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Increased effector–target cell conjugate formation due to HLA restricted specific antigen recognition

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Abstract

The T cell receptor (TCR) orchestrates T cell mediated-cytotoxicity through a complex interaction that results in an antigen-specific effector–target cell conjugate formation. While it is well recognized that specific TCR/antigen interactions generate the immunological synapse, their direct contribution to the effector–target cell conjugate has not been conclusively demonstrated. Moreover, since human cytotoxic T lymphocyte (CTL) clones are also susceptible to antigen-independent adhesion to target cells, it remains unclear whether effector–target cell conjugate formation can serve as an indicator of specific antigen recognition by the TCR. To address this question, a well-characterized epitope-specific CTL clone recognizing the melanoma-associated antigen epitope gp100:209–217 in association with HLA-A*0201 was tested against melanoma cell lines lacking or expressing the HLA-A*0201 allele and/or gp100. In this model, TCR/HLA/antigen interactions cooperated with accessory/adhesion molecules to facilitate effector–target cell conjugate formation. HLA-restricted antigen recognition played a dominant role resulting in up to 2-fold increases in conjugate frequency, and a 50% increase of CTL binding to tumor cells over background. The increased number of CTL contained in conjugates correlated with the number of IFN-γ producing CTL. These results warrant further investigation to evaluate conjugate assays as a potential tool to detect and isolate viable and functionally active CTL. Since conjugate formation analysis does not require knowledge of the target antigen, this assay could potentially be used for enrichment of CTL directed against novel antigens.

Keywords

Effector–target conjugate formation; HLA-restricted antigen recognition; TCR/HLA/epitopes; Intracellular cytokines; Adhesion molecules

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Introduction

Cytotoxic T lymphocyte (CTL)-target cell interaction involves antigen recognition, effector–target cell binding, and delivery of cytotoxic granule content. Effector/target cell conjugate formation is a critical step for T cell-mediated cytotoxicity prior to lethal hit delivery and cytolysis [1]. A number of studies have provided insights into the complexity of this process at the biological, biophysical, and molecular levels. Two decades ago, selected studies showed that the specificity of conjugate formation generally paralleled that expected from cytotoxicity studies and suggested the utility of conjugate formation for enumeration of cytotoxic T cells by flow cytometry [1, 2]. However, the underlying mechanism(s) associated with conjugate formation remains unclear and the requirement for a specific TCR/human leukocyte antigen (HLA)/epitope interaction remains controversial due to the finding that human CTL clones are susceptible to antigen-independent adhesion to target cells [3, 4]. Currently, this remains unresolved, even though increasing evidence supports that TCR-specific antigen recognition is key to the stabilization of effector–target cell binding [5]. It was noted that specific antigen recognition by TCR triggered an amplification of adhesion mechanisms converting lymphocyte functional antigen (LFA-1) into a high-avidity state within 5–10 min inducing a transient TCR/LFA-1 coupling that resulted in intracellular signaling pathway activation [6]. More recently, Mueller et al. demonstrated that LFA-1 integrin-dependent T cell adhesion was regulated by both Ag specificity and sensitivity [7]. Another accessory/adhesion molecule, CD2, was found to synergize with TCR for the activation of the phospholipase Cγ1/calcium pathway at the immunological synapse [8, 9].

The role of TCR/HLA/antigenic epitope interactions as modulators of cytotoxic T cell/target adhesion is revisited in this study adopting a set of T cell and tumor cell clones well characterized for relevant HLA allele and antigen expression [10–12]. The primary aim of the study was a conclusive analysis of the requirement for TCR/HLA/antigenic epitope interactions for conjugate formation; although intuitive such demonstration has never been conclusively provided. Validation of this hypothesis may provide insights about the requirements necessary for full T cell activation and at the same time suggest the practical utilization of this phenomenon to detect and isolate viable and functionally active T lymphocytes against antigens whose identity may not as yet be identified. Our results demonstrate that HLA-restricted antigen recognition consistently contributes to an increased frequency of effector–target cell conjugates that can be measured by flow cytometry. The frequency of the conjugate formation is paralleled by enhanced antigen-specific cytokine expression as detected by intracellular cytokine analysis.

Materials and methods

Cytotoxic T cell clone and culture

The 1520-tumor infiltrating lymphocyte (TIL) clone was derived from a metastatic lesion of a patient with cutaneous melanoma treated with immunotherapy at the Surgery Branch, National Cancer Institute (NCI), Bethesda, MD, under an Institutional Review Board-approved protocol; clone 1520-TIL has been amply characterized as reactive to the gp100:209–217 peptide in association with HLA-A*0201 [13]. The 1520-TIL clone was maintained in Iscove's Modified Dubelcco's Medium (IMDM) (Biofluids, Rockville, MD) supplemented with 10% Heat Inactivated Human AB Serum (Gemini, Bio-products, Woodland, CA), 10 mM Hepes Buffer (Biofluids), 0.03% Glutamine, 100 units/ml Penicillin-Streptomycin (Biofluids), 10 μg/ml Ciprofloxacin (Bayer, West Haven, CT), and 0.5 μg/ml Amphotericin B (Biofluids) (IMDM complete medium) with addition of Interleukin 2 (IL-2) (Novartis Chiron, Emeryville, CA) at final concentration of 6000 IU.

Melanoma cell lines and culture

The human melanoma cell lines used in this study were established at the Surgery Branch, NCI, and cultured as previously described [10]. 624.38-MEL (HLA-A2$^+$, gp100$^+$) and 624.28-MEL (HLA-A2$^-$, gp-100$^+$) were cloned from a bulk culture characterized by high heterogeneity of HLA-A*0201 allelic expression and have been amply characterized, in particular, while both cells express similar level of melanoma differentiation antigens including gp100/PMel17. They were selected among other clones for their extreme differences in expression of the HLA-A*0201 allele responsible for the presentation of several gp100/PMel epitopes including the one recognized by 1520-TIL, gp100:209–217 [10, 11]. The melanoma cell line 1390-MEL (HLA-A2$^+$, gp100$^-$) was selected among several other HLA-A*0201 expressing cell lines that we have previously characterized [12] because of its high expression of the HLA-A*0201 allele and the complete depletion of gp100/PMel 17 at the messenger RNA and protein level. All cell lines were maintained in monolayer culture in RPMI1640 (BIofuids) supplemented with 10% Heat-Inactivated Fetal Calf Serum (FCS) (Biofluids), and Hepes Buffer, Glutamine, Penicillin-Streptomycin, and Ciprofloxacin as described above.

Peptides and peptide pulsing of melanoma cell line

The gp100:209–217 (210M) (IMDQVPFSV, gp100–209 2M) peptide was commercially synthesized by Princeton Biomolecules (Columbus, OH). The peptide was purified by gel filtration to more than 95% purity, and its identity was confirmed by mass spectral analysis [14]. Peptide gp100–209 2M has been shown to enhance the recognition by gp100–209 reactive cytotoxic T cell clones including 1520-TIL recognizing also the naturally processed and presented endogenous gp100:209–217 [15]. Flu-M1$_{58–66}$ (GILGFVFTL) from the influenza matrix protein was used as control where relevant (Multiple Peptide System, San Diego, CA). The melanoma cell line 1390-MEL was pulsed with gp100–209 2M peptide or Flu peptide at final concentration of 1 μg/ml/1 × 10^6 cells in a 37°C incubator for 1 h with gentle shaking every 20 min. The cells were then washed twice in complete medium at 1,400 rpm (420 × G) for 5 min immediately before the conjugate assay.

Antibodies

The following monoclonal antibodies (MAB) were used: W6/32 (mouse IgG2a) reacting with a HLA Class I molecule (Abcam, Cambridge, UK); anti-human LFA-1 clone 25.3.1 (mouse IgG1) (Immunotech, Marseille Cedex, France); anti-human CD2 clone SFC13Pt2H9 (T11, mouse IgG1) (Beckman Coulter, Fullerton, CA); monoclonal anti-human CD54 (BD Pharmingen, San Diego, CA); monoclonal anti-human CD58/LFA-3 (BD Pharmingen); PE conjugated monoclonal anti-human IFN-γ antibody (BD Pharmin-gen); and PE-Cy5 conjugated anti-human CD8 (BD Pharmingen).

Intracellular cytokine assays

At the beginning of the effector/target cell co-culture, 500 μl IMDM complete medium containing Brefeldin A (Sigma) at 2 μg/μl was added for intracellular cytokine assay. After staining with extra cellular antibodies, the cells were treated using Fix & Perm Medium A (Caltag Laboratories, Burlingame, CA) for 15 min at room temperature. The cells were washed with Phosphate Buffered Saline (PBS) (GIBCO), containing 5% FCS at 1500 rpm (600 × G) for 5 min. The supernatant was aspirated, and 100 μl of Fix and Perm Medium B (Caltag Laboratories) was added along with 20 μl PE conjugated anti-human IFN-γ. After 30 min of incubation at room temperature, the cells were washed twice in PBS containing 5% FCS prior to flow cytometry analysis.

Fluorescent labeling of cells

Fluorescent dye-5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR), was used to label target cells as previously described [16]. Briefly, 5×10^6 target cells were washed with and re-suspended in 500 μl PBS. The cells were incubated with CFSE at final concentration of 0.31 μM for 7 min, followed by addition of equal volumes of human AB serum for 1 min. The cells were then washed twice with complete medium and cultured for 72 h prior to the conjugate assay.

Formation and measurement of conjugates

The 1520-TIL at cell density 1×10^6 cells/ml in IMDM complete medium was mixed with an equal volume (50 μl) of target cells at the same cell density in a 5 ml polystyrene round-bottom tube (BD, Falcon, Franklin Lakes, NJ), i.e., effector (E) to target (T) ratio of 1:1. The cells were centrifuged at 800 rpm (137 × G) for 10 s, and then incubated in 5% CO_2 at 37°C for 15 min without agitation. At the end of incubation, 100 μl of FACS medium (Miltenyi Biotec, Auburn, CA) containing PE-Cy5 conjugated anti-human CD8 antibody was added into each tube with gentle mixing. After incubation for 10 min at 4°C, the cells were washed once in FACS medium at 1400 rpm (420 × G) for 5 min. The supernatant was aspirated, and the cell pallet was re-suspended in 200 μl of PBS containing 1% formaldehyde and vortexed for 15 s immediately before analysis. Flow cytometry (BD FACSort) was utilized to measure conjugate frequency: free effector cells were stained red alone (labeled by Cy5-PE), free target cells were stained green alone (labeled by CFSE), while conjugates were double positive staining both red and green.

Calculation of percentage of lymphocytes in conjugates

The binding of multiple effector cells to one target cell was observed in our studies corroborating the findings by others [17, 18]. An equation established by Segal et al. [17] was adapted to calculate the percentage of T cells bound to target cells in this study:

$$X = PL^0 - PL^f / P^c(1 - FL^0)$$

X, Number of effecter cells (lymphocytes) bound to one target cell; PL^0, Percentage of lymphocytes at 0 time point of incubation. In this study E:T ratio = 1:1, and therefore = 50%; PL^f, Percentage of free lymphocytes after conjugate formation; P^c, Percentage of conjugate measured by flow cytometry; FL^0, Fraction of lymphocytes at 0 time point of incubation. In this study, E: T ratio = 1:1, therefore $FL^0 = 0.50$.

The percentage of lymphocytes in conjugates was calculated by the percentage of conjugates multiplied by the number of effector cells bound to each target cell (X), i.e.,

$$\%\text{lymphocyte bound} = X \times \%\text{of conjugate obtained from flow data}$$

Statistical analysis

A two-tailed, paired Student *t*-test was utilized for comparison of experimental groups.

Results

Characterization of effector–target conjugate by light microscopy and flow cytometry examination

Morphological examination by light microscopy was employed to screen for the cellular composition of conjugates in addition to flow cytometry analysis. The characteristics of conjugates involving different targets is noted based on the adherence of varying numbers of effector T cells to a single target cancer cell (Fig. 1a, b). Formation of conjugates occurs preferentially in tumor cells expressing the relevant epitope which in this case is represented by the HLA-A*0201/gp100 endogenous antigen combination. The adherence of multiple effector T cells to one target cell led to application of the formula developed by Segal et al. [17] for the assessment of the percentage of T cells contained in the conjugates (see materials and methods).

Figure 1c and d illustrates the representative distribution of free lymphocytes (upper left quadrant), free target cells (lower right quadrant), and effector/target conjugates (upper right quadrant) in a flow cytometry scatter plot when the relevant HLA/epitope combination is absent (c) or present (d). These data demonstrate that effector–target conjugates can be maintained during flow cytometry procedure and be readily identified and isolated.

Increased effector–target conjugate formation through HLA-A2-restricted antigen recognition

The antigen specificity in effector/target cell conjugate formation was tested in two model systems. The first utilized target cells endogenously expressing the gp100/PMel17 antigen recognized by 1520-TIL; the specific requirement for HLA phenotype as well as specific antigen recognition was determined in this model system (Table 1) by the lack or presence of expression of the HLA-A*0201 allele by two clones (624.28-MEL and 624.38-MEL) from the same bulk cultures. Although the two clones originated from the same culture and, to our knowledge, differ only in HLA-A*0201 expression, it could be argued that other unknown factors may contribute to differential adherence by T cells. Therefore, a second, more stringent, model system was utilized in which the same cell line was employed. The 1390-MEL cell line expresses normal levels of HLA-A*0201 but it cannot be recognized by 1520-TIL because it lacks expression of the target antigen gp100/PMel17 at the messenger RNA and protein level [11]. The addition of the minimal epitopic determinant gp:100:209–217 2M is sufficient to re-establish the recognition of this tumor cell by 1520-TIL and promote formation of conjugates (Table 2). This exogenous antigen loading system irrefutably confirms that conjugate formation is dependent upon productive TCR/HLA/antigenic epitope interaction.

Comparing the frequency of conjugate between the 1520-TIL and cell lines lacking or presenting the appropriate HLA-A*0201/antigenic peptide combination, we observed that HLA-A*0201-restricted antigen recognition contributes to a significantly higher frequency of conjugate formation in both model systems (Tables 1, 2). Calculation of effector cells bound to each target suggested that up to 52% of the cytotoxic lymphocytes participated in conjugate formation, and may be identified and isolated by flow cytometry.

Percentage of effector cells in conjugates paralleled with the number of effector Cells producing intracellular IFN-γ

The average number of lymphocytes that participated in conjugates occurring when 1520-TIL and 624.38-MEL were co-cultured was 43.6 ± 7.2% above the background, which was consistent with the percentage of 1520-TIL that displayed increased expression of intracellular IFN-γ under the identical culture condition (40.8 ± 9.7%, *t* test P_2 value >0.05,

$n = 8$). The average 1520-TIL participating in conjugates when co-cultured with 1390-MEL exogenously pulsed with the gp100 peptide was 21.8 ± 7.5% above the background that paralleled the percent of lymphocytes that displayed increased expression of intracellular IFN-γ (23.9 ± 2.5%, t test P_2 value >0.05, $n = 5$). Therefore, the proportion of lymphocytes producing intracellular IFN-γ in response to cognate stimulation corresponded generally to the number of lymphocytes participating in conjugate formation supporting the sequential relationship of conjugate forming, TCR triggering, and effector T cell activation in these test groups (Fig. 2). Our results also revealed that antigen-independent adhesions resulted in minimum intracellular TFN-γ production (Fig. 2b, d).

HLA-Class I blockade partially inhibits conjugate formation and intracellular IFN-γ production

The HLA-A*0201-restricted antigen-dependent conjugate formation associated with intracellular IFN-γ production was further confirmed by blocking experiments with an anti-HLA Class I monoclonal antibody, which could partially block both conjugate formation and intracellular IFN-γ production (Table 3).

Contribution of different accessory/adhesion molecules to conjugate formation

Since HLA Class I blockade only partially inhibited conjugate formation, we further tested the blocking activity of various MABs against cell surface molecules known to promote adhesion between T cells and their target. Partial inhibition of conjugate formation occurred when 1520-TIL was co-cultured with 624.38-MEL or 624.28-MEL in the presence of MABs against human LFA-1, CD54, CD58, and CD2 (Fig. 3). Synergistic blockade was noticed with a combination of anti-human CD54 and anti-human CD58, which resulted in a nearly complete blockade of non-specific binding and virtually eliminated conjugate formation between 1520-TIL and 624.28-MEL. In contrast, conjugate formation was abrogated to a lesser extent by the same MAB combination added to 1520-TIL/624.38-MEL co-cultures further supporting the additional and independent role that TCR/HLA/peptide interactions play in conjugate formation. However, the triple combination of anti-human CD54, -CD58, and -HLA-Class I MABs induced additional reduction in conjugate formation between 1520-TIL and 624.38-MEL, although it did not induce complete blockade. These results support the involvement of antigen-specific interactions between effector T cells and their targets in inducing congregations around their targets that may strengthen other, non-antigen-specific, modalities of cell-to-cell adhesion. Several adhesion molecules appeared to be responsible for the antigen-independent interactions; however, in this model, the combination of anti-CD54 and anti-CD58 blockade appeared to play a prevalent role compared with other combinations (data not shown).

Discussion

While the independent roles of TCR and relevant adhesion molecules in immunological synapse formation and T lymphocyte activation have been well studied, there is limited information regarding the net outcome from the interplay of these molecules at different stages of effector/target cell interaction. In the present study, the individual and cooperated effects of TCR/HLA/antigenic epitope interaction and participating adhesion molecules are dissected at the stage of effector/target cell conjugate formation, in order to determine if the conjugate assay can be utilized as a real time indicator for the specific antigen recognition by TCR. A consistently increased proportion of T cells involved in conjugates is seen in the test groups consisting of an epitope-specific T cell clone and target cells expressing appropriate HLA/antigenic epitope combination. Both HLA allele and specific antigenic epitope are required, because the enhanced binding is diminished when either the appropriate HLA allele or specific antigenic epitope is not expressed or is blocked by a

specific MAB. These results provide strong and conclusive evidence to support that spe-cific TCR/HLA/antigentic epitope interaction is responsible for the increased effector/ target cell conjugate formation. The parallel relationship between the number of effector cells in conjugates and the number of lymphocytes with enhanced intracellular IFN-γ production further confirm the sequential events of TCR engagement, conjugate formation, and T cell activation. Taken together, the increased frequency of effector–target cell conjugate formation reflects the early events of specific antigen recognition by TCR, although considerable background does exist.

In addition to TCR involvement, accessory/adhesion molecules also appear to play a role in conjugate formation as demonstrated by MAB blocking experiments. These data are not contradictory to the dominant role of TCR antigen recognition in conjugate formation, since previous studies have revealed that antigen recognition by TCR triggers the rapid conversion of adhesion molecules to a high-avidity state [7–9]. This process is important to strengthen the immunological synapse and likely plays a role in the engagement of additional effectors per target. Also as illustrated in this study, adhesion molecule binding alone without TCR/ HLA/antigenic epitope involvement does not lead to intracellular cytokine production (Figs. 2, 3).

Antigen-independent adhesions by cytotoxic T cells were reported previously [3, 4], and our data identify involvement of the same adhesion molecules in the antigen non-specific process as are involved in antigen-specific binding (Fig. 3). However, the relative contribution of the various adhesion molecules differs when targets express appropriate HLA/ peptide complex, compared to targets lacking HLA/peptide complex (Figs. 2, 3). In addition, not all lymphocytes bound to target cells bearing specific antigen/HLA complex produce intracellular IFN-γ. The finding that antigen-independent adhesions accompany conjugate formation with target cell lines presenting and lacking HLA/specific antigen epitopes supports the “two-step” model theory for TCR recognition [5, 19], i.e., a TCR scanning process preceding and/or concurring with specific antigen recognition by TCR [9].

Our study demonstrates that increased frequency of effector/target cell conjugates is due to HLA-restricted specific antigen recognition, and this phenomenon is measurable using standard flow cytometry. This finding combined with current consensus that cytotoxic T lymphocytes are able to engage in repeated cycles of target cell binding, lethal hit delivery, and detachment from targets [6, 20] validates the hypothesis that conjugate formation could be used as an approach to identify and isolate antigen-specific cytotoxic T cells. The current approaches to identify antigen-specific T cells include analysis of tetramers or multimers that requires fully characterized antigenic epitopes; while intracellular cytokine production requires fixation and cell membrane permeabilization. Conjugate formation, although not perfect due to significant background, may prove complementary to the forgoing technologies with the following potential advantages: (1) it may be useful in the situations where the target antigen is unknown or multiple antigens are involved; (2) it may provide a means to identify novel immunogenic peptides capable of eliciting potent cytotoxic T cell response; and (3) it may allow isolating cytotoxic T cells in a viable state for further functional characterization and possible in vitro amplification. The model system established in this study is technically easy and provides results in a short time line.

The apparent problems with the current model include: (1) there is a considerable background and (2) not all HLA-gp100 tetramer positive epitope-specific T cells bind to targets. In this study, we identified that MAB against human CD54 and CD58 result in nearly complete elimination of conjugates in negative control groups, which may provide a way to reduce the background. We and others [2, 4] noted that the percentage of T cells from a particular T cell clone bound to a specific target is relatively stable regardless of the

changing E:T ratio (data not shown). Consistent with our finding, previous in vivo and in vitro studies demonstrated that only a portion of epitope-specific T cells producing intracellular cytokines and causing target cell lysis suggested that tetramer positivity did not completely match the effector functionality [14, 21, 22]. Our preliminary results from phenotypic evaluation of effector cells show variable levels of perforin, TCR-$\alpha\beta$, and CD25 expressions in 1520-TIL (data not shown). The significance of these minor variations and their impact on T cell effector function are beyond the scope of the present discussion.

The results from current study provide further information on the commandant effect and mechanisms of TCR regulation, and establish that conjugate formation can serve as a physical point to evaluate TCR/HLA/antigenic epitope interaction. This finding also suggests that conjugate assay may provide a useful tool to monitor and isolate viable and functionally active cytotoxic T cells, particularly when the antigens of interest have not yet been fully characterized.

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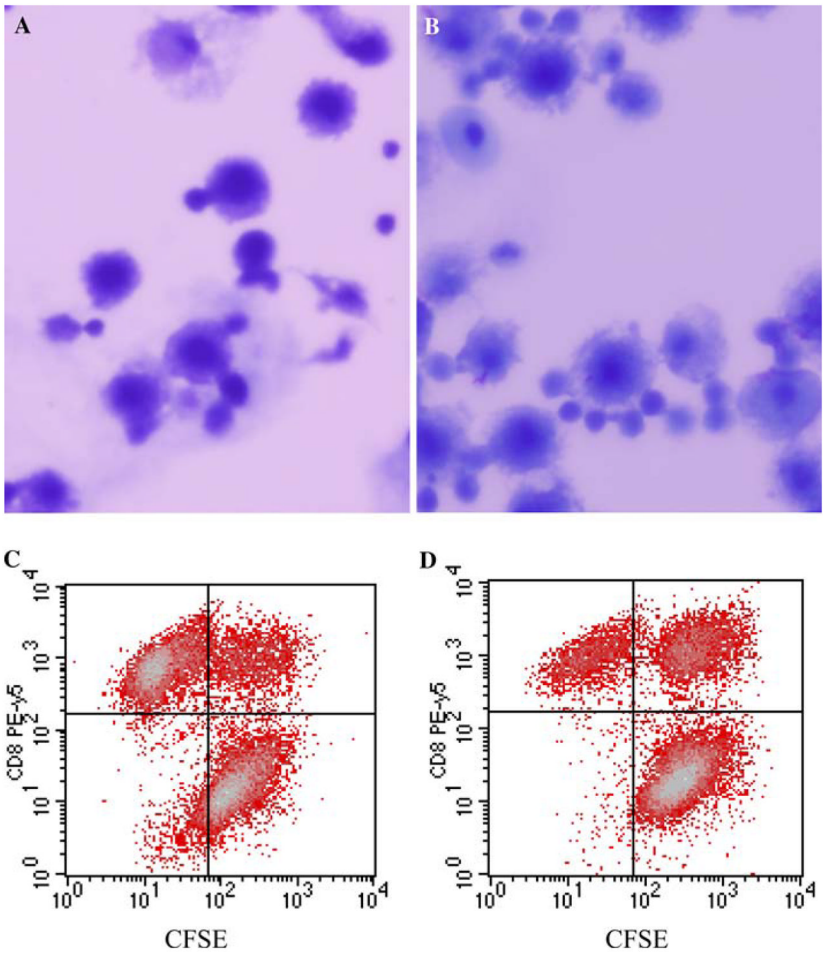

Fig. 1.

Morphologic and flow cytometry characteristics of conjugates in gp100 positive melanoma cell lines without and with HLA- A*0201 allele. (**a**) 400×, less 1520-TIL bound to a single 624.28-MEL; (**b**) 400×, more 1520-TIL bound to a single 624.38-MEL; dot plot analysis of co-culture consisting of: (**c**) 1520-TIL and 624.28-MEL and (**d**) 1520-TIL and 624.38-MEL

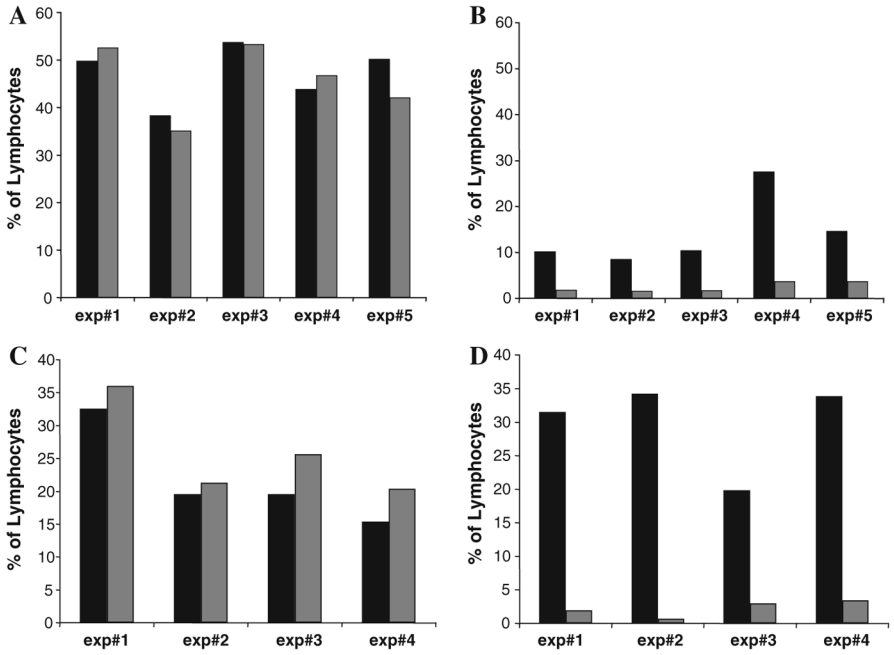

Fig. 2.

Correlation between percentage of lymphocytes in conjugates and those with increased intracellular IFN-γ production. The dark bars represent the percentages of lymphocytes in conjugates, and the shaded bars represent the percentages of lymphocytes producing intracellular IFN-γ. The representative results from the following co-cultures are summarized in (**a**). 1520-TIL and 624.38-MEL; (**b**) 1520-TIL and 624.28-MEL; (**c**) 1520-TIL and 1390-MEL pulsed with gp100–209 2M peptide; and (**d**) 1520-TIL and 1390-MEL without peptide pulsing. The background from antigen-independent adhesions has been subtracted from the values in Fig. 2a and c

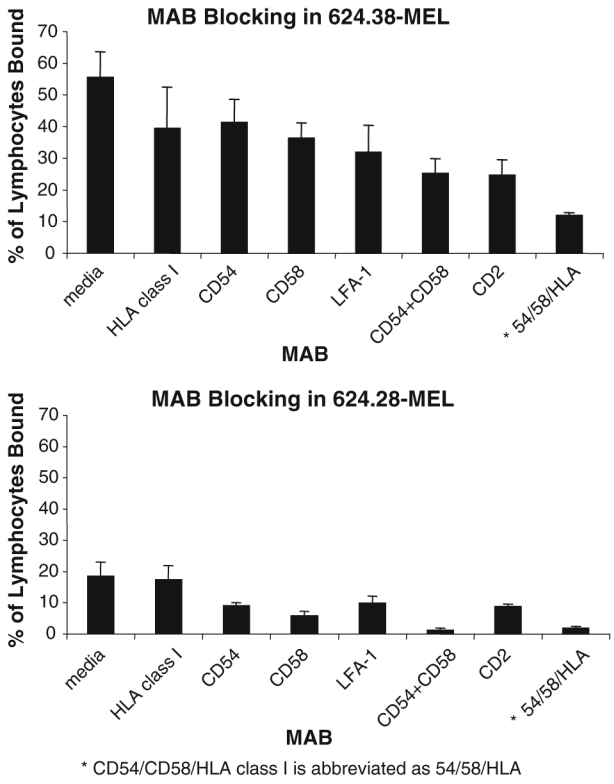

Fig. 3.

Adhesion molecules involved in conjugate formation between 1520-TIL and 624.38-MEL or 624.28-MEL. Melanoma cell lines were pre-incubated with individual or combinations of MAB as indicated for 15 min at room temperature, followed by the routine conjugate formation assay. Anti-HLA Class I (W6/ 32), anti-LFA-1, and anti-CD2 MAB were used as purified IgG at 12.5 μg/ml final concentration, and anti-human CD54-PE and anti-human CD58-PE were added at 10 μg/ml final concentration. Top Panel: Significant inhibition of conjugate formation between 1520-TIL and 624.38-MEL (paired, two-tailed Student *t* test; $P_2 < 0.01$, $n = 4$) by MAB against HLA-Class I, CD54, CD58, LFA-1, and CD2. Bottom panel: Significant inhibition of antigen-independent binding between 1520-TIL and 624.28-MEL (paired, two-tailed Student *t* test; $P_2 < 0.01$, $n = 4$) by MAB against CD54, CD58, LFA-1, and CD2, but not by HLA-Class I ($P_2 > 0.05$)

Table 1

Percentages of free and conjugate particles in gp100 positive melanoma cell lines with or without HLA-A*0201 allele

Experiments	Samples (MEL)	Free lymphocytes (%)	Conjugates (%)	X	Lymphocytes bound to targets (%)
Exp. 1	624.38	21.0	18.1	3.2	58.0
	624.28	44.4	9.7	1.2	11.1
Exp. 2	624.38	18.2	20.2	3.2	63.6
	624.28	41.1	12.6	1.4	17.8
Exp. 3	624.38	16.5	22.8	2.9	67.0
	624.28	42.8	13.5	1.1	14.4
Exp. 4	624.38	25.2	23.4	2.4	55.1
	624.28	44.8	9.3	1.1	10.4
Exp. 5	624.38	31.3	20.9	3.4	71.6
	624.28	48.9	14.3	1.9	27.7
P value		0.0001	0.002		0.000007

Table 2

Percentages of free and conjugate particles in 1390-MEL pulsed with soluble gp100 peptide, control flu peptide, or no peptide

Experiments	1390-MEL (± peptide)	Free lymphocytes (%)	Conjugates (%)	X	Lymphocytes bound to targets (%)
Exp. 1	+ gp100	18.0	41.9	1.5	64.1
	− Peptide	34.2	31.2	1.0	31.2
	+ Flu	33.9	29.1	1.1	32.2
Exp. 2	+ gp100	23.1	14.9	3.6	53.8
	− Peptide	32.9	12.3	2.8	34.3
	+ Flu	32.9	10.5	3.2	34.2
Exp. 3	+ gp100	25.4	15.4	3.2	49.3
	− Peptide	33.1	11.6	2.9	33.9
Exp. 4	+ gp100	21.5	39.5	1.4	55.2
	− Peptide	34.2	31.2	1.0	31.2
Exp. 5	+ gp100	30.3	18.5	2.1	39.5
	− Peptide	40.1	12.3	1.6	19.9
P value		0.001	0.01		0.001

Table 3

Inhibition of conjugate formation and intracellular IFN-γ production by HLA Class I MAB

MEL	% Lymphocytes bound			% Lymphocytes with intracellular IFN-γ		
	Control	With MAB	Inhibition	Control	With MAB	Inhibition
624.38 (n = 5)	37.8 ± 5.0	25.7 ± 5.7	42.5 ± 13.3	34.7 ± 7.0	24.6 ± 10.4	31.5 ± 7.4
P value		=0.0001			=0.0004	
1390 + gp100 (n = 4)	19.8 ± 2.5	15.4 ± 2.6	21.8 ± 4.5	22.5 ± 2.3	15.4 ± 3.1	27.6 ± 7.2
P value		=0.006			=0.01	

Conjugates between 1520-TIL and 624.38-MEL and between 1520-TIL and 1390-MEL pulsed with gp100 peptide were allowed to form for 15 min. at 37°C in the presence of anti HLA-Class I monoclonal antibody (1:25). Percentage of inhibition is relative to the number of conjugates formed in the presence of irrelevant control antibody. The background has been subtracted from the value in this table