Construction, and *In Vitro* and *In Vivo* Analyses of Tetravalent Immunoadhesins

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Received: January 17, 2012 / Revised: April 5, 2012 / Accepted: April 14, 2012

Previous observations demonstrated that various immunosuppressive agents and their combination therapies can increase allograft survival rates. However, these treatments may have serious side effects and cannot substantially improve or prolong graft survival in acute graft-versus-host disease (GVHD). To improve the therapeutic potency of divalent immunoadhesins, we have constructed and produced several tetravalent forms of immunoadhesins comprising each of cytotoxic T-lymphocyte-associated antigen-4 (CTLA4), CD2, and lymphocyte activation gene-3 (LAG3). Flow cytometric and T cell proliferation analyses displayed that tetravalent immunoadhesins have a higher binding affinity and more potent efficacy than divalent immunoadhesins. Although all tetravalent immunoadhesins possess better efficacies, tetravalent forms of CTLA4-Ig and LAG3-Ig revealed higher inhibitory effects on T cell proliferation than tetravalent forms of TNFR2-Ig and CD2-Ig. *In vitro* mixed lymphocytes reaction (MLR) showed that combined treatment with tetravalent CTLA4-Ig and tetravalent LAG3-Ig was highly effective for inhibiting T cell proliferation in both human and murine allogeneic stimulation. In addition, both single tetravalent-form and combination treatments can prevent the lethality of murine acute GVHD. The results of this study demonstrated that co-blockade of the major histocompatibility complex class (MHC)II:T cell receptor (TCR) and CD28:B7 pathways by using tetravalent human LAG3-Ig and CTLA4-Ig synergistically prevented murine acute GVHD.

**Keywords:** Cytotoxic T lymphocyte antigen-4, lymphocyte activation gene-3, tetravalent immunoadhesin, graft-versus-host disease, mixed lymphocyte reaction

The immunological rejection of transplanted organs or tissues is a natural process of immune responses to protect self from non-self [31, 42]. Graft-versus-host disease (GVHD) is a primary T-cell-mediated complication of allogeneic bone marrow transplantation, occurring when mature T cells in bone marrow grafts are stimulated by host antigen-presenting cells (APCs), enhanced by proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) [41]. Current immunosuppressive agents for acute rejection of allografts are targeting T cell activation, cytokine production, and clonal expansion subsequently [52]. Although many immunosuppressive agents such as cyclosporine A (CsA), methotrexate (MTX), sirolimus (rapamycin), anti-CD3 antibody (Ab) (muromonab), anti-TNF- Ab (infliximab), and CTLA4-Ig (abatacept) have been demonstrated as potential therapeutic options to prevent acute rejection of allografts [3, 4, 16, 26, 43, 49], they still need to be developed further for their functional achievements clinically.

T cell activation is mandatory for rejection of allografts, and requires minimum two signals for optimal induction of IL-2 production and clonal expansion [47]. Antigen specificity is delivered by the first signal, which involves engagement of T cell receptor (TCR) with a major histocompatibility complex (MHC)–peptide complex on the APCs. Secondary signals are delivered by binding of co-stimulatory receptors on T cells to the ligands on APCs. In addition, cell-adhesion molecules such as intercellular adhesion molecules-1 (ICAM-1), ICAM-2, ICAM-3, lymphocyte function-associated antigen-1 (LFA-1), CD2, and LFA-3 are also important in the interaction of T cells with APCs [15, 46]. After T cells become activated, they produce various effector molecules, cytotoxin, cytokines, and related membrane-associated protein [17, 23]. Therefore, the interaction of these multiple pathways is a distinct advantage in the immunological environment.

Immunoadhesin, a soluble Ig fusion protein [9], is widely used as an immunosuppressive agent for various human diseases. The platform of this molecule comprises a hinge and a constant region of the immunoglobulin (Ig) Fc
region (domains of hinge, CH2, and CH3) following the target binding region of a receptor, a ligand, an enzyme, or an adhesion molecule. Immunoadhesin has various advantages including increased avidity, lower dosage, lower neutralizing antibody induction, slower in vivo clearance, and convenient process of the protein purification [9].

CTLA4-Ig, the best characterized co-stimulatory blockade immunoadhesin, blocks the B7/CD28 signaling pathway by binding to CD80 and CD86 [35]. CTLA4-Ig (abatacept) is approved for use in rheumatoid arthritis [8, 12, 40]. This molecule can induce tolerance and has low in vivo toxicity. However, immunosuppressive drugs currently in use, such as CsA, FK506, and rapamycin, are generally considered to be nonspecific, are not tolerogenic, and are often toxic [5]. To increase the suppressive effect, many researchers have shown that combined treatment with more than two suppressive agents leads to prolonged graft survival. The combination of CTLA4-Ig with CsA and other molecules such as MTX, rapamycin, anti-CD2 Ab, anti-LFA-1 Ab, anti-CD40L Ab, anti-CD4 Ab, and anti-ICAM-1 Ab, has been reported to have a synergistic effect on acute GVHD [20, 28, 38, 56, 57, 62, 63]. However, these treatments may have serious side effects such as hypertension, dyslipidemia, nephrotoxicity, marrow suppression, and hirsutism, and cannot substantially improve graft survival in acute GVHD.

Antibody-engineering technology involves multimerization of binding Fab domains such as diabody, triabody, and tetrabody. These technical platforms are widely in use to develop better antibodies with higher avidity and potency, and some of the products have been approved for clinical applications, especially in cancer therapy and viral retargeting fields [2, 29, 45, 58, 59]. Recently, it has been demonstrated that tetravalent immunoadhesins engineered using inline fusion of the same or different extracellular domains has a higher avidity and more potency than divalent immunoadhesins in chronic disease models [11, 61]. However, there has not been any attempt to examine the effect of tetravalent immunoadhesins alone or in combination with other immunoadhesins in disease models. In this study, we have constructed four different tetravalent immunoadhesins of CTLA4-Ig, CD2-Ig, TNFR2-Ig, and LAG3-Ig to inhibit interactions of B7:CD28, LFA-3:CD2, TNF-α/β:TNFR, and MHC II:TCR, respectively. These studies showed that tetravalent forms of immunoadhesins have higher affinity, and more potent suppressive effect in vitro and in vivo. Furthermore, co-blocking of the MHC II:TCR and CD28:B7 pathways could be a better strategy for preventing acute GVHD.

### MATERIALS AND METHODS

#### Bacterial Strain, Plasmids and DNA Manipulation

The bacterial strain and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in Luria–Bertani broth. When necessary, antibiotics were added to the growth media as follows: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml. Restriction enzyme and DNA modification enzymes were purchased from Takara. DNA isolation and manipulations in *E. coli* were carried out according to standard procedures [21]. Plasmid DNA was extracted with the Wizard miniprep DNA purification system (Promega) according to the manufacturer’s instruction. The primers used in this study are listed in Table 2. DNA fragments were PCR-amplified with the pfu DNA polymerase (Stratagene) and the PCR products were purified with a QIAquick Gel Extraction kit (Qiagen).

#### Isolation of Peripheral Blood Mononuclear Cells (PBMC) and Cell Culture Conditions

Unless stated otherwise, all cell culture reagents were purchased from Life Technologies Gibco/BRL. Heparinized venous blood was obtained from healthy volunteers. PBMC were isolated by Ficoll–Hypaque (GE Healthcare) density gradient centrifugation at 400 × g for 20 min at 22°C. The cells were washed 2× with HBSS containing 2% FBS (HBSS-2), and resuspended at 1 × 10^6 cells/ml in RPMI-1640 supplemented with 10% FBS (RPMI-10). PBMC were stimulated with phytohemagglutinin (PHA; Sigma) at 2 µg/ml at 37°C in a humidified 5% CO₂ atmosphere for 3 days. PHA-stimulated PBMC are referred to as PHA blasts.

#### Construction of Plasmids

The divalent forms of CTLA4-Ig [35], LAG3-Ig [24], and CD2-Ig were constructed by the method described previously [61]. Total RNA was isolated from PHA blasts or PBMC using an RNeasy kit.

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<th>Strain or plasmid</th>
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<tr>
<td><strong>Strains</strong></td>
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<td>DH5</td>
<td>Plasmid-free, restriction-deficient</td>
<td>Stratagene</td>
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<td><strong>Plasmids</strong></td>
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<td>pCI-neo</td>
<td>Mammalian expression vector</td>
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<td>pCI-CTL4A4-Ig</td>
<td>pCI-neo carrying divalent CTLA4-Ig</td>
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<td>pCI-(LAG3)-Ig</td>
<td>pCI-neo carrying tetravalent CTLA4-Ig</td>
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(Qiagen) and reverse-transcribed with a random hexamer primer by Superscript II reverse transcriptase (Invitrogen Inc). DNA fragments encoding a soluble extracellular domain including CTLA4, CD2, and LAG3 were amplified by PCR from cDNA of PHA blasts with primer pairs P113/P114 (CTLA4), P117/P118 (CD2), and P121/P122 (LAG3) (Table 2). A DNA fragment encoding the IgG Fc region was amplified by PCR from cDNA of PBMC with P125/P127 or P125/P127 (LAG3) (Table 2). The PCR products were digested with PstI and SpeI, and ligated with T, DNA ligase (Invitrogen). The product of the sub-fragments were further digested with EcoRI and XbaI, and inserted into the pCI-neo mammalian expression vector (Promega).

The tetravalent immunoadhesins were constructed as previously described with minor modifications [61]. Two DNA fragments encoding a soluble extracellular domain of CTLA4, CD2, and LAG3 were amplified from divergent constructs by PCR with following primer sets: P113/P116 (CTLA4), P117/P120 (CD2), and P121/P124 (LAG3) for the first fragment; P115/P127 (CTLA4), P119/ P127 (CD2), and P123/ P127 (LAG3) for the second fragments. The first and second fragments were blunt-ligated and further subjected to PCR application with the primer pairs P113/P116 (CTLA4), P117/ P118 (CD2), and P121/P122 (LAG3). The resulting fragments were digested with EcoRI and XbaI, and inserted into the plasmid pCI-neo. TNFR2-Ig fusions (etanercept) were produced as described previously [61].

### Expression and Purification of Immunoadhesins

CHO/dhfr cells (ATCC, CRL 9096) were cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% FBS, 0.1 mM sodium pyruvate, 0.016 mM thymidine, 50 µg/ml penicillin, and 50 µg/ml streptomycin. CHO/dhfr cells were transfected with 2 µg of plasmid and 0.2 µg of pSv2-dhfr (ATCC 37146) using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. After two days, transfected cells were selected by replacing the media with minimal essential medium (MEM) deficient in deoxyribonucleosides and ribonucleosides containing 10% dialyzed FBS and 400 µg/ml G418 (Invitrogen). For MTX (Sigma) selection, G418-resistant cells were grown in deoxyribonucleosides- and ribonucleosides-deficient MEM supplemented with 10% dialyzed FBS and 20 nM MTX. The MTX concentration was increased stepwise to 4-fold. The cells were allowed to adapt for at least 1 month at each step of MTX concentration. Production of soluble immunoadhesins from the transfecteds was detected by a goat anti-human IgG (KPL)-specific ELISA as described previously [61]. Produced immunoadhesins were purified from culture supernatants by using HiTrap protein A and HiPrep Sephacryl S300 HR columns (GE Healthcare). Purified proteins were analyzed for their purities by running on SDS-PAGE gels under reducing and nonreducing conditions.

### Flow Cytometry Analysis

PHA blasts (5 × 10^6 cells/sample) were incubated with various immunoadhesins (10 µg/ml) or control human Ig (10 µg/ml) for 30 min on ice in HBSS-2 containing 0.1% NaN3 (Sigma), followed by washing with the same buffer. The cells were then stained with FITC-conjugated anti-hIgG (Dako). All stained cells were analyzed by flow cytometry using a FACSScalibur, and data captured were analyzed with CellQuest software (BD Biosciences).

### In Vitro Immunosuppressive Assay

PHA blasts were plated at 1 × 10^6 cells/well in 96-well microplates and varying concentrations of purified immunoadhesins and control CsA (Sigma) were added. Plates were incubated at 37°C and 5% CO2 for 3 days. Proliferation of the cells was determined by adding [3H] thymidine (1 µCi/well; GE Healthcare) for the last 18 h of the assay. T lymphocyte proliferation was measured by β-scintillation counting on a MicroBeta TriLux model 1450 counter (Wallac).

### Human and Mouse Allogeneic MLR

For the human allogeneic MLR assay, responder cells were plated at 1 × 10^6 cells/well (donor A PBMC), and γ-irradiated (30 Gy; 60Co source) stimulator cells at 5 × 10^5 cells/well (donor B PBMC), in 96-well microplates. For the murine allogeneic MLR assays, C57BL/6 splenocytes (1 × 10^6 cells/well) were used as responders and irradiated...
(20 Gy) CBF₁ splenocytes were used as stimulators (5 × 10⁵ cells/well). Responder and stimulator cells were incubated with serial dilutions of the tetravalent CTLA4-Ig, tetravalent LAG3-Ig, or combination with tetravalent CTLA4-Ig and tetravalent LAG3-Ig at the start of the assay. The cell mixture was incubated for 3 days at 37°C in 5% CO₂ and the cultures were pulsed with [³H] thymidine (1 µCi/well) for 18 h before harvesting. Proliferation of the cells was determined by a β-scintillation counter as described above.

Murine Acute GVHD
C57BL/6 (H-2ᵇ) donor mice and BALB/c (H-2ᵈ) were purchased from SLC (Hamamatsu, Shizuoka, Japan) and CBF₁ (C57BL/6 × BALB/c; H-2ᵇ/d) recipient mice were generated and kept under standard conditions in the animal facility of Hanyang University, Korea. Murine GVHD model was carried out as described previously [19]. Briefly, recipient CBF₁ (H-2ᵇ/d) mice were irradiated with 7.0 Gy γ-rays and were treated with 40 mg of trimethoprim and 200 mg of sulfamethoxazole (Roche) at one day before transplantation. In order to induce acute GVHD, 2.5 × 10⁷ viable splenocytes from allogeneic C57BL/6 (H-2ᵇ) donor mice were transplanted into the irradiated CBF₁ (H-2ᵇ/d) recipient mice by i.v. injection. Thereafter, the LAG3-Ig, CTLA4-Ig, tetravalent LAG3-Ig, and tetravalent CTLA4-Ig were individually dissolved in PBS at a concentration of 200 µg, and i.p. injected into the recipient mice developing GVHD 0, 2, 4, and 6 days post-transplantation. To investigate the combinational effect on acute GVHD, LAG3-Ig (100 µg) + CTLA4-Ig (100 µg), and tetravalent LAG3-Ig (100 µg) + CTLA4-Ig (100 µg) were treated in the same manner as described above. Control recipient mice were administered with PBS. Survival was monitored daily, and recipients’ body weights were measured every two days.

Statistical Analysis
All results are expressed at the mean ± SD. Student’s t-test for populations with normal distribution and equal variance or two-sample t-test with the Welch correction was used. Differences were considered statistically significant at P < 0.05.

RESULTS

Construction and Purification of Immunoadhesins
Conventional divalent (Fig. 1A left) and tetravalent (Fig. 1A right) immunoadhesins were produced from CHO cells by transfecting each construct encoding the extracellular domains of CTLA4, CD2, and LAG3 fused to the Fc portion of human IgG₁. The molecular mass of each immunoadhesin was estimated in SDS-PAGE reducing condition to be approximately 40–45 kDa (CTLA4-Ig), 50–60 kDa (tetravalent CTLA4-Ig), 45–50 kDa (CD2-Ig), 55–65 kDa (tetravalent CD2-Ig), 50–60 kDa (LAG3-Ig), and 75–85 kDa (tetravalent LAG3-Ig), respectively (Fig. 1B).

Analyses of Immunoadhesin Binding to Activated PBL
Flow cytometric analyses showed that all immunoadhesins bound efficiently to PHA blasts (Fig. 2). Comparing with each divalent immunoadhesin, the fluorescence intensities of each tetravalent immunoadhesin showed significant increase in any case of each immunoadhesin.

In Vitro Immunosuppressive Effects of Immunoadhesins
To examine the immunosuppressive effects of immunoadhesins, a bioassay using human PBL PHA blasts was performed at various concentrations of immunoadhesins. This experiment included both divalent and tetravalent forms of TNFR2-Ig used in a previous study as the control [61]. All immunoadhesins were able to inhibit the T cell proliferation by the immunoadhesin in a concentration-dependent manner (Fig. 3). The T cell proliferation was inhibited 45.2 ± 1.2% (LAG3-Ig), 51.5 ± 1.5% (CTLA4-Ig), 54.5 ± 1.5% (CD2-Ig), and 60.6 ± 2.1% (TNFR2-Ig) when 10 µg/ml of divalent immunoadhesins was treated in culture (Fig. 3, open circles).

Fig. 1. Construction of immunoadhesins.
(A) Schematic structures for divalent (left) and tetravalent (right) immunoadhesins. Extracellular domains of each CTLA4, CD2, or LAG3 are fused to the Fc portion of human IgG₁. The extracellular domains are indicated with white oval shapes and the hIgG Fc regions (domains of hinge and CH₂–CH₃) are gray filled oval shapes. (B) SDS-PAGE analysis of the purified immunoadhesins. Immunoadhesin constructs were transfected into CHO/dhfr cells and the soluble immunoadhesins were purified by protein-A chromatography. Purified immunoadhesins including divalent CTLA4-Ig (lane 1), tetravalent CTLA4-Ig (lane 2), divalent CD2-Ig (lane 3), tetravalent CD2-Ig (lane 4), divalent LAG3-Ig (lane 5), and tetravalent LAG3-Ig (lane 6) were analyzed by SDS-PAGE under reducing condition. M, Molecular weight marker proteins.
However, when treated with tetravalent immunoadhesins (10 µg/ml) in the culture, the T cell proliferation was further inhibited 24% (tetravalent LAG3-Ig; 21.2 ± 1.5), 24.3% (tetravalent CTLA4-Ig; 27.2 ± 1.2), 15.2% (tetravalent CD2-Ig; 39.3 ± 2.4), and 15.1% (tetravalent TNFR2-Ig; 45.4 ± 2.7), respectively (Fig. 3 closed circles). Moreover, tetravalent CTLA4-Ig and tetravalent LAG3-Ig revealed higher inhibitory effects on T cell proliferation than tetravalent TNFR2-Ig and tetravalent CD2-Ig did. These data demonstrated that the tetravalent immunoadhesins have more potent effect than their divalent immunoadhesins in vitro.

Combination Effect of Tetravalent CTLA4-Ig and Tetravalent LAG3-Ig In Vitro MLR
In order to examine the immunosuppressive potentials of each immunoadhesin in allogeneic stimulation, we performed the in vitro primary MLR assay. Since the data above showed that tetravalent CTLA4-Ig and tetravalent LAG3-Ig were potent candidates for the immunosuppressants, further study focused on whether combination treatment of tetravalent CTLA4-Ig and tetravalent LAG3-Ig would enhance the suppressive function of in vitro allogeneic MLR assay. Inhibition of alloantigen-specific T cell proliferative responses was dramatically increased in the presence of either tetravalent CTLA4-Ig or tetravalent LAG3-Ig in a concentration-dependent manner (Fig. 4A). More importantly, the combined treatment with tetravalent CTLA4-Ig and LAG3-Ig (88 ± 2%) resulted in a significant inhibition of alloantigen-specific T cell proliferation when compared with responses in the concentration at 1 µg/ml of either tetravalent CTLA4-Ig (64 ± 2%) or tetravalent LAG3-Ig (65 ± 3) alone (Fig. 4A). Similar results were observed when CBF1 (H-2b/d) responders were incubated with C57BL/6 (H-2b) stimulators in the presence of tetravalent CTLA4-Ig, tetravalent LAG3-Ig or both tetravalent CTLA4-Ig and tetravalent LAG3-Ig (Fig. 4B). Although single treatment of either tetravalent CTLA4-Ig or tetravalent LAG3-Ig was able to inhibit the mouse allogeneic MLR efficiently, the combination treatment with both tetravalent immunoadhesins exhibited more potent inhibition of T cell proliferation (Fig. 4B).

Combination Effect of Tetravalent CTLA4-Ig and Tetravalent LAG3-Ig In Vivo GVHD
To examine whether treatment of CTLA4-Ig and LAG3-Ig suppresses the development of acute GVHD, we transplanted splenocytes from C57BL/6 (H-2b) mice into sublethally irradiated CBF1 (H-2b/d) mice and treated with LAG3-Ig, CTLA4-Ig, or control PBS. Results showed that CBF, mice treated with control PBS died by day 15 (Fig. 5A), with a severe decrease in body weight (Fig. 5C). However, like CTLA4-Ig-treated mice, the CBF, mice treated with LAG3-Ig further survived by 36 days (Fig. 5A), but with a more significant (Fig. 5C), and treatment of LAG3-Ig significantly reduced GVHD mortality. Treatment with tetravalent CTLA4-Ig or tetravalent LAG3-Ig led to significant prolongation of survival compared with CTLA4-Ig or LAG3-Ig treatment (median survival time (MST), tetravalent CTLA4-Ig (38 d) vs. CTLA4-Ig (22 d); tetravalent LAG3-
Ig (35 d) vs. LAG3-Ig (20 d), respectively; p<0.005; Fig. 5A] further demonstrated that treatment with the tetravalent form of CTLA4-Ig or LAG3-Ig prevented GVHD mortality than treatment with the divalent form of CTLA4-Ig or LAG3-Ig.

Although treatment with the tetravalent form of CTLA4-Ig or LAG3-Ig prolonged the survival, it was not optimal because all mice died within day 50 post transplantation. We therefore examined the synergistic effects of combined treatment with either divalent forms or tetravalent forms of CTLA4-Ig and LAG3-Ig in acute GVHD. As shown in Fig. 5B, combined treatment with LAG3-Ig and CTLA4-Ig led to significantly prolonged allograft survival for a maximum of 80 days, with borderline increases in body weight as compared with the pre-transplanted mice (Fig. 5C). Furthermore, combined treatment with tetravalent LAG3-Ig and tetravalent CTLA4-Ig could synergistically prolong allograft survival, with 80% of the GVHD free of rejection in recipients (Fig. 5B) and a consistent increase of body weight (Fig. 5C). These data demonstrated that combinations of divalent LAG3-Ig and CTLA4-Ig or tetravalent LAG3-Ig and CTLA4-Ig synergistically improve the efficacy of each molecule to prolong allograft survival in murine acute GVHD.

**DISCUSSION**

We have previously shown that the tetravalent forms of TNF receptor-type immunoadhesins have a high binding affinity and a potent efficacy to their target receptors [61]. Further to these findings, in this study, we have generated
Fig. 4. Effects of tetravalent CTLA4-Ig, tetravalent LAG3-Ig, and their combination in primary MLR. (A) For the human allogeneic MLR assay, PBMC (1 × 10^5 cells/well) from donor A as a responder and γ-irradiated PBMC (5 × 10^4 cells/well) from donor B as a stimulator were co-cultured in a 96-well microplate. Cells were incubated with the presence of varying concentrations of tetravalent CTLA4-Ig (■), tetravalent LAG3-Ig (□), tetravalent (CTLA4-Ig + LAG3-Ig) (▲), or control CsA (△). (B) For the murine allogeneic MLR assay, responder splenocytes (1 × 10^6 cells/well) from C57BL/6 and γ-irradiated stimulator splenocytes (5 × 10^5 cells/well) from CBF1 were incubated with the presence of varying concentrations of tetravalent CTLA4-Ig (■), tetravalent LAG3-Ig (□), tetravalent (CTLA4-Ig + LAG3-Ig) (▲), or control CsA (△). Three days after stimulation, the proliferation rate of the cells was determined by adding [3H] thymidine (1 µCi/well) for the last 18 h of the assay and measured by a scintillation counter. Data are shown as the mean of inhibition rate ± SD of triplicate wells and representative of three independent assays. R, responders only; S+R, stimulators + responders; CsA, Cyclosporin A. *p < 0.05.

Fig. 5. Dose and combination effects of tetravalent CTLA4-Ig and LAG3-Ig in murine acute GVHD. Acute GVHD was induced by an i.v. injection of 2.5 × 10^7 viable C57BL/6 splenocytes into CBF1 recipient mice. Recipients were injected i.p. with divalent LAG3-Ig (200 µg; ○), divalent CTLA4-Ig (200 µg; ▼), divalent [LAG3-Ig + CTLA4-Ig (each 100 µg; ▲)], tetravalent LAG3-Ig (200 µg; ■), tetravalent CTLA4-Ig (200 µg; □), and tetravalent [LAG3-Ig + CTLA4-Ig (each 100 µg; ◆)] at days 0, 2, 4, and 6. Control recipient mice were administered with PBS (●). Ten mice per group were tested. Survival rate (A and B) and mean body weights (C) are plotted. (A and B), Median survival time (MST): ●, 14 d; □, 20 d; ○, 22 d; ▼, 35 d; ▲, 38 d; ▼, 50 d; ■, > 80 d (p < 0.005)
various tetravalent immunoadhesins and their functional superiorities were tested in a murine acute GVHD model. In addition, we have demonstrated that the combined treatment of tetravalent LAG3-Ig and tetravalent CTLA4-Ig induces long-term survival (more than 80 days) of mice receiving allogeneic donor cells that without the combined treatment would induce acute lethal GVHD.

In this study, we have evaluated the contribution of LAG3:MHCII, CTLA4:B7, CD2:LFA-3, and TNF-α/α TNF:TNFR2 interactions in T cell activation via (i) nonspecific PHA stimulation, (ii) allogeneic stimulation in vitro mixed lymphocyte reactions of human and mouse systems, and (iii) allogeneic stimulation in vivo graft-versus-host disease of mice. LAG3, a MHC class II ligand structurally related to CD4, is expressed in activated T cells and natural killer (NK) cells and is able to block MHC II:TCR interaction in an adhesion assay [25, 53]. Subramanyam et al. [50] have shown that LAG3-Ig inhibits allosponses of naïve human CD4+ T cells in in vitro MLR assay. Interestingly, on further analysis, LAG3-Ig can bind to MHC II (Fig. 2) and can inhibit alloreactive T cell proliferation in both human and mouse MLR (Fig 4), suggest that LAG3-Ig is a potential candidate of immunosuppressive agents for transplantation control. CTLA4, another CD28 family member, is a glycoprotein expressed on only activated T cell and shares B7 ligands, with CD28 [36]. Previous studies showed the efficacy of CTLA4-Ig in several animal models of T-cell-dependent antibody response, autoimmunity, GVHD, and transplantation [18, 22, 33]. More recently, LEA29Y, a mutant form of CTLA4-Ig developed by Bristol-Myers Squibb (BMS-224818), has proven to be a potential agent of next-generation therapeutics and can prolong islet allograft survival indefinitely under cover of FK506/rapamycin in nonhuman primates [1]. Our results showed that although all immunoadhesins efficiently bound to PHA blasts, LAG3-Ig and CTLA4-Ig have higher binding affinities than CD2-Ig (Fig. 2). Furthermore, these LAG3-Ig and CTLA4-Ig have more potent effector function to inhibit T cell proliferation in vitro than CD2-Ig and TNFR2-Ig did (Fig. 3). Findings of this study suggest that blockade of MHCII:TCR and B7:CD28 by LAG3-Ig and CTLA4-Ig, respectively, could be key factors to control the T cell tolerance.

Although the CD2-Ig and TNFR2-Ig showed lower potential to inhibit T cell proliferation (Fig. 3), the blockade of CD2:LFA-3 or TNF:TNFR interaction was commonly used to prevent GVHD [26, 27, 44, 51, 60]. Currently, in some clinical trials, anti-CD2 (MEDI-507; humanized BTI-322) and LFA3-Ig (alefacept; Amevive, Biogen) have been used as immunosuppressive agents to control graft or host rejections in allogeneic bone marrow or blood stem cell and skin or cardiac allografts [27, 44, 51]. TNFR2-Ig (etanercept; Enbrel, Amgen), which is a TNF antagonist that decreases the excessive levels of TNF associated with inflammatory diseases down to physiologic levels, is now widely used for decreasing the clinical signs and symptoms of rheumatoid arthritis (RA), polyarticular course juvenile RA, ankylosing spondylitis, psoriasis, and psoriatic arthritis [13, 14, 37, 48]. In addition, treatment with infliximab (TNF-Ab), which is a genetically constructed IgG1 murine–human chimeric monoclonal antibody, has been reported to not only affect chronic GVHD, but also affect the steroid-resistant severe acute GVHD [26, 60]. Our results also showed that LFA-3 and TNF co-blockade using tetravalent CD2-Ig and tetravalent TNFR2-Ig is more potent to inhibit the T cell proliferation than CD2-Ig or TNFR2-Ig alone (Fig 3). Findings of this study have suggested that tetravalent immunoadhesins would be one of the better options in immunosuppressive treatments, and combinations of at least two of them would expect to show a better therapeutic outcome. Tetravalent CTLA4-Ig + tetravalent LAG3-Ig would be one of the best candidates to consider for immunosuppressive agents for preventing acute GVHD.

To enhance the potency of mAbs that exert their effect through the clustering of target molecules, several creative multivalent Ab have been generated [2, 11, 29, 45, 58, 61]. Typically, these mAbs were generated by linking three or four Fabs in a linear form; by using linear Fabs to generate tetravalent F (ab’)2 forms; and by fusing the linear Fab to an IgGFc. These engineered antibodies are all designed to increase the functional binding affinity to target antigens that lead to potently activating the immune system. In addition, tetravalent forms of immunoadhesins were reported as potent blockers of immune reaction by several researchers including us [11, 61]. These studies showed that the tetravalent form had a higher affinity in binding to their target molecules than divalent TNFR-Ig in competition binding assay using the tetravalent TNFR-Igs binding to TNF-α. Tetravalent forms were also associated with better potency in cytotoxicity assay and collagen induced arthritis (CIA). Further to our previous findings, this study presents several lines of data supporting our claims that tetravalent immunoadhesins is a more potent platform of immunosuppressive agents. First, in flow cytometry, tetravalent immunoadhesins have higher binding affinity than divalent immunoadhesins (Fig. 2). Second, tetravalent immunoadhesins have more potent effect to inhibit T cell proliferation in vitro (Fig. 3 and 4). Lastly, treatment of tetravalent immunoadhesins enhances to prolong graft survival in an acute murine GVHD model.

The in vitro MLR assay demonstrated that co-blockade of the MHCII:TCR and B7:CD28 pathways using LAG3-Ig and CTLA4-Ig showed the best immunosuppressive outcome. In combinational effect, CTLA4-Ig or anti-B7 antibodies with other molecules including CsA [10], anti-CD40 Ab [28], anti-CD4 Ab [30], anti-CD2 Ab [57], and anti-LFA-1 Ab [7] were reported to show synergistic inhibition effect.
on primary MLR. We also demonstrated that the combined treatment of tetravalent CTLA4-Ig with tetravalent LAG3-Ig could significantly inhibit T cell proliferation on allogeneic primary MLR. It means that those 2 human recombinant proteins showed similar results in both human and murine assay systems. These results can be explained by the conserved structure, the sequence homologies, of CTLA4 and LAG3 between human and murine (71% and 70%, respectively) and their conserved functionality across species as shown in various in vitro studies [34, 39].

Immunoadhesin has properties with high therapeutic efficacy and low toxicity compared with other immunosuppressants such as complete antibody type therapeutics. Although CTLA4-Ig treatment has been validated for use in many transplantation models to induce long-term allograft survival, it has not substantially improved prolonged graft survival in acute GVHD. Thus, many researchers have tried to combine it with other agents and led to long-term graft survival. The combination of CTLA4-Ig with CsA and other molecules including MTX, rapamycin, anti-CD2 Ab, anti-LAF-1 Ab, anti-CD40L Ab, anti-CD4 Ab, and anti-ICAM-1 Ab has been reported to have synergistic effect on acute GVHD [20, 28, 32, 38, 56, 57, 62, 63]. Our studies also demonstrated that combined treatment with CTLA4-Ig and LAG3-Ig markedly induced prolonged allograft survival (Fig 5B), whereas treatment with either CTLA4-Ig or LAG3-Ig alone did not (Fig. 5A). Although the combination of CTLA4-Ig and LAG3-Ig markedly prolonged the allograft survival, it was not able to produce a permanent engraft. In combination of tetravalent CTLA4-Ig with tetravalent LAG3-Ig, however, it can significantly decrease acute GVHD induced in lethally irradiated recipients of fully allogeneic donor grafts (Fig. 5B), suggesting that the simultaneous blockade of CD28/CTLA4: B7 and MHC II:TCR pathways by potent immunosuppressants such as tetravalent CTLA4-Ig and tetravalent LAG3-Ig would be a better approach for preventing acute GVHD.

In conclusion, the tetravalent immunoadhesin approach described in this report suggests the versatility to design a novel target protein to potentially enhance its therapeutic utility. In addition, along with previously described combined treatment, co-blockade of CD28/B7 and MHC II:TCR by using tetravalent immunoadhesins would likely be an effective method that may be able to modulate the pathogenesis of acute GVHD.

Acknowledgment

This study was supported by a grant of the Korea Health 21 R & D Project, Ministry of Health & Welfare, Republic of Korea (Project No. A040004).

References


IMMUNOSUPPRESSIVE EFFECT OF TETRAVALENT IMMUNOADHESINS


