA4–Ig did not augment anti-CTLA4–Ig antibody titers in psoriasis or RA patients \[27,101\]. This suggests that CTLA4–Ig has little immunogenicity and/or may suppress its own immunogenicity in humans and is unlikely to cause hypersensitivity reactions.

**Expert opinion**

CTLA4–Ig fusion proteins represent a novel class of immunosuppressive agents whose mode of action stems from their high binding avidity for B7 molecules on APCs. While initially viewed as a way to disrupt the CD28 costimulation pathway of T-cell activation, occupancy and cross-linking of the B7 ligands by CTLA4–Ig may exert additional T-cell inhibitory effects through the induction of IDO expression in a subset of APCs. The binding of CTLA4–Ig to B7 molecules may, however, also block their interaction with CTLA4 on T-cells, which is known to downregulate T-cell activation and may be involved in the generation of regulatory T-cells. This raises the concern that CTLA4–Ig might exacerbate immune responses in certain situations. Despite this concern, the bulk of data obtained in several experimental models of transplant rejection and autoimmune diseases showed that CTLA4–Ig effectively stifles the activation of naïve CD4+ T-cells and the secondary recruitment of B-cells and macrophages, resulting in beneficial immunosuppression. Any immunoenhancing effect arising from the blockade of CTLA4 may thus be counterbalanced by the blockade of CD28 and the induction of IDO, causing overall T-cell hyporesponsiveness without obvious short-term alterations of peripheral T-cell homeostasis. Whether such alterations would occur upon prolonged CTLA4–Ig treatment remains to be determined. In any case, it has become evident that the immunosuppressive action of CTLA4–Ig \textit{in vivo} is more complex than initially envisioned. In particular, the early hope that CTLA4–Ig treatment may result in antigen-specific tolerance was substantiated in only a few instances, indicating that tolerance induction by costimulation blockade may depend on the context of the immune response. Evidence was nonetheless obtained that, although CTLA4–Ig is not sufficient to induce transplantation tolerance when administered alone, it may help achieve this goal when combined with other immunomodulating procedures.

As far as the safety of CTLA4–Ig proteins is concerned, preclinical studies made it clear that, due to their unique specific target, these agents act in a lymphoid specific manner, with no detectable adverse side effects. All three forms of CTLA4–Ig constructs have also proven to be well tolerated, without limiting toxicity, in human trials. This compares favorably with many of the currently available immunosuppressive drugs, which affect more ubiquitous targets and can cause mechanism-based toxicity (Table 1). Moreover, several of the recently approved biological agents targeting T-cell antigens or cytokines have broad immunosuppressive effects, resulting in increased risks of infectious complications and malignancies (Table 1). In contrast, CTLA4–Ig proteins appear to neutralize T-cells that initiate harmful immune responses without compromising the body’s ability to fight off infections, although this needs to be substantiated by more extensive investigations in the clinical setting.

Preliminary results from the first human study in renal transplantation suggested that LEA29Y may be as effective as standard therapy with CsA in preventing acute rejection, but with improved kidney function, blood pressure and total cholesterol levels. This warrants further evaluation of LEA29Y to examine its long-term effectiveness when administered with minimal adjunct immunosuppression, and whether it may alleviate the development of chronic rejection, a major problem of transplantation medicine for which no adequate treatment currently exists \[79\]. Moreover, the possibility of substituting LEA29Y for CsA or other calcineurin inhibitors might prove useful in transplant tolerance induction protocols, since calcineurin function appears required for T-cell anergy \[110\].
The studies in RA patients further revealed that both LEA29Y and CTLA4–Ig are capable of ameliorating an ongoing autoimmune process. The latter agent was also found to be potentially useful for the treatment of psoriasis. Interestingly, sustained clinical improvement of psoriasis was observed in some cases for several months after administration of CTLA4–Ig [27], suggesting a protracted inhibition of pathogenic immune processes. Notwithstanding these encouraging results, there is no immediate plan to further develop CTLA4–Ig in psoriasis. Presently, the greatest potential therapeutic utility of CTLA4–Ig fusion proteins may therefore be for the treatment of RA. Indeed, despite the introduction of new biological agents targeting inflammatory cytokines, RA remains an intractable disease where remission is induced in only a small subset of patients and for which there is no cure [99]. For instance, about only 70% of RA patients are responsive to TNF-α inhibitors, with fewer than 30% experiencing a dramatic and sustained disease improvement. As CTLA4–Ig appeared effective in patients who were refractory to TNF-α blockade or methotrexate, it would represent a welcome therapeutic option with a distinct and complementary mode of action for such patients. Combination therapy with other anti-R A drugs may further extend the prospective utility of CTLA4–Ig for the treatment of RA. Ongoing and future trials may also tell us whether CTLA4–Ig proteins administered alone or in conjunction with standard therapies will be useful for the treatment of other autoimmune conditions, such as SLE and multiple sclerosis.

CTLA4–Ig fusion proteins share with other biological agents the common drawbacks of high production costs, relatively short shelf-life and lack of oral bioavailability requiring parenteral routes of administration. Such disadvantages may nonetheless be offset by the prolonged duration of action of these agents, allowing for intermittent dosing schedules. Significant efficacy was attained when CTLA4–Ig or LEA29Y were administered at monthly intervals in transplantation and autoimmune diseases. However, it remains unclear as to whether such treatments will need to be given for life in order to provide long-term benefit. One may anticipate that as more clinical studies are conducted with CTLA4–Ig proteins, a better understanding of their optimal use will emerge.

Overall, the current findings indicate that CTLA4–Ig fusion proteins may provide a much needed novel paradigm for therapeutic immunosuppression that would facilitate the prophylaxis of transplant rejection and the therapy of immune-mediated inflammatory disorders. Pending confirmation of the data in extended clinical trial, the approval of these agents for medical use would thus represent an important milestone, which might well beget a revolution in immune intervention strategies.

Outlook
Immunosuppressive strategies are likely to keep evolving over the next decade as several new drugs are poised to reach the clinic [1] and deeper insights into the molecular mechanisms of immune regulation are to be expected [119]. The use of multi-drug regimens tailored to the individual patient is a probable trend that will require a wide diversity of therapeutic options.

In transplantation, the goal will remain to maximize graft survival in a context of organ supply shortage and especially to prevent chronic organ deterioration while minimizing drug side-effects [120]. Current attempts at reducing or eliminating the use of calcineurin inhibitors will probably intensify to favor sirolimus- and/or mycophenolate mofetil-based regimens where CTLA4–Ig molecules may play an important role [87,201]. Among the newer agents in advanced clinical trials, the sphingosine analog FTY720 may also become one of the most innovative adjunct to such immunosuppressive regimens in the near future, owing to its unique mode of action, reflecting an alteration of the trafficking of lymphocytes rather than of their activation or proliferation, and its favorable safety profile [121,122]. Approval of FTY720 for marketing would significantly impact the field of immunotherapy, but would not eclipse the therapeutic potential of CTLA4-Ig molecules since preclinical evidence suggested beneficial effects of combining these two types of agents in transplantation [123]. Although the disruption of Janus kinase 3 signaling is another very interesting novel immunosuppressive modality with potential in transplantation [124,125], it may take many years before such an approach graduate to the clinic.

The quest for clinically effective transplant tolerance induction protocols is also likely to gain momentum in the coming years [126,127], and this should be greatly facilitated by the identification of surrogate markers predicting long-term graft survival [128]. While the manipulation of DCs and regulatory T-cells may not be therapeutically practical before long [129,130],
Highlights

- Soluble CTLA4–immunoglobulin (Ig) fusion proteins bind to B7 (CD80 and CD86) molecules on antigen-presenting cells, thereby blocking the CD28/B7 costimulation pathway and preventing full T-cell activation.
- CTLA4–Ig proteins may also induce the expression of indoleamine-2,3-dioxygenase in dendritic cells, resulting in further inhibition of T-cell activation.
- They exert immunosuppressive effects manifested in vivo as decreased cell-mediated immunity and T-dependent antibody production.
- They may help achieve antigen-specific immunosuppression and contribute to immune tolerance induction.
- Owing to their immunoglobulin (Ig)G moiety, CTLA4–Ig proteins have prolonged half-life in vivo, which may confer a prolonged duration of action.
- CTLA4–Ig proteins are well-tolerated, without major side-effects and have low immunogenicity in humans.
- Data suggest that these agents may not cause general immunosuppression, but this needs to be verified in long-term clinical studies.
- Preliminary evidence for clinical efficacy in kidney transplantation was obtained, as was more substantiated evidence for efficacy in psoriasis and rheumatoid arthritis.
- The therapeutic potential of CTLA4–Ig proteins for other autoimmune diseases was suggested in preclinical studies and deserves to be further evaluated.
- Optimal protocols of administration and of combination of CTLA4–Ig proteins with other immunosuppressive agents still remain to be determined.
- The long-term consequences of CTLA4–Ig treatment on immune homeostasis also remain to be ascertained in human.
- Disadvantages of CTLA4–Ig proteins, that are inherent to biological agents, include high cost of production and need for parenteral administration.

Costimulation blockade in conjunction with other methods may help promote tolerance in the foreseeable future [126,127]. As outlined in this review, the combination of CTLA4–Ig with anti-CD40L mAbs would appear particularly useful [131]. However, development of the latter reagents has been marred with thromboembolic complications in preclinical and early clinical studies [132–134], such that CTLA4–Ig molecules may remain the only clinically relevant costimulation blockers for some time. Another strategy targeting T-cell activation, which may have the capacity to induce immune tolerance, is provided by a novel generation of humanized FcR nonbinding anti-CD3 mAbs whose clinical evaluation is presently underway [135].

For autoimmunity, efforts will focus on disease prevention and reversal of disease progression rather than merely symptomatic relief [136]. Most efficient strategies for therapeutic intervention in this area may rely more and more on individualized medicine that takes into account the patient's own genotypic and etiologic factors. This is well demonstrated in the case of RA, where variable responses to virtually any treatment modality imply marked heterogeneity in the pathogenic processes [99]. Besides CTLA4–Ig and FTY720, other emerging drugs that target activation ligands or adhesion molecules on immune cells, or act as anticytokines may prove invaluable to confront this heterogeneity [1]. One of the novel agents with significant potential in that respect is natalizumab (Antegren™, Elan Corporation and Biogen Idec), a humanized mAb to α4-integrin adhesion molecule, which recently completed phase III trials in Crohn's disease and multiple sclerosis [137,138].

By and large, there is therefore much hope that, along with the possible application of CTLA4–Ig-based approaches, the next 5 to 10 years will bring substantial advances in the medical treatment of transplant rejection and autoimmune diseases.

Disclaimer
Francis J Dumont does not have any propriety interest in any pharmacologic agent or company discussed in this review.

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- Demonstrates that for optimal suppression of cardiac allograft rejection in rats, CTLA4-Ig needs to be present on day 2 post-transplantation, at the time of alloantigen recognition by host T-cells.


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The addition of CTLA4-Ig to etanercept therapy in patients with active RA was well tolerated and resulted in a significant dose-dependent improvement of disease signs and symptoms as compared with placebo plus methotrexate after 6 months of treatment.


**110. After 6 months of treatment, CTLA4-Ig added to etanercept produced significantly greater improvement in health-related quality of life than placebo plus methotrexate in RA patients.**


**112. The addition of CTLA4-Ig to etanercept therapy in patients with active RA was well tolerated and resulted in a significant improvement of disease signs and symptoms as compared with placebo plus etanercept after 6 months of treatment.**


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