A Role for CD2 Antibodies (BTI-322 and its Humanized Form) in the in vivo Elimination of Human T Lymphocytes Infiltrating an Allogeneic Human Skin Graft in SCID Mice: An Fcγ Receptor-Related Mechanism Involving Co-Injected Human NK Cells

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Background. Pilot clinical studies have shown that the rat anti-human-CD2 monoclonal antibody, LoCD2a/BTI-322, can efficiently prevent and treat acute kidney rejection. However, the in vivo mechanism by which it prevents allograft rejection has not been studied. BTI-322 and its humanized form have been shown to mediate in vitro antibody-dependent cell-mediated cytotoxicity (ADCC) against $CD2^+$ cells through the activation of monocytes or natural killer (NK) cells.

Methods. Human fetal skin samples were grafted into severe combined immunodeficient/nonobese diabetic mice. Five weeks later (day 0), the mice were injected with human allogeneic peripheral blood lymphocytes (PBL). Either on day 0 or on day 14, mice were treated with BTI-322, hu-BTI-322, or their $F(ab')_2$ fragments. Peripheral blood mononuclear cells (PBMC) thoroughly devoid of NK cells were also assayed.

Results. After injection of PBL, the human skins became heavily infiltrated with activated human T lymphocytes, resulting in dermal microvascular injuries indicative of graft rejection. Early treatment with BTI-322 and hu-BTI-322 prevented all these events. These CD2 antibodies rapidly eliminated human T lymphocytes that had already infiltrated the grafts, with no evidence of recirculation toward the spleen. Their $F(ab')_2$ fragments were, in contrast, ineffective. Elimination of NK cells from injected PBMC prevented the curative effect exerted by whole CD2 antibodies. It also abrogated their cytotoxicity potential against $CD2^+$ cells in ADCC assays.

Conclusion. $F(ab')_2$ fragments of the CD2 antibodies could not prevent allograft rejection, whereas whole immunoglobulin G could, and human NK cells were required for the curative effect exerted by these antibodies. The results are consistent with an FcγR-dependent ADCC mechanism mediated in vivo by human NK cells.

Keywords: CD2 monoclonal antibodies, SCID/NOD mice, Skin transplantation, Acute rejection, Fcγ receptors, NK cells.

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All human mature αβ-T-lymphocytes and natural killer (NK) cells express at their surface the CD2 receptor, a 50 kDa transmembrane glycoprotein, the ligand of which is the widely expressed CD58 (lymphocyte function-associated antigen [LFA]-3) protein (*1*). CD2 is essential in promoting adhesion between T cells and antigen-presenting cells (APCs) through its interaction with LFA-3, resulting in the formation of the "immunologic synapse" (*2*). Nondepletive CD2 monoclonal antibodies (mAb) have been successfully used to target the CD2 to disrupt T-lymphocyte/APC interactions in several animal models of organ transplantation (*3–6*). CD2 is also a signaling molecule that contributes to T-cell activation by recruiting the $p56^{lck}$ or the $p59^{fyn}$ tyrosine kinases at its intracytoplasmic tail (*7*, *8*). The induction of CD2 signaling by specific pairs of CD2 mAb can, in addition, result in the apoptosis of activated T lymphocytes through a Fas- and caspase-independent pathway (*9–12*). $FcγR^+$ cytotoxic cells (e.g., NK cells) can cause the apoptosis of $CD2^+$ cells in vitro by an antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism when incubated with the CD2 receptor-specific LFA-3/immunoglobulin (Ig)G-1 fusion protein, alefacept (*13*).

Several studies have concentrated on the cytotoxic effects of a rat anti-human CD2 mAb named LoCD2a/BTI-322 and of its humanized form, hu-BTI-322, a fusion protein expressing human IgG-1k (*9*, *14*, *15*). Both of these mAb induce the down-modulation of CD2 molecules in vitro and kill activated T cells through an ADCC reaction involving interferon–γ-activated monocytes (*16*) and NK cells (*14*, *16*). One pilot clinical study (phase II) using BTI-322 to prevent or to treat acute rejection in kidney-transplant patients has provided encouraging results (*16*). BTI-322 has also been shown to be efficient for the treatment of steroid-resistant graft-versus-host-disease in allogeneic bone-marrow recipients (*17*). Hu-BTI-322, commonly known as siplizumab (or MEDI-507), is currently being tested in clinical trials for the treatment of psoriasis by MedImmune Inc. Our in vitro studies have shown that very low concentrations of BTI-322 can trigger the apoptosis of alloreactive human T cells in primary

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mixed lymphocyte cultures. The surviving T cells do not respond to a secondary challenge with the same set of alloantigens but do respond to other alloantigens or to nominal antigens such as protein purified derivative (PPD) (*9*, *13*).

In the above mentioned clinical study, the amount of CD2 molecules and peripheral T lymphocytes decreased dramatically after BTI-322 treatment (*16*). However, it is not clear whether the lymphocytes had been killed by BTI-322 or trapped in lymphoid organs. In addition, the activity of the humanized mAb has never been evaluated in clinical or experimental in vivo transplantation studies.

Therefore, the purpose of our study was to explore the in vivo mechanism of action of BTI-322 and hu-BTI-322. For this, we used an experimental transplantation model that resembles human allotransplantation, consisting of severe combined immunodeficient/nonobese diabetic (SCID/NOD) mice grafted with human fetal skin and subsequently injected with human allogeneic peripheral blood lymphocytes (PBL) or peripheral blood mononuclear cells (PBMC). The NOD-LtSz-*scid/scid* mouse strain is characterized by a lack of functional T and B lymphocytes, a functional deficiency in monocyte/macrophage and NK cell activity, and an absence of hemolytic complement (*18*). Our animal model was derived from that developed by Pober et al. (*19*, *20*) with adult human skin. It was developed concurrently with that of Erdag et al. (*21*), also using fetal skin. In both models, dermal microvascular injuries are the most specific histologic signs of skin-graft rejection.

Our immunohistochemical analyses show that BTI-322 and hu-BTI-322 can efficiently prevent or reverse ongoing rejection of human skin grafts by targeting alloreactive T lymphocytes. Importantly, both CD2 antibodies rapidly and completely eliminated the numerous activated T lymphocytes infiltrating the skin grafts in the course of acute rejection. In this respect, the $F(ab')_2$ fragments of these antibodies were ineffective. The removal of NK cells from human PBMC, before their injection into mice, almost completely prevented the curative effect of hu-BTI-322. These results strongly suggest that the mechanism of in vivo action of the CD2 antibodies is an ADCC mediated by human NK cells.

MATERIALS AND METHODS

Animals

Eight-week-old male or female NOD-LtSz-*scid/scid* mice were obtained from INSERM U506 (Dr Peault, Villejuif, France). All experimental protocols were approved by the Groupe de Réflexion sur l'Ethique Biomédicale de Bicêtre (GREBB).

Antibodies

Lo-CD2a/BTI-322 mAb (a rat IgG-2bκ), a humanized version of BTI-322, hu-BTI-322 (IgG1-κ), their $F(ab')_2$ fragments, and the control isotype matched rat IgG2b anti-DNP mAb (Lo-DNP-57) were given by BioTransplant Inc. (Charlestown, MA). These antibodies were injected subcutaneously (SC) on the opposite side of the graft. The $F(ab')_2$ fragments of BTI-322 were prepared by pepsin digestion and chromatography procedures including anion exchange and gel filtration. The $F(ab')_2$ fragments of human BTI-322 were prepared by pepsin digestion and passage on protein A and anion exchange columns. We assessed the purity of the $F(ab')_2$ preparations by Western blot analysis using horseradish peroxidase (HRP)-conjugated mouse IgG directed against human IgG (clone GG-5, Sigma) and HRP-conjugated rabbit IgG against rat IgG (Sigma). Immunoblots were developed using enhanced chemiluminescence reagents (ECL kit, Amersham Biosciences). The antigen-binding capacity of the CD2 preparations was analyzed by flow cytometry after incubation with $CD2^+$ Jurkat cells. Secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat IgG and FITC-conjugated rabbit anti-human kappa light chains (Dako, Trappes, France).

mAb used for immunohistochemistry were mouse anti-human CD3 (OKT3, Orthoclone, Bridgewater, NJ), mouse anti-human CD4 (Diaclone, Besançon, France), mouse anti-human CD8 (OKT8, ATCC, Rockville, MD), mouse anti-human CD2 ($T11_1$ from Dr A. Bernard, INSERM U343, Nice, France), mouse anti-human human leukocyte antigen (HLA)-DR (L243, from Dr G. Sterkers, Hôpital Robert Debré, Paris, France), mouse anti-human CD31 (Dako) and rat anti-mouse CD31 (Dako), control mouse IgG (Vector Laboratories, ABCYS, Paris), and biotin-conjugated horse anti-mouse IgG and goat anti-rat IgG (Vector). Mouse macrophages were stained with the rat anti-mouse F4/80 (clone CI:A3–1, Caltag, Tebu, Le-Perray-en-Yvelines, France). mAb used for flow cytometry were anti-human CD3, CD4, CD8, CD45, and CD16 from Diaclone (Besançon, France) and anti-human CD2 from Immunotech (Beckman-Coulter, Villepinte, France).

Skin Grafts

Human fetal skin was obtained from tissues discarded after voluntary abortions, carried out between the 9th and the 12th week of pregnancy at the Kremlin Bicêtre Hospital, by use of a protocol approved by the GREBB. The fetal skin was cut into approximately 5 by 5 mm pieces. The SCID/NOD mice were anesthetized, shaved, and a 1 cm incision made on the left flank. Two pieces of human skin were grafted SC. Grafts were allowed to heal for 5 weeks before human PBL injection.

Isolation and Injection of Human PBL

Blood samples from healthy adult volunteers were provided by the Centre de Transfusion Sanguine, Hôpital Saint-Louis, Paris. PBMCs were isolated by centrifugation on Ficoll-Hypaque density gradient. Most adherent monocytes were removed by incubation on plastic dishes for 30 minutes at 37°C. Five weeks after skin grafting, 2×10^8 nonadherent cells, referred to as PBL, were injected intraperitoneally (IP) into each mouse. In some experiments, NK cells were eliminated from PBMC by two rounds of negative selection performed with an anti-CD56 (clone 3B8, a mouse IgM given by Dr. Hercend, Institut Gustave Roussy, France) and an anti-CD16 (clone B-E16, an IgG2a from Diaclone) in the presence of rabbit complement (Cedarlane, Tebu).

Histology and Immunohistology

Mice were killed 10 to 21 days after injection of human PBL. Human skin grafts and mouse livers were embedded in Tissue-Tek OCT compound (Miles, Elkart, IN) and frozen at −80°C. Immunohistochemistry was conducted using a bi-

otin-avidin based immunologic staining. Six micrometer cryostat (Leïca) sections of the skin specimens were fixed in acetone and incubated for 30 minutes with phosphate-buffered saline (PBS) supplemented with 10% human AB serum to saturate Ig receptors. The sections were then incubated with a primary mAb followed by a biotinylated horse anti-mouse or goat anti-rat IgG. Finally, tissues were incubated with streptavidin-conjugated alkaline phosphatase (Caltag) and enzymatic activity was revealed using Fast-Red and Naphtol AS-MX phosphate (Sigma). Endogenous phosphatase activity was blocked by adding levamisole (Sigma).

The degree of PBL infiltration was scored using the following grading system established by Sultan and coworkers (*22*): grade 0: 1 to 10 mononuclear cells scattered around vessels; grade 1: more than 10 sparse perivascular mononuclear cells; grade 2: patchy perivascular infiltrate with interstitial involvement; grade 3: dense perivascular and diffuse interstitial dermal infiltrate; grade 4: sheets of mononuclear cells filling the dermis. The density of the dermal microvascular system was determined by counting the number of vessels within a 0.16 cm^2 square grid put on the skin sections (stained with anti-human CD31). We also determined by this method the percentages of injured microvessels.

Flow Cytometry Analysis of Human Lymphocytes

When the mice were killed, 1 mL of blood was obtained by cardiac puncture, and the spleens were harvested. PBMC were isolated from blood and spleen by centrifugation on Ficoll-Hypaque density gradient. PBMC were suspended in PBS supplemented with 10% human AB serum to saturate Ig receptors. Human cells were detected by immunofluorescent double staining using a phycoerythrin-conjugated human-specific anti-CD45 and FITC-conjugated anti-human CD2, -CD4, -CD8, -CD14, or -CD16. After incubation for 30 minutes at 4°C, cells were analyzed with a FACScan (Becton Dickinson).

Protocols for BTI-322 and hu-BTI-322 Injections

The protocol to prevent skin graft rejection consisted of four SC injections of 1.5 to 10 μg of BTI-322 antibodies at 3-day intervals, starting the day of human PBL injection. Mice were killed 10 to 14 days later and skin grafts examined by immunohistochemistry. The "curative" protocol was used to treat acute rejection. It consisted of two SC injections of 10 μg BTI-322 or hu-BTI-322 at a 3-day interval, starting on day 14 postPBL injection. $F(ab')_2$ fragments of both antibodies were also tested at cumulative doses of 20 to 100 μg. Mice were killed 7 days after the first antibody injection (on day 21 postPBL injection) and skin grafts examined. Control mice received PBS or the isotype control mAb, LoDNP57.

In Vitro ADCC Assays

Human PBL or NK–cell-depleted PBMC were stimulated for 4 and 5 days with OKT3 and interleukin (IL)-2 and incubated for 4 hours with PBS or hu-BTI-322 (100 ng/mL). Changes in the inner mitochondrial transmembrane potential $\Delta\Psi m$ of the cells, indicative of apoptosis, was measured by cytofluorometry with the potential sensitive green fluorescent dye 3.3'-diethyloxacarbocyanine ($DiOC_6$ (3) Molecular Probes, Eugene, OR) as previously described (*23*). When using $CD2^+$ Jurkat cells as target cells, whole or NK–cell-depleted PBMC were mixed for 4 hours at 37°C with Jurkat cells at various effector/target (E:T) ratios, in the presence or absence of BTI-322 and hu-BTI-322 (100 ng/mL). To distinguish the target from the effector cells, the former were loaded with 5 mg/mL FITC-dextran (77 kDa, Sigma) for 2 hours at 37°C. Changes in $\Delta\Psi m$ of FITC-dextran$^+$ target cells were evaluated by cytofluorometry with the potential sensitive red fluorescent probe chloromethyl-X-rosamine (CMXRos, Molecular Probes).

RESULTS

Development and Differentiation of Human Fetal Skin Engrafted in SCID/NOD Mice

Human fetal skin fragments engrafted SC in SCID/NOD mice were allowed to develop and to grow in thickness for 5 weeks. At this time, the grafts were already well differentiated, consisting of three layers (Fig. 1A): the epidermis (including the superficial corneal sheet, the keratinocytes, and the basement membrane), the dermis containing follicles and microvessels, and the hypodermis made of lipid islets. Immunostaining with both anti-human and anti–mouse-CD31 revealed that skin grafts were vascularized by both human and mouse vessels (Fig. 1, B and C). The densities of human and mouse microvessels were similar ($112.3\pm44.0/mm^2$ vs. $89.3\pm22.9/mm^2$, respectively, $P=0.22$). Immunohistologic staining of frozen skin sections indicated that the grafts were devoid of resident human T lymphocytes before human PBL infusion (not shown).

Activated Human Allogeneic T Lymphocytes Circulate in SCID/NOD Mice and Heavily Infiltrate Human Skin Grafts

Five weeks after skin transplantation, human PBL were injected IP into mice (2×10^8/ mouse). The mice were killed 14 days later. Flow cytometry showed that $10.1\pm4.2\%$ of circulating cells and $12.7\pm5.7\%$ of splenocytes were positive for human CD45 at this time (Table 1). More than 95% of these human T cells were CD2-positive. Less than 1% of the cells expressed the human CD16 NK cell marker or the human CD14 monocyte/macrophage marker.

All human skin grafts contained large mononuclear cell infiltrates consisting almost exclusively of CD4 or CD8-positive $CD3^+$ T lymphocytes, a large percentage of which ex-

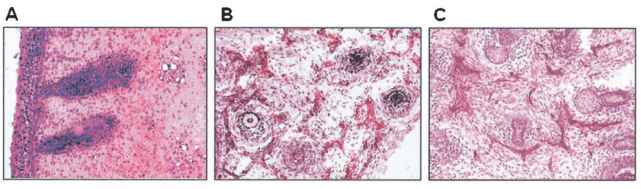

FIGURE 1. Examination of human fetal skin grafts. (A) Control human fetal skin graft 7 weeks after transplantation in severe combined immunodeficient/nonobese diabetic (SCID/NOD) mice (hematoxylin-eosin staining, magnification ×100). (B) Fetal grafted skins with anti-human CD31 and (C) with anti-mouse CD31 to show the dual vascularization (magnification ×250).

TABLE 1. Phenotype of human cells assessed by flow cytometry analysis in SCID/NOD mice 14 days after PBL injection

	Percentages of human CD45$^+$ cells[a]	Percentages of cell subtypes among human CD45$^+$ cells				
		CD2$^+$	CD4$^+$	CD8$^+$	CD14$^+$	CD16$^+$
Blood (n=6)[b]	10.1±4.2	97.8±2.4	31.4±6.5	65.7±5.6	0.4±0.7	1.0±1.4
Spleen (n=6)	12.7±5.7	96.6±5.8	36.9±9.8	59.0±10.2	0.2±0.4	0.4±0.6
Skin grafts (n=4)	38.8±10.1	99.7±0.2	54.5±6.8	33.0±3.6	0.5±0.4	0.6±0.3

[a] Percentages of human CD45$^+$ cells detected within a forward scatter/side scatter lymphocyte gate.
[b] Number of mice analyzed.
SCID/NOD, severe combined immunodeficient/nonobese diabetic; PBL, peripheral blood lymphocytes.

pressed CD25 and HLA-DR (Fig. 2, A and C). Human CD16$^+$ NK cells and CD14$^+$ monocytes/macrophages were hardly detected in the skin grafts by immunohistochemistry (not shown). However, low percentages of such cells were shown to leak from the skin fragments when they were left in culture medium (Table 1).

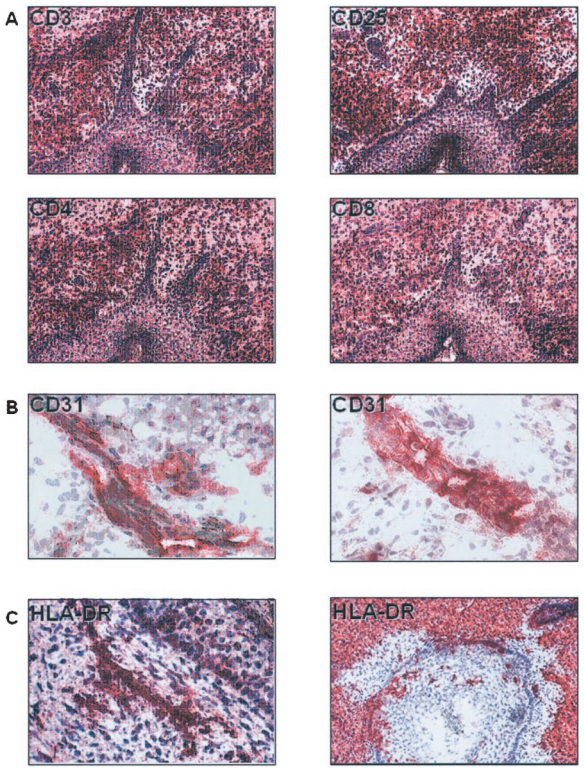

FIGURE 2. Infiltration of human skin grafts with injected human T lymphocytes and resulting vascular injuries. Examination of human skins (7 weeks after grafting) engrafted into mice that had been injected intraperitoneally for 14 days with 2×10^8 peripheral blood leukocytes (PBL). (A) Mononuclear cells that infiltrate the grafts with anti-human CD3, CD25, CD4, and CD8 monoclonal antibodies (mAb) (magnification ×100). Grafts from control mice (i.e., mice that had not received PBL) were not stained with these mAb (not shown). (B) Anti-human CD31 staining to assess dermal microvascular injuries: fibrin deposition (left) and vascular thickening (right; magnification ×250). (C) Human leukocyte antigen (HLA)-DR expression by dermal endothelial cells (left, magnification ×250) and keratinocytes (right, magnification ×100) resulting from interaction of the epidermis with activated human T lymphocytes (also expressing HLA-DR).

Dermal microvessel injuries, indicative of rejection, were visible in skin grafts. They consisted of parietal fibrin deposits, thickening of the vascular wall caused by endothelial cell proliferation (Fig. 2B), and expression of HLA-DR by endothelial cells (Fig. 2C). No HLA-DR staining was seen in the skin grafts of mice that did not receive PBL (not shown). Other signs of rejection were the dilation of the vascular lumen and the disruption of the endothelial cell layer (not shown). Such injuries were visible on day 10, when the human lymphocytes were just beginning to infiltrate the skin. They were more severe on day 21. In 9 of 10 mice, cells of the basal keratinocyte layer strongly expressed HLA-DR on their surface, provided they were infiltrated by human CD8$^+$ T lymphocytes or simply in contact with them, which is another sign of rejection (Fig. 2C).

Prevention of Human Skin Allograft Rejection by Early Injection of the Anti-CD2 mAb BTI-322

Early treatment with BTI-322 prevented human T-cell infiltration and subsequent vascular injury of the skin graft (Fig. 3). The mean degree of lymphocyte infiltration was significantly lower in BTI–322-treated mice than in control mice: 0.2±0.6 versus 2.6±0.8, respectively ($P<10^{-5}$). The percentage of damaged microvessels was reduced from 30.8±9.7% in control mice injected with the isotypic control antibody, LoDNP57 (n=10), to 7.6±10.3% in mice injected with BTI-322 ($P<0.0005$, n=8) Fig. 3C). The latter percentage was similar to that obtained from mice that did not receive PBL (10.9±6.9%, n=7). Little or no HLA-DR was expressed by endothelial cells and by keratinocytes of grafted skins obtained from mice injected with BTI-322. The effect of BTI-322 was dose-dependent: when the cumulative dose was reduced from 40 μg to 5 μg, skin infiltration and vascular injuries were prevented in only 50% of the mice tested (n=4), whereas lower doses (1.5 μg, n=4) had no preventive effect (not shown). Flow cytometry analysis revealed that only 0.2±0.2% and 0.7±0.6% of human CD45$^+$ cells remained in the blood and spleens of BTI-322-treated mice, compared with 10.1±4.2% and 12.7±5.7%, respectively, in control mice ($P<0.005$, n=6 in each group). Similar results were obtained using a small cohort of mice (n=4) treated with hu-BTI-322 (data not shown).

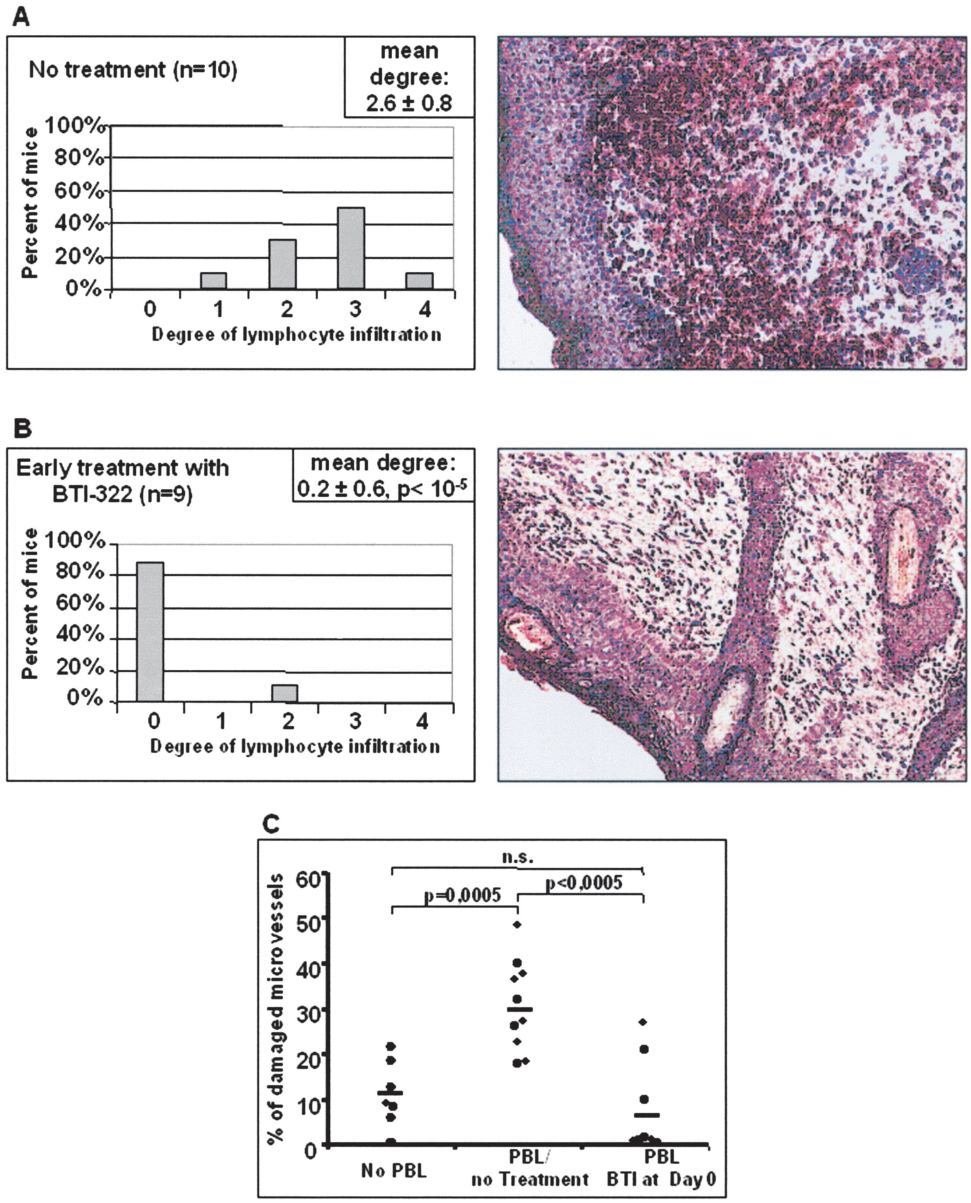

FIGURE 3. BTI-322 prevents the infiltration of grafted skins with human T lymphocytes and subsequent dermal microvascular injuries. Five weeks after human fetal skin grafting, mice were injected (A) with allogeneic PBL or B) with allogeneic PBL+BTI-322. Skin grafts were harvested 14 days after the PBL infusion. The degree of T-lymphocyte infiltration was scored using the five point scale (left panels) after immunostaining with an anti-human CD4 mAb (right, magnification ×100). The data indicate the number of mice in each group (upper left corner) and the mean degree of infiltration±SD (upper right corner). Groups were compared using Student's *t* test. (C) Dermal microvascular injuries in skin grafts harvested from control mice (that did not receive human PBL) and from mice 14 days after PBL injection, with or without preventive therapy. The percentage of damaged human vessels (stained with an anti-human CD31) was determined using a square grid.

Acute Rejection Therapy: BTI-322 and hu-BTI-322 Lead to Rapid and Complete Elimination of Infiltrating T Cells through Fcγ Receptor-Mediated Mechanism

The ability of BTI-322 and hu-BTI-322 to reverse ongoing rejection was tested using the "curative" protocol. BTI-322 or hu-BTI-322 were first injected 14 days postPBL infusion. At this time, skin grafts were significantly infiltrated with human T lymphocytes (Figs. 2A and 3A). BTI-322 rapidly led to the complete elimination of the infiltrating human T cells, as seen on day 21 postPBL infusion (n=11) (Fig. 4A). Conversely, lymphocyte infiltration progressed massively in control animals (n=11) treated with the isotype-matched mAb, LoDNP57. This curative treatment was again accompanied by a strong decline in peripheral lymphocytes: 1.1±2.2% and 1.5±2.5% of human $CD45^+$ cells remained in the blood and spleens of BTI–322-treated mice compared with 13.5±6.0% and 24.5±20.2%, respectively, in control mice ($P<0.05$, n=6 in each group). Hu-BTI-322 was also very efficient when injected curatively, strongly reducing the mean

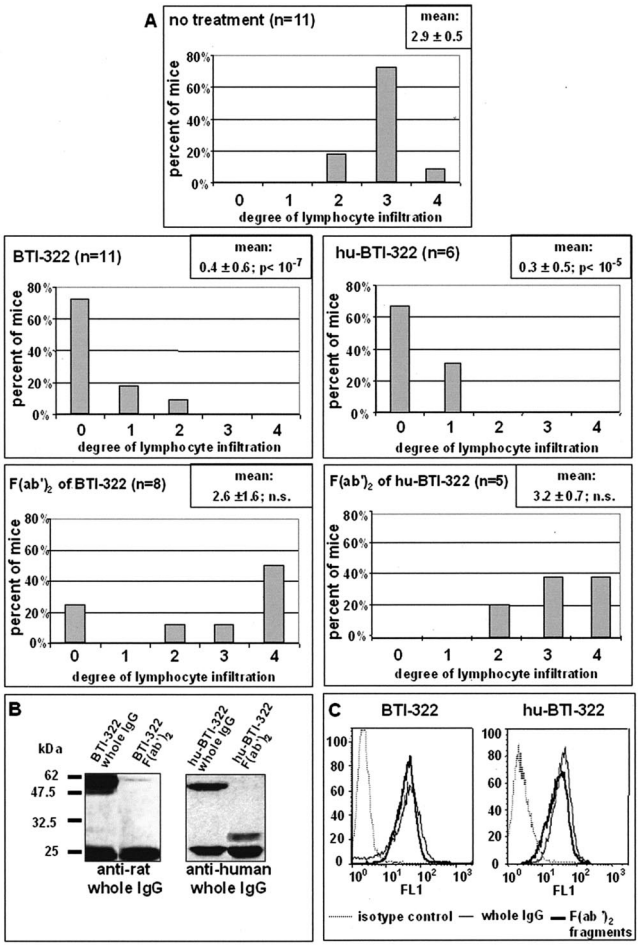

FIGURE 4. Effects of BTI-322, hu-BTI-322, and their $F(ab')_2$ fragments on human T-cell infiltration. (A) Curative protocol: 14 days after PBL injection, skin grafted mice received two subcutaneous injections performed at a 3-day interval of vehicle or isotype-matched control antibody LoDNP 57, 10 μg of BTI-322 or hu-BTI-322, or 50 μg of the corresponding $F(ab')_2$ fragments. Mice were killed 7 days after the first antibody injection. The degree of human lymphocyte infiltration was scored using the 5-point scale. Groups were compared using Student's *t* test. (B) Immunoblot analysis of whole and $F(ab')_2$ fragments of BTI-322 and hu-BTI-322 revealed by horseradish peroxidase (HRP)-conjugated rabbit immunoglobulin (Ig)G specific for rat Ig and by a mouse HRP-conjugated IgG specific for human IgG, respectively. (C) Antigen-binding capacity of whole BTI-322, hu-BTI-322, and of their $F(ab')_2$ fragments was evaluated by staining $CD2^+$ Jurkat cells with 1 μg of these reagents. Secondary antibodies were fluorescein isothiocyanate-conjugated rabbit anti-rat IgG and rabbit anti-human kappa light chains.

degree of infiltration (Fig. 4A). Kinetics studies, performed after hu-BTI-322 injection, indicated that infiltrating T lymphocytes were almost completely eliminated within 48 hours, with no redistribution toward the spleen, the blood (Table 2), or the liver (not shown). This argues for a deletion mechanism operating in situ within the skin grafts.

We tested the ability of the $F(ab')_2$ fragments of BTI-322 and hu-BTI-322 to treat acute rejection. The $F(ab')_2$ preparations of BTI-322 were contaminated with 0.1% of whole IgG, whereas the $F(ab')_2$ preparations of hu-BTI-322 were totally devoid of whole IgG (as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Fig. 4B). Cytofluorometry indicated that $F(ab')_2$ preparations reacted with CD2-positive Jurkat cells to the same extent as their whole IgG counterparts (Fig. 4C). When tested in vivo at 20 μg, $F(ab')_2$ fragments of BTI-322 had no effect (not shown). Increasing the cumulative doses up to 100 μg did not significantly modify the mean degree of lymphocyte infiltration, but the infiltration completely regressed in 25% of treated mice (Fig. 4A). The small contamination with whole IgG seen in the $F(ab')_2$ preparations of BTI-322 may explain why they displayed a depleting effect at high doses. In contrast, the highly purified $F(ab')_2$ preparations of hu-BTI-322 did not eliminate the lymphocytes infiltrating the human skin grafts, even when the cumulative dose was increased up to 100 μg (Fig. 4A). These results show that an Fcγ-related mechanism was involved.

TABLE 2. Percentages of human cells and grades of lymphocyte infiltrates after hu-BTI-322 treatment

	Percent human $CD45^+$ cells[a] (time after hu-BTI-322 injection)			
	0 hr	8 hr	24 hr	48 hr
Blood (n=3)	6.8±4.8	5.6±1.4	4.1±0.1	1.4±1.4
Spleen (n=3)	10.8±3.8	5.8±0.1	2.5±0.2	0.8±0.3
	Lymphocyte infiltration grade			
	0 hr	**8 hr**	**24 hr**	**48 hr**
Human skin (n=3)	2.7±0.5	1.7±0.5	1.0±0.0	0.3±0.5

Mice were injected subcutaneously with a single 10 μg dose of hu-BTI-322, 21 days after peripheral blood lymphocyte infusion.

[a] As detected by flow cytometric analysis within the FSC/SSC lymphocyte gate.

Human NK Cells Required for Curative Effect of CD2 hu-BTI-322

Previous experiments from our group have indicated that elimination of monocytes from human PBMC, before in vitro stimulation with OKT3+IL-2, did not prevent the apoptotic effect exerted by BTI-322 on activated T lymphocytes (*9*). We examined here the role of NK cells in this model. CD3-activated lymphocytes were no more susceptible to the in vitro apoptotic effect of hu-BTI-322, provided human NK cells had been removed from PBMC preparations (Fig. 5A). Similar results were obtained when using BTI-322 (not shown). This prompted us to inject NK– cell-depleted PBMC into skin grafted SCID/NOD mice and to examine whether, in these conditions, hu-BTI-322 would still eliminate the infiltrating T lymphocytes. Figure 5B shows that $CD56^+$-$CD16^+$-depleted PBMC (less than 0.5% $CD16^+$ cells) were devoid of spontaneous natural cytotoxicity against Jurkat cells. Importantly, they were unable to use hu-BTI-322 against these target cells in an ADCC assay. When injected into the skin-grafted mice, NK– cell-depleted PBMC gave rise to alloreactive T lymphocytes that infiltrated the grafts. However, hu-BTI-322 was no more able to eliminate these lym-

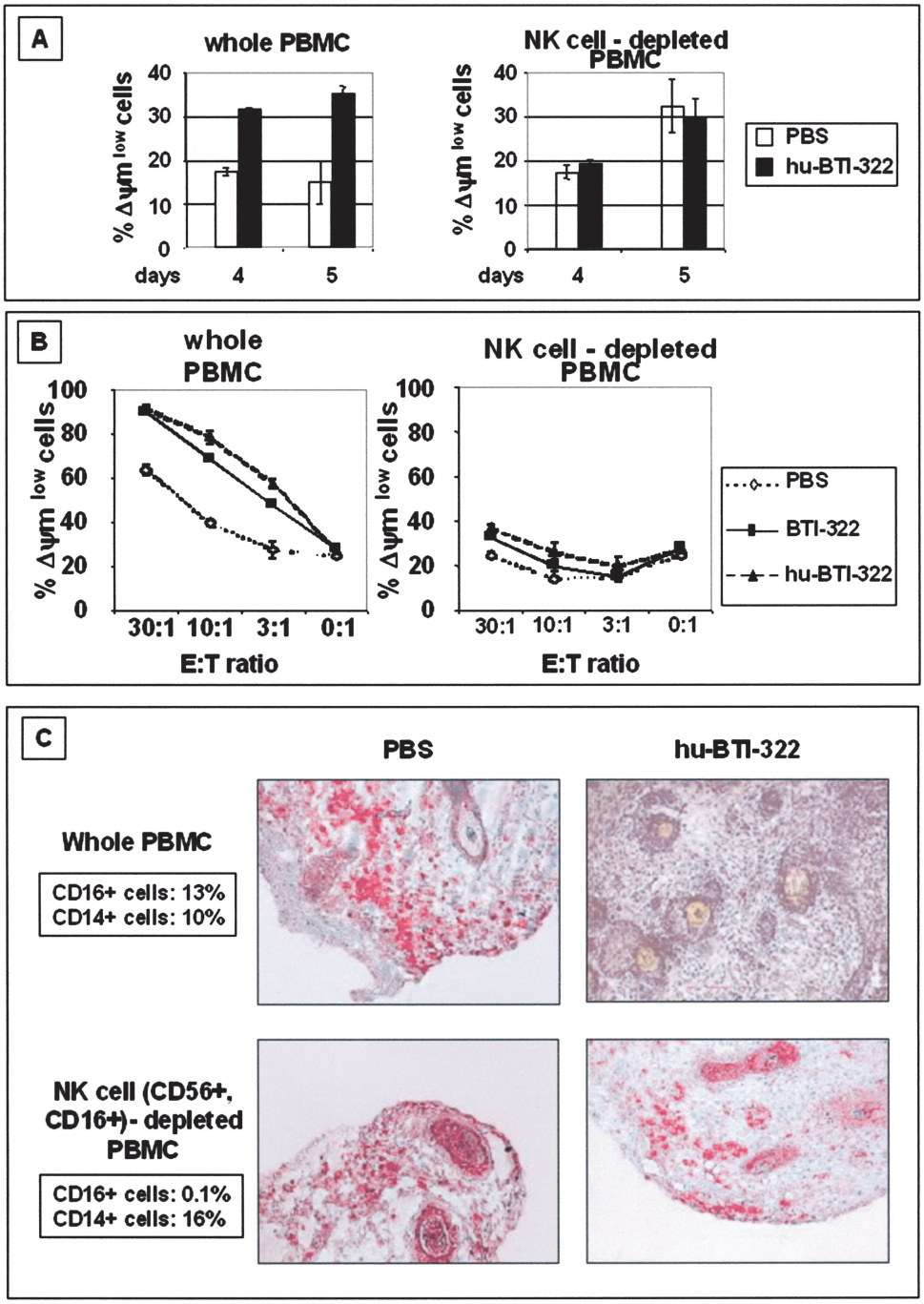

FIGURE 5. Natural killer (NK) cells are involved in the elimination of graft-infiltrating T cells by hu-BTI-322. (A) Human PBL or NK cell-depleted peripheral blood mononuclear cells (PBMC) were stimulated for 4 or 5 days with OKT3+interleukin-2. Hu-BTI-322 (100 ng/mL) was then introduced into the cultures for 4 hours. Percentages of apoptotic ($\Delta\Psi m^{low}$) cells were evaluated by flow cytometry analysis using the $DiOC_6$ (*3*) fluorescent probe. (B) Before injection into SCID/NOD mice, samples of whole PBMC and NK cell-depleted PBMC were used as effector cells in antibody-dependent cell-mediated cytotoxicity (ADCC) assays at various effector:target cell (E:T) ratios. Targets were Jurkat $CD2^+$ cells efficiently loaded with FITC-dextran and exposed for 4 hours to effector cells, with or without BTI-322 or hu-BTI-322 (100 ng/mL). Apoptosis was evaluated with the fluorescent CMXRos dye. Percentages of apoptotic ($\Delta\Psi m^{low}$) red cells among dextran-positive (*green*) target cells are represented. (C) Five weeks postskin grafting, mice were injected with whole allogeneic PBMC or NK–cell-depleted PBMC. Before injection, the percentages of $CD16^+$ NK cells and $CD14^+$ monocytes were monitored by flow cytometry. Mice received two subcutaneous injections (14 and 17 days after PBMC infusion) of 10 μg hu-BTI-322 or phosphate-buffered saline and were killed on day 21. Skin frozen sections were stained with an anti-human CD4 mAb (magnification $\times$100). The data shown are representative of three mice tested in each group.

phocytes (Fig. 5C). Human NK cells were therefore the effectors mediating the depleting effect exerted by hu-BTI-322 on infiltrating T lymphocytes.

DISCUSSION

Various anti-CD2 mAb induce prolonged or indefinite graft survival in animal models by different mechanisms of action: immune deviation of helper T cells to a Th2 phenotype (IL-4 secretion) (*6*), down-modulation of CD2 (*3*, *5*), and depletion of $CD2^+$ cells (*5*). A phase II trial showed that LoCD2a/BTI-322 can be used to prevent and to treat acute kidney-allograft rejection (*16*). In this trial, two groups of 20 patients were given a preventive treatment consisting of a triple immunosuppressive regimen alone or in combination with 5 μg/day of BTI-322 for 10 days. Importantly, this antibody was also able to cure acute rejection. We and others had previously shown that BTI-322 can trigger the apoptosis of activated T cells in vitro (*9*, *15*). BTI-322 and its humanized form, MEDI-507, kill activated lymphocytes in vitro through an ADCC mechanism (*14*, *16*). This provided a good rational basis to investigate the in vivo mechanism(s) of action of these CD2 antibodies.

As a model of allograft rejection, NOD-LtSz-*scid/scid* mice were grafted with human fetal skin and then injected with human allogeneic PBL. In our experiments, PBL injection also caused lesions of the dermal microvasculature, associated with the infiltration of human T $CD4^+$ and $CD8^+$ lymphocytes displaying the activation marker CD25. Use of both anti-human CD31 and anti-mouse CD31 mAb confirmed that vascular lesions only affected human vessels. The lack of ischemic necrosis is probably explained by the dual mouse/human vascularization.

Our animal model confirms that BTI-322 can both prevent and treat allograft rejection as in human trials (*14*, *16*). It shows that hu-BTI-322 is also efficient in treating allograft rejection. The injection of these CD2 antibodies into the mice led to a dramatic decrease in the number of human T lymphocytes circulating in the blood or residing in the spleen of the animals. In the "curative" protocol, this decrease began rapidly (within a few hours) and was concomitant with the disappearance of the lymphocyte infiltrate. In no instance were human cells found in the liver. These observations argue against the possibility of alloreactive T lymphocytes escaping from the skin and homing in the spleen. In the "preventive" protocol, lymphocytes must have disappeared before they could reach the skin graft, given that human endothelial cells did not express HLA-DR, the first sign of attack by PBL (*19*).

$F(ab')_2$ preparations of BTI-322 and hu-BTI-322 had no significant curative effect on skin graft rejection. A complement-dependent cytotoxicity, which involves the Fc portion of IgG, can be ruled out, although rat IgG2b has a high affinity for human complement (*24*). Indeed, the NOD/LtSz-scid/scid mouse strain is devoid of hemolytic complement activity (*18*). In vitro, BTI-322 and hu-BTI-322 have been shown to induce the deletion of activated lymphocytes (*9*, *14*, *16*). An ADCC mechanism was the cause, requiring effector cells bearing FcγRIII receptors (NK cells and macrophages). In our hands, NK cells, but not monocytes, were the main effectors mediating the in vitro BTI-322 and hu-BTI-322-induced ADCC against CD3/IL–2-activated T lymphocytes (*9*) (as also shown in this study). This is in line with the study of Branco et al. (*14*), who showed that activated T cells generated during a mixed lymphocyte reaction were eliminated by NK cells in the presence of MEDI-507. We show that in vivo, human NK cells coinjected with alloreactive T lymphocytes were in charge of the curative effect of hu-BTI-322. Engagement of FcγRIII (CD16) receptors by target-specific IgG activates the potent cytolytic mechanism of NK cells. Most probably, such an event occurred within the grafted skins because human cells were apparently not redistributed toward the spleen and the liver. Thus, even if NK cells appeared to account for less than 1% of human cells infiltrating the grafts, this may be sufficient for ADCC.

Human monocytes, accounting for 16% of NK–cell-depleted PBMC, were ineffective in mediating the in vivo curative effect induced by CD2 antibodies. It is possible that activated monocytes need high E:T cell ratios, as suggested perviously (*16*), to activate in response to FcγR cross-linking, the secretion of soluble toxic molecules as tumor necrosis factor-α and radical oxygen species. Mouse Fcγ receptor-bearing cells did not intervene in our model. Yet, it has recently been shown that an ADCC reaction can occur with the cells of SCID/NOD mice (*25*). Also, KM966, an anti-ganglioside GM2 mAb, can block metastasis formation of GM2-positive human lung cancer cells injected in NK–cell-depleted SCID mice. This was controlled by an ADCC reaction mediated by mouse macrophages (*26*). While writing this article, we became aware of the work of Zhang et al. (*27*), who showed that MEDI-507 can kill adult T-cell leukemia cells in SCID/NOD mice through the activity of $Fc\gamma R^+$ murine cells (polymorphonuclear leukocytes and monocytes). Note that long-term treatments, with high concentrations of MEDI-507, were carried out (100 μg/week for 4 weeks or for 6 months). In our study, the lower efficient dose of hu-BTI-322 was 10 μg. This dose was, however, sufficient to activate the cytolytic mechanism of NK cells and to destroy within 48 hours an alloreactive T-cell infiltrate (Table 2). It should be stressed that murine macrophages were readily detected by immunohistochemistry in the periphery but not inside the human lymphocyte infiltrates (not shown). This distribution pattern might explain why these macrophages were not involved in the destruction of alloreactive lymphocytes.

Our results may encourage clinicians to use siplizumab in human transplantation trials because this should prevent the anti-rat immunization observed with BTI-322 (*16*). Siplizumab is being tested in phase I/II trials for the treatment of psoriasis. The recombinant protein alefacept (human LFA-3-IgG1 fusion protein), which also targets CD2, has been shown to be efficient in this indication. Its mechanism of action is also ADCC, involving FcγRIII receptors of NK cells (*28*, *29*). Thus, reagents targeting CD2 molecules and triggering the death of alloreactive T lymphocytes by an ADCC involving NK cells may represent a powerful clinical tool.

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