

Anti-CD2 Receptor and Anti-CD2 Ligand (CD48) Antibodies Synergize to Prolong Allograft Survival

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Summary

Indefinite graft survival was obtained with murine cardiac allografts using the combined administration of monoclonal antibodies (mAbs) directed against the receptor ligand pair CD2-CD48. Although each antibody could prolong graft survival when given alone, neither resulted in the indefinite graft survival seen with the combination. Combined mAb administration is associated with inhibition of T cell priming and help and subsequent cytotoxic T lymphocyte generation. This indicates that the interaction between CD2 and its ligand is important for antigen priming and recognition, and combined mAbs may prove to be a useful therapeutic regimen for transplantation.

Tolerance induction is the major goal in transplantation. It has been obtained with several regimens including the administration of pairs of mAbs to CD4 and CD8 (1), CD2 and CD3 (2), and CD11a/CD18 and CD54 (3). The combined use of antireceptor plus antiligand mAbs may be advantageous in blocking both T cells and APCs, and in blocking interactions with as yet unknown ligands. Recent findings have identified murine CD48 (4), and human CD59 (5, 6), and sulfated carbohydrates (7) as potential ligands for CD2, in addition to the well-characterized human ligand CD58 (LFA-3). Murine CD48 (Blast-1, BCM1) has structural homology to human CD58, including glycosyl-phosphatidylinositol linkage to the cell membrane, and functions in signal transduction and activation of lymphoid cells (4, 8-10). CD2 is important in adhesion and signal transduction (11, 12), and anti-CD2 mAbs suppress cell-mediated immunity (13, 14) and prolong allograft and xenograft survival (15). With the recent availability of mAbs defining this receptor-ligand pair, the combination of anti-CD2 plus anti-CD48 mAbs was administered in a murine cardiac allograft model and indefinite graft survival was observed. The induction of indefinite graft survival is associated with inhibition of T cell priming and help and subsequent CTL generation, while maintenance of tolerance is associated with a lack of T cell help. This indicates that CD2-CD48 interactions and signals are integral to antigen priming and recognition, and combined mAbs are an important therapeutic regimen for transplantation.

Materials and Methods

Animals. CBA/J (H-2^k) and BALB/cByJ (H-2^d) female mice, 8-10-wk-old, were purchased from The Jackson Laboratory (Bar

Harbor, ME). Timed pregnant C57BL/6 (H-2^b) and BALB/c mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and were used as the donor strains.

Reagents. The 12-15 rat IgG1 anti-murine CD2 hybridoma (16), a gift of Dr. Peter Altevogt (Immunology and Genetics Institute, Heidelberg, Germany), and the HM48-1 hamster IgG anti-murine CD48 (4) were grown in culture and purified over protein G (Pharmacia, Piscataway, NJ). For control mAbs the YTS-259 rat IgG1 anti-ras (17) was used. Abs were injected intravenously in PBS or were used in vitro at specified concentrations.

Cardiac Transplantation. Donor embryonic (E 18-21) or neonatal (P 1-2) C57BL/6 or BALB/c mice were killed, whole hearts removed, and placed in a subcutaneous pocket in the ear pinnae of CBA/J recipients as previously described (15, 18, 19). Survival of allografts was followed by EKG monitoring (Polygraph 78 Series with preamp and filters; Grass Instruments Co., Quincy, MA) every other day. Cessation of cardiac electrical activity was the determinant of rejection. Statistical comparison of results is with Wilcoxon Sign Rank. Recipients received intravenous injections of mAbs in 0.5-ml volumes of PBS at specified times.

Cytotoxic T Lymphocytes. Spleens were removed and gently dissociated into single cell suspensions. Red blood cells were removed from responder cell populations by centrifugation over a Ficoll (Sigma Chemical Co., St. Louis, MO)-Metrizoate (Nycomed Pharmaceuticals, Oslo, Norway) discontinuous gradient (specific gravity 1.09, 24°C, 15 min, 1,200 g). Red blood cells were removed from stimulator cells by Tris-NH₄Cl lysis. The CTL culture and assay were performed as described previously (13). Responders and stimulators were placed in culture and harvested after 7 d. A standard 4-h, Cr⁵¹ release assay was performed in triplicate wells. Control always included irrelevant targets (P815 or EL-4) and were always ≤5% specific lysis. Spontaneous release was 5-15% of total incorporated counts. CTL generated by this protocol were CD8⁺, H-2 restricted, and antigen specific with peak alloantigen-specific, primary CTL responses (CBA/J anti-B6 or CBA/J anti-BALB/c) noted

after 7 d of culture (13). Results are expressed as: specific lysis = (experimental - spontaneous)/(100% lysis - spontaneous). The SEM was always $\leq 2\%$.

Mixed Lymphocyte Reaction. 2×10^5 responder spleen cells were cocultured in triplicate with 2×10^5 1,500-rad γ -irradiated stimulator cells for 5 d. 18 h before termination of culture the wells were pulsed with 0.5 μCi of [^3H]thymidine and incorporation was quantitated on a scintillation counter. Results are expressed as mean; SEM $\leq 5\%$.

Mitogen-induced Proliferation Assays. Splenic lymphocytes were isolated by mechanical dissociation and Tris-NH₄Cl lysis of red blood cells, and plated at 10^5 cells in 200- μl 96-well round-bottomed plates. Con A and PHA were added to 5 $\mu\text{g}/\text{ml}$. Anti-CD3 mAb (2C11) was coated onto wells in 50 μl at 10 $\mu\text{g}/\text{ml}$. mAbs were each added to culture to 5 $\mu\text{g}/\text{ml}$. Cultures were incubated for 72 h, pulsed with [^3H]TdR for the last 18 h, harvested, and counted. Data are expressed as mean counts per minute of triplicate samples; SEM $\leq 5\%$. For separate CD4 and CD8 populations, splenic T lymphocytes were enriched over nylon wool columns and separated on antibody columns (R & D Systems, Minneapolis, MN) according to manufacturer's instructions. After column separation, flow cytometric analysis showed $<1\%$ contamination of responder cell population with the opposite T cell subset.

Cytokine Assays. Culture supernatants from 4-d alloantigen-stimulated cultures were harvested and tested for IL-2 in the CTL-L assay (20) or for IL-4 in the HT-2 assay (21). Standard curves were run concurrently with rmIL-2 or rmIL-4 (Genzyme, Cambridge, MA).

Results and Discussion

Animals receiving anti-CD2 mAb alone had graft survival of 24.4 ± 1.0 d, and those receiving anti-CD48 mAb alone had graft survival of 19.9 ± 0.6 d. These were mildly prolonged and significantly different ($p < 0.05$) from control animals receiving no mAb, or control mAb, which rejected allografts by 13.4 ± 0.5 d. However, animals that received the combination of anti-CD2 plus anti-CD48 mAbs showed

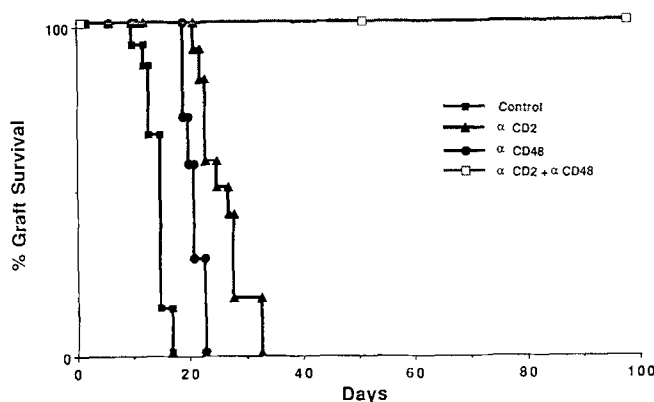


Figure 1. Survival of cardiac allografts. C57BL/6 neonatal hearts were transplanted into CBA/J mice. Recipient mice (seven/group) were injected intravenously with either 100 μg of anti-CD2, isotype control mAb, or anti-CD48 alone or in combination at the time of allografting (day 0) and on day 1 after transplantation. Survival time of the combined regimen was significantly greater ($p < 0.001$) than either mAb alone or control mAb.

prolongation of graft survival for >100 d (Fig. 1). These animals showed no apparent toxic side effects and no mortality associated with the mAb regimen. Dose-response and kinetic data showed 100 μg of mAb to be the maximal and optimal dose for immunosuppression and that mAb had to be administered at the time of transplantation (3, 15, and our unpublished results). A delay in administration with respect to transplantation by a few days was not effective. Thus the mAbs were more effective as prophylactic than therapeutic reagents.

Since CTLs are a major effector arm of graft rejection, CTL cultures were used to probe the mechanism of tolerance induction and the level at which the combination of mAbs was acting. Previous work showed that anti-CD2 and anti-CD48 mAbs can inhibit the priming of CTL in vivo (13 and our unpublished observations). Therefore, additional experiments focused on the effects of the mAbs in vitro. The mAbs were added at the initiation of primary allogeneic CTL culture or at the time of CTL assay. The doses of mAbs used were known to be saturating from previous experiments (not shown). Both anti-CD2 and anti-CD48 mAbs partially inhibited the generation of CTL activity when added alone to culture (Table 1). However, when combined, CTL induction was completely inhibited. The mAbs had minimal effect alone, or in combination, when added 7 d later to the CTL lytic assay. These results show that interference with receptor-ligand interactions at the time of priming is an essential component for tolerance induction. This suggests that the mAbs interfere more with T cell helpers or CTL precursors than with cytotoxic effectors.

Since proliferative responses are often considered a reflection of normal immunity, the combination of mAbs was also

Table 1. Anti-CD2 Plus Anti-CD48 mAbs Inhibit CTL Stimulation Cultures but Not the Lytic Assay

mAb added to:		Percent specific cytotoxicity (E/T ratio)		
		100:1	30:1	10:1
Stimulation culture	-	68.5	54.9	42.4
	αCD2	45.1	31.2	18.9
	αCD48	18.9	9.6	3.0
	$\alpha\text{CD2}/\alpha\text{CD48}$	2.5	4.4	0
Lytic assay	-	71.8	63.1	58.9
	αCD2	74.0	60.0	52.2
	αCD48	74.9	55.6	49.5
	$\alpha\text{CD2}/\alpha\text{CD48}$	70.4	57.1	54.7

BALB/c splenic lymphocytes were placed in culture with C57BL/6 stimulators for 7 d. Anti-CD2, anti-CD48, or the combination of mAbs were added at the initiation of culture, or at the time of assay, at a concentration of 5 $\mu\text{g}/\text{ml}$. Similar data were obtained with CBA/J responder cells.

Table 2. *Anti-CD2 Plus Anti-CD48 mAbs Partially Inhibit Mitogen-stimulated T Cell Proliferation*

Cells	Mitogen	mAbs				
		Control mAb	α CD2	α CD48	α CD2/ α CD48	
			<i>cpm</i>			
CD4 ⁺	-	409	273	308	278	
	Con A	37,391	14,707	7,656	20,300	
	PHA	15,092	5,480	4,353	4,950	
	α CD3	78,937	92,838	69,047	67,523	
	MLR	57,680	60,536	36,820	38,058	
CD8 ⁺	-	315	261	122	364	
	Con A	19,184	14,270	14,481	14,844	
	PHA	21,905	9,025	4,968	6,229	
	α CD3	33,864	34,716	23,422	25,971	
	MLR	11,447	9,391	9,906	10,680	

BALB/c splenic lymphocytes were stimulated with the indicated mitogen as described in Materials and Methods. C57BL/6 was the stimulator in MLR.

assessed for its ability to block mitogen-induced T cell proliferation. The mAbs partially inhibited *in vitro* proliferative responses to Con A, PHA, MHC alloantigen, and anti-CD3 mAb in CD4⁺ and CD8⁺ splenic lymphocytes (Table 2). For CD4⁺ cells, anti-CD2 did not inhibit anti-CD3 or alloantigen-driven proliferation, while anti-CD48 had a marginal effect on anti-CD3-induced proliferation and a more significant effect on alloantigen-induced proliferation. For CD8⁺ cells, anti-CD2 again did not inhibit anti-CD3-driven proliferation, while anti-CD48 was effective in this regard. Both mAbs effectively inhibited CD4⁺ and CD8⁺ responses to both Con A and PHA. This demonstrates that these antibodies can affect responsiveness of both major T cell subsets via multiple different activation pathways which may (alloantigen, anti-CD3) or may not (Con A, PHA) depend on direct T cell receptor stimulation. These mitogens are, however, all antigen-presenting cell dependent with the culture conditions used (22–24), and this may be a determinant of mAb inhibition. As a correlate to inhibition of mitogen- or alloantigen-driven proliferation, supernatants from these stimulation cultures were harvested and assayed for cytokine content. The results in Table 3 show that CD8⁺ cells produce little cytokine in response to alloantigen and that anti-CD2 and/or anti-CD48 mAbs have little effect on these levels. Conversely, CD4⁺ cells produce both IL-2 and IL-4 in response to alloantigen, and cytokine production is inhibited by both mAbs. In general, anti-CD48 is more effective in this regard than anti-CD2, and the combination of mAbs is at least additive in its inhibitory effect. Assays for IL-10 and TGF- β showed no changes as a result of mAb treatment of separated or unseparated cells (not shown). For anti-CD3-stimulated cultures, neither anti-CD2 nor anti-CD48 inhibited IL-2 or IL-4 production by CD4⁺ or CD8⁺ subsets (not shown), which parallels the findings with the prolifer-

ative responses in Table 2. Therefore the mAbs inhibit the alloantigen-stimulated production of helper-derived cytokines responsible for driving effector proliferation and maturation.

To evaluate the maintenance of the tolerant state, animals with long-term surviving grafts received a second donor-specific graft of the identical H-2 type to the contralateral ear pinna. Surprisingly, rejection of both the first and second grafts ensued at 10.0 ± 1.0 d. This indicated that maintenance of the tolerant state most likely was not due to clonal deletion, suppression, or graft adaptation. Previous flow cytometry data showed that these mAbs do not deplete T cells,

Table 3. *Anti-CD2 Plus Anti-CD48 mAbs Inhibit Alloantigen-Stimulated IL-2 and IL-4 Production*

Cells	mAb	Cytokine response	
		IL-2	IL-4
		<i>U/ml</i>	
CD8 ⁺	Control mAb	3.0	0
	α CD2	2.4	0
	α CD48	1.2	0
	α CD2/ α CD48	1.0	0
CD4 ⁺	Control mAb	19.8	3.2
	α CD2	14.0	0.9
	α CD48	6.4	0
	α CD2/ α CD48	2.8	0

Day 4 MLR culture supernatants from unseparated or separated cell subsets were assayed for cytokine content.

but induce transient changes in cell surface expression of CD2 or CD48 without affecting CD3, CD4, or CD8 expression (3, 13, 14, and our unpublished observations). Therefore, these data suggest that anergy or alterations in T cell populations at the time of initial antigen and mAb exposure result in indefinite graft survival. In particular the data demonstrate that effector cells or their precursors are present and therefore suggest that effective T cell help is lacking. The data in Table 3 showing inhibition of cytokine production support this notion further. Furthermore, this effect was antigen specific since CBA recipients that tolerated a first C57BL/6 graft for >60 d rejected a third party BALB/c graft in a first set fashion (12.0 ± 0.6 d) while maintaining the first C57BL/6 graft intact. To test this hypothesis further, CTL activity was measured in recipients of either one or two allografts. Splenic lymphocytes from animals that had received anti-CD2 mAb alone and had rejected a single allograft showed normal CTL activity to both donor H-2-specific (Table 4) and third party target cells (not shown). Lymphocytes from animals treated with the combination anti-CD2 plus anti-CD48 mAbs, which had initially accepted a first graft but had subsequently rejected both first and second grafts after a second transplant, also showed normal donor-specific and third party CTL activity. Likewise, lymphocytes from animals treated with the combination of mAbs, and which retained one long-term surviving graft, also showed normal donor-specific and third party CTL activity. Similarly the mixed lymphocyte proliferative responses were essentially the same for all groups, or even slightly augmented in the group retaining its single graft. The presence of CTL precursors, and the ability to stimulate the response by regrafting, suggest that tolerance is main-

tained by a lack of effective T cell help in vivo. This help could be generated by restimulation with lymphocytes in vitro or a second graft in vivo.

The combination of anti-CD2 and anti-CD48 mAbs is able to induce indefinite graft survival. Although each mAb alone can induce immune suppression, each does not result in the same state of long-term graft survival seen with the combination. The mechanism of induction is related to inhibition of initial priming to antigen. The CTL (Table 1), proliferative (Table 2), and cytokine data (Table 3) indicate that inhibition affects both precursor CTL and helper cells. It is not clear if the mechanism of tolerance induction is related to direct signaling effects of the mAbs themselves on T cells, to blocking of CD2-CD48 receptor-ligand interactions, or to inhibition of interactions of these molecules with other potential ligands (5-7). These are not mutually exclusive mechanisms and all may be important. Since anti-CD2 and anti-CD48 induce long-term graft survival in the putative absence of blockade of other receptor-ligand pairs (e.g., LFA-1-ICAM-1, CD28-B7), this suggests that some sort of "negative" signal is generated by mAb or receptor blockade that subsequently inhibits second messenger pathways of alternative coreceptors. Since CD2-knockout mice (25) are immunologically intact, the results here support a signaling role for mAb. Anti-CD2 also impairs NK function (15) and this may further contribute to inductive effects.

While our results indicate that reduced T cell help may be responsible for indefinite graft survival, they do not indicate how this reduction is maintained long term. It may be considered that at the time of initial grafting both class I and class II MHC and other costimulatory molecules are ex-

Table 4. Tolerant Recipients Can Generate Normal CTL and MLR Activity

	In vivo treatment	Cardiac transplant	Rejection	Target	Percent specific cytotoxicity (E/T ratio)		
					100:1	30:1	10:1
CTL	α CD2	$\times 1$	+	EL-4(H-2 ^b)	72.4	65.6	43.4
	α CD2/ α CD48	$\times 2$	+		73.2	67.9	59.1
	α CD2/ α CD48	$\times 1$	-		60.0	47.6	36.1
					Stimulator		
					-	H-2 ^b	H-2 ^d
MLR	-	$\times 1$	+		1,823	16,805	16,869
	α CD2	$\times 1$	+		4,620	17,861	16,727
	α CD2/ α CD48	$\times 2$	+		6,720	28,925	36,070
	α CD2/ α CD48	$\times 1$	-		4,701	37,273	35,185

CBA/J recipients were originally transplanted with C57BL/6 and received 100 μ g anti-CD2 or 100 μ g anti-CD2 plus 100 μ g anti-CD48 on days 0 and 1 after transplantation. Recipients were retransplanted without further immunosuppression 100 d after the initial transplant, and killed at least 60 d after the first or second transplant. Splenic lymphocytes were stimulated in culture with donor-specific (C57BL/6) or third party (BALB/c) splenocytes and CTL or MLR activity was assayed. CTL responses of untransplanted or transplanted and untreated recipients were similar to those of the anti-CD2-treated recipients (not shown).

pressed on the graft as a result of nonspecific inflammatory events (26–28). Anti-CD2 plus anti-CD48 may interfere with the priming of helpers and generation of effectors at this stage. As initial inflammation wanes, class II MHC and costimulatory molecule expression disappear, resulting in a graft that expresses class I MHC but is unable to generate effective help. A second graft would express costimulators and provide effective help in this situation. Likewise, the allogeneic stimulator lymphocytes used in culture may also provide effective costimulatory help in vitro for MLR and CTL responses. The approach of combined mAbs may prove to be an impor-

tant regimen for induction therapy for allografting. The results here and elsewhere (1–3) may imply that the complete inhibition of a single receptor–ligand pair with a combination of mAbs is enough to abrogate the stimulatory effects of other coreceptors. This may be achieved because receptors are more completely blocked by this regimen. Alternatively, because many of these receptor–ligand pairs are coexpressed on the same cell, the combination of mAbs may be providing more potent negative signals than single mAbs at the individual cell level.

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