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Coreceptor Function of CD4 in Response to the MHC Class I Molecule

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Abstract—The specificity of the T-cell receptor (TCR) and its interaction with coreceptors play a crucial role in T-cell passing through developmental checkpoints and, eventually, determine the efficiency of adaptive immunity. The genes for the α and β chains of TCR were cloned from T-cell hybridoma 1D1, which was obtained by fusion of BWZ.36CD8α cells with $CD8^+$ memory cells specific for the H-$2K^b$ MHC class I molecule. Retroviral transduction of the 1D1 TCR genes and the CD4 and CD8 coreceptor genes was used to obtain 4G4 thymoma variants that exposed the CD3/TCR complex together with CD4, CD8, or both of the coreceptors on their surface. Although the main function of CD4 is to stabilize the interaction of TCR with MHC class II molecules, CD4 was found to mediate the activation of transfected cells via TCR specific for the H-$2K^b$ MHC class I molecule. Moreover, CD4 proved to dominate over CD8, since the response of $CD4^+CD8^+$ transfectants was suppressed by antibodies against CD4 and the A^b MHC class II molecule but not to CD8. The response of $CD4^+$ transfectants was not due to a cross-reaction of 1D1 TCR with MHC class II molecules, because the transfectants did not respond to splenocytes of H-2^b knockout mice, which were defective in the assembly of the MHC class I molecule/β2 microglobulin/peptide complex and did not expose the complex on cell surface. The domination was not due to sequestration of $p56^{lck}$ kinase, since CD4 devoid of the kinase-binding site was functional in 4G4 thymoma cells. The results were used to explain some features of intrathymic cell selection and assumed to provide an experimental basis for developing new methods of anticancer gene therapy.

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Key words: T cell, cloning, retrovirus, transduction, coreceptor, CD4, CD8, MHC, TCR

INTRODUCTION

The repertoire of T cells is formed via random rearrangements of T-cell receptor (TCR) gene segments and positive and negative selection within the thymus. These processes yield clones whose TCRs are capable of interacting with cognate major histocompatibility complex (MHC) molecules associated with endogenous peptides of the thymic environment (positive selection) and eliminate clones capable of high-affinity interactions with cognate MHC/peptide complexes (negative selection) to prevent autoimmune reactions. The T-cell clones whose TCRs meet these conditions, mature and cease producing the "unnecessary" coreceptor [1]. Recent studies have shown that the choice of a coreceptor is determined by the strength and duration of the signal that the T cell receives as its TCR and CD4 coreceptor interact with MHC molecules in the thymus. Prolonged strong signaling induces expression of the Th-POK and GATA-3 transcription factors, which trigger differentiation of the $CD4^+$ T-cell lineage with helper functions [2, 3]. Weak intermittent signaling causes Runx3 expression, Runx3 binding to the CD8 enhancer and CD4 silencer, and default differentiation of $CD8^+$ killers [4, 5]. This normally yields two T-cell subpopulations, $CD4^+$, recognizing MHC class II molecules, and $CD8^+$, recognizing MHC class I molecules. The problem is that both of the coreceptors occur on the T cell at the moment when the further differentiation program is selected in the thymus, and it is unclear why prolonged signaling caused by the interaction of MHC class I molecules with TCR and the CD8 coreceptor does not trigger the same differentiation program of the $CD4^+$ T-cell lineage. In this work, we used transfected 4G4 thymoma cells expressing the genes for MHC class I-specific TCR and the coreceptors and showed that the CD4 coreceptor is able to perform the CD8 functions and to play a role in T-cell activation, provided that the natural coreceptor ligands are available. Moreover, we found that the CD8 coreceptor is silent in double-positive T cells, expressing both CD4 and CD8 on their surface. The silencing is not associated with sequestration of the $p56^{lck}$ kinase by CD4 molecules and

probably results from competition of the coreceptors during the formation of the immunological synapse.

EXPERIMENTAL

Animals. Mice of the C57BL/6 ($K^bI\text{-}A^bD^b$), C57BL/10 ($K^bI\text{-}A^bD^b$), B10.D2 ($K^dI\text{-}A^dI\text{-}E^dD^dL^d$), B10.D2(R101) ($K^dI\text{-}A^dI\text{-}E^dD^b$), B6.C-H-$2^{bm1}$($K^{bm1}I\text{-}A^bD^b$), C57BL/6J-H-$2^{bm3}$ ($K^{bm3}I\text{-}A^bD^b$), and B6.C-H-2^{bm12} ($K^bI\text{-}A^{bm12}D^b$) strains were obtained from the breeding facility of the Blokhin Cancer Research Center. Strains knocked-out in the genes for β_2-microglobulin (C57BL/6J-*B2m*^{tm1Unc} (H-2^b)) and transporters associated with antigen processing (TAP) (C57BL/6J-*Tap1*^{tm1Arp}) were obtained from the Jackson Laboratory (Bar Harbor, Main, United States) and maintained in the Laboratory of Regulatory Mechanisms in Immunity, Institute of Carcinogenesis, Blokhin Cancer Research Center. Genetically modified strains were maintained and genotyped according to the instructions available at http://www.jax.org/imr/tech_info.html.

Antibodies. The following antibodies were from Pharmingen (United States): anti-CD3ε-FITC (Cat. No. 01084A), anti-CD4-PE (Cat. No. 09425A), anti-CD8-APC (Cat. No. 01049A), anti-TCR β-chain-FITC (Cat. No. 01304A), and anti-rat Ig (mouse adsorbed)-FITC (Cat. No. 12314D). FITC-conjugated goat antibodies against mouse Ig (Cat. No. F1010) and secondary FITC-conjugated antibodies against Fc of mouse Ig (Cat. No. F2772) were from Sigma. The supernatants of hybridomas GK1.5 (IgG2a) and 3.168 (IgG2a) were used as a source of monoclonal antibodies against mouse lymphocyte CD4 and CD8, respectively, in experiments with immunomagnetic cell separation. Antibodies against mouse CD3 (clone 145-2C11) were used in the form of a hybridoma supernatant.

Cell lines. MC-11 (K^bD^b) sarcoma and EL4 thymoma cells were transplanted in syngenic C57BL/10 mice at (3–5) $\times 10^6$ per mice and grown as ascites tumors. P815 (K^dD^d) mastocytoma cells were grown in vitro by passages in RPMI 1640 supplemented with 4 mM L-glutamine and 10% fetal calf serum (FCS). The EL4 cell line with a high spontaneous production of mouse interleukin 2 (IL-2) was used as a source of IL-2 (the line was kindly provided by A.V. Chervonsky, Jackson Laboratory, Bar Harbor, Main, United States). EL4 cells were maintained in DMEM supplemented with 4 mM L-glutamine and 10% FCS. IL-2 was quantified by the MTT catabolism assay with IL-2-dependent CTLL-2 cells as in [6] with minor modification. The CTLL-2 cell line was maintained in RPMI 1640 supplemented with 4 mM L-glutamine, 10% FCS, and 5 MU/ml IL-2. The BWZ.36CD8α cell line was kindly provided by N. Shastri (Department of Molecular and Cell Biology, University of California, United States) [7].

Immunization. Mice were immunized via intraperitoneal injection of 25×10^6 allogeneic tumor cells per mouse. Splenocytes of immunized mice were used as a source of memory cells 2 months after the immunization.

Cell suspensions. Stimulator and responder lymphocytes were gently squeezed from mouse spleen in a Potter homogenizer with a conic pestle. To separate the T-cell subpopulations by complement-dependent cytolysis, RPMI 1640 (Institute of Poliomyelitis and Virus Encephalitides, Moscow) supplemented with 0.2% BSA (Sigma, Cat. No. 84F-9435) was used as a washing medium. Viable cells were counted after staining with trypan blue–eosin.

Cell staining with antibodies and flow cytometry. Staining with antibodies was carried out at 4°C for 20–30 min. Cells were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, United States) with argon (480 nm) and helium–neon (630 nm) lasers, using the CellQuest program. Dead cells and their fragments were excluded from the analysis via staining with propidium iodide (PI) and measuring the forward and side scatters. At least 40 000 events were recorded to characterize the peripheral T-cell populations and cell cultures. The results were analyzed using the WinMDI 2.8 program.

Generation and specificity of T-cell hybridomas. Memory T cells, obtained by enrichment in response to heated stimulators for 4 days, and BWZ.36CD8α cells were washed twice with a serum-free medium, mixed in glass vials at a 3 : 1 ratio, centrifuged at 1500 *g* for 4 min to achieve a physical cell-to-cell contact, and incubated in a CO_2 incubator at 37°C for 20 min. The supernatant was removed with a surgical aspirator. Cells were combined with preheated (37°C) 50% polyethylene glycol 1500 (Merck) in 75 mM HEPES (pH 7.0), incubated for 70 s, and washed three times with a serum-free medium, centrifuging the suspension at 1000 *g* for 2 min. After the last centrifugation, cells were combined with 20 ml of a serum-containing medium and incubated in a CO_2 incubator at 37°C for 45 min. Cells were counted, and a suspension of 0.4×10^6 cells/ml was plated in 96-well plates. The medium was replaced after 2 days with a selective medium containing a hypoxanthine–aminopterin–thymidine mixture (HAT), cells were grown for 2 weeks, and the resulting clones were transferred into a selective medium containing a hypoxanthine–aminopterin mixture (HA). To test the T-cell hybridomas, hybridoma cells were plated in flat-bottom wells with antigen-presenting cells (APCs) expressing various allelic forms of MHC molecules. The plates were frozen after 1 day. The supernatants were tested for the capability of sustaining the growth and viability of CTLL-2 cells [8].

Expression analysis and cloning of TCR gene variants. A pellet of (5–10) × 10^6 cells was lyzed in 0.5 ml of TRIZOL LS (Gibco BRL, United States) at room temperature for 5 min. The lysate was combined with 0.1 ml of chloroform, stirred well, incubated at room temperature for 2–15 min, and centrifuged in a Minispin centrifuge (Eppendorf) at 12000 rpm for 15 min with chilling. The aqueous phase, containing RNA, was combined with 0.5 ml of isopropyl alcohol and incubated at room temperature for 10 min; RNA was precipitated as above. The RNA pellet was washed with 70% ethanol and dissolved in water to 1 μg/μl. To synthesize cDNA, 5 μg of the RNA solution in water were mixed with 5.6 μl of water treated with diethyl pyrocarbonate (DEPC), and the mixture was combined with 0.4 μl of 25 mM dNTPs and 1 μl of 500 μg/ml oligo(dT), incubated at 65°C for 5 min, and chilled in ice for 2 min. Then, the mixture was combined with 4 μl of a 5× first-strand synthesis buffer (Invitrogen), 2 μl of 0.1 M DTT (Invitrogen), and 1 μl of 40 units/μl RNase inhibitor; incubated at 42°C for 2 min; combined with 1 μl (200 units) of Superscript II reverse transcriptase (Invitrogen); and incubated at 42°C for 50 min. The reaction was terminated by heating the mixture at 70°C for 15 min. The resulting cDNA solution was diluted threefold, and 0.2 μl of the dilution was used for PCR. The primers and RT–PCR conditions were as in [9]. The mixture was analyzed in 1.5% agarose gel containing ethidium bromide; electrophoresis was carried out in 40 mM Tris-acetate (pH 8.0) at 8 V/cm for 45 min. Gel was photographed and analyzed using a UV transilluminator equipped with a CD camera.

Analysis of the V, D, and J segments and complementarity-determining regions (CDRs) of the TCR gene. The amplified TCR gene fragments were cloned in pTZ57RT; the clones were sequenced at the Genom Center, Institute of Molecular Biology. Nucleotide sequences were analyzed using the Chromas v. 1.45 program. Amino acid sequences were deduced, and stop codons were identified in the rearranged region with the DNAssist v. 1.0 program. The BLAST package was used to identify the V-, D-, and J-segment sequences, template-dependent palindromic (P) nucleotides, and non-template-dependent (N) nucleotides added by terminal deoxynucleotidyl transferase. The same package was used to assemble the hypothetical full-length cDNA sequences of the identified TCR α and β chains with the help of the best characterized genomic sequence of the α/δ and β loci of the 129 mouse cell line. The full-length cDNAs were cloned in retroviral vectors.

Cloning of the full-length cDNAs of the TCR chains in expression vectors. The cDNAs coding for the α- and β chains of hybridoma TCRs were amplified with primers specific to the full-length TCR chain genes and containing the *Xho*I (in the case of the α chain) or *Bgl*II (in the case of the β chain) restriction sites, digested with the corresponding enzyme (all restriction enzymes were from Fermentas, Lithuania), and purified from low-melting agarose with a QIAGEN kit. The chain cDNAs were cloned in the retroviral expression vectors pMigRI (Invitrogen), which contained the green fluorescent protein (GFP) gene with the internal ribosome entry site (IRES) and was used for α-chain cDNAs, and pMINV (Invitrogen), which contained the neomycin resistance gene with the IRES and was used for β-chain cDNAs. The vectors were linearized with *Xho*I and *Bgl*II, respectively, treated with CIAP (Fermentas), and purified from low-melting agarose gel with a QIAGEN kit. The α- and β-chain cDNAs and the vectors were ligated with T4 DNA ligase (Fermentas), and the resulting constructs were sequenced. The procedure was repeated several times to obtain sequences free from mutations of the coding region.

Cloning of the coreceptor chain-coding sequences in expression vectors. The sequences coding for the α and β chains of the CD8 coreceptor in pBS were kindly provided by D. Littman (the Kimmel Center for Biology and Medicine, Skirball Institute, New York University School of Medicine, United States). The CD8α and CD8β sequences were obtained as *Sal*I and *Apa*I–*Sac*II fragments of pBS, respectively. The CD4-coding sequence in pcDNA3.1(+/–) was kindly provided by Chervonsky. The sequence was isolated from pcDNA3.1(+/–) with *Apa*I and *Hin*dIII. The pMSCVpuro and pMSCVhygro were digested with *Xho*I. The lentiviral expression vector pLenti6/V5-D-TOPO was linearized with *Apa*I. The single-stranded ends were filled in with complementary nucleotides; the fragments were digested with alkaline phosphatase, purified, and cloned as above. We cloned the CD8α cDNA in pMSCVpuro, the CD8β cDNA in pMSCVhygro, and the CD4 cDNA in pLenti6/V5-D-TOPO.

Construction of truncated CD4. A stop codon was introduced in the CD4 gene sequence in place of the triplet coding for Cys420, responsible for the interaction with Lck tyrosine kinase, via amplification with primers containing complementary sequences. The mutant CD4 cDNA was cloned in pLenti6/V5-D-TOPO.

Transfection of packaging fibroblasts. Fibroblasts (1 × 10^6 cells) of the 293.1 line (Invitrogen) were plated in a Petri dish with 4–5 ml of complete DMEM (Sigma) supplemented with 20 mM HEPES (Sigma), 4 mM L-glutamine (Serva), and 10% FCS (Pan Eko). After 24 h, the medium was replaced with 1.6 ml of DMEM. A DNA mixture with accessory plasmids (total volume no less than 30 μl) was prepared and combined with 0.2 μg of pEGFPN1 (Invitrogen) to estimate the transfection efficiency. PACK (Invitrogen) was used as an accessory plasmid in the case of

pMINV and pMigRI. The molar ratio of the accessory plasmid to a vector was 2 : 1. In the case of pLenti6/V5-D-TOPO, transfection was performed with the 293FT packaging fibroblast line. The molar ratio of the accessory plasmids pLP1, pLP2, and pLP3 (Invitrogen) to a vector was 3 : 1. In the case of pMSCVpuro and pMSCVhygro, transfection was performed with GP293 fibroblasts. The molar ratio of accessory pVSVG (Invitrogen) to a vector was 1 : 1. Transfection was carried out with Escort V as recommended by Sigma. After 8–16 h, the medium was replaced with 5 ml of DMEM supplemented with 20 mM HEPES, 4 mM L-glutamine, and 5% FCS. The virus-containing supernatant was collected 48 and 72 h after transfection and stored at –70°C.

Infection of 4G4 thymoma cells. Thymoma cells (10^5) were combined with 1 ml of a virus-containing supernatant and 1 μl of 8 mg/ml polybrene (Sigma) and plated in a 24-well plate. After 1 day, the medium was replaced with complete RPMI1640 supplemented with a selective antibiotic: 1 μg/ml neomycin (Sigma) in the case of β chains, 1 μg/ml puromycin (Sigma) in the case of CD8α, and 600 μg/ml hygromycin (Sigma) in the case of CD8β. Since pMigRI lacked an antibiotic resistance gene and coded for GFP, cells expressing TCR α chains were analyzed by flow cytometry and cloned by the limited dilution method. CD4 and CD8 were detected on the surface of the resulting clones by flow cytometry. Clones that displayed stable expression of the coreceptors and an intense staining with the corresponding antibodies were selected for further experiments.

Functional analysis of TCR transfectants. The TCR function was evaluated using anti-CD3ε antibodies sorbed on plastic. Antibodies were diluted with sterile PBS to 25 μg/ml. Antibodies were incubated in a 96-well flat-bottom plate at 4°C overnight. The medium was removed, and the wells were washed three times with cold PBS. Transfected cells were plated at 50×10^3 per well and incubated at 37°C for 1 day. The supernatants were tested for IL-2, using the IL-2-dependent CTLL-2 cell line.

IL-2 assays with CTLL-2 cells were performed according to the original protocol [8] with minor modification. The supernatant of a cell culture was placed in wells of a 96-well plate at 100 μl/well. The wells contained $(5–10) \times 10^3$ CTLL-2 cells, which were preliminarily washed from IL-2 three times with PBS. After 18 h, 15 μl of Alamar Blue (Gibco) were added to each well. Cell viability was inferred after 18–24 h from the reduction of the oxidized dye, using a MULTISCAN instrument. The percent reduction was calculated as $[OD^{exp}_{540} - OD^{exp}_{620} \times (OD^{AB}_{540} - OD^{med}_{540})/(OD^{AB}_{620} - OD^{med}_{620})] \times 100\%$, where OD^{exp}_{540} and OD^{exp}_{620} are the optical densities in test wells, OD^{AB}_{540} and OD^{AB}_{620} are the optical densities of 10% Alamar Blue in 100 μl of the medium, and OD^{med}_{540} and OD^{med}_{620} are the optical densities of 100 μl of the medium at 540 and 620 nm, respectively.

Statistical analysis. The mean and mean error were calculated for each test group. The significance was evaluated by Student's *t*-test.

RESULTS

Cloning of cytotoxic lymphocytes (CTLs) and memory cells is necessary for subsequent molecular identification of their antigen-specific receptors. However, it is rather difficult to clone such cells because the resulting clones are short lived and unstable, individual conditions should be selected for their restimulation, and expensive cytokines are necessary. These difficulties can be overcome by generating T-cell hybridomas. Our protocol of selective activation of $CD8^+$ memory cells make it possible to achieve their proliferation, which is essential for their fusion with tumor partners [10–12]. Hybrid cells resulting from the fusion lose both cytotoxic activity and the CD8 coreceptor, which stabilizes the interaction of TCR with the MHC molecule. Hence, BWZ.36 CD8α thymoma, which is a stable transfectant of BW5147 thymoma and carries a genetic construct coding for the CD8 α subunit, is commonly used as a tumor partner. Activation of hybrid cells can be estimated from the production of IL-2 or the T-cell transcription factor NF-AT (nuclear factor of activated T cells) [7].

To obtain memory T cells specific for H-$2K^b$ MHC class I molecule, B10.D2(R101) ($K^dI^dD^b$) mice were immunized with EL4 thymoma cells, originating from mice of the C57BL/6 ($K^bI^bD^b$) strain. Such immunization induces an immune response to the only allelic product, the H-$2K^b$ MHC class I molecule. $CD8^+$ T cells of the recipient are selectively activated in this case, which leads to production of CTLs with peak activity observed after 12 days. Two months after the immunization, memory cells, lacking cytolytic activity, remain in the recipient spleen and can be selectively restimulated in vitro with C57BL/6 splenocytes killed by severe heat shock. We fused such restimulated memory cells with BWZ.36CD8α to obtain T-cell hybridomas. Half of the resulting clones (28 out of 60) produced IL-2 in response to splenocytes or immortalized fibroblasts of C57BL/6 mice. Antibodies against the H-$2K^b$ molecule blocked the response of the clones, and only one-third of them remained capable of responding to allogenic splenocytes killed by acute heat shock. This capability was probably associated with a high TCR affinity, since the response of clones was incompletely blocked by antibodies against H-$2K^b$ and was detectable upon stimulation

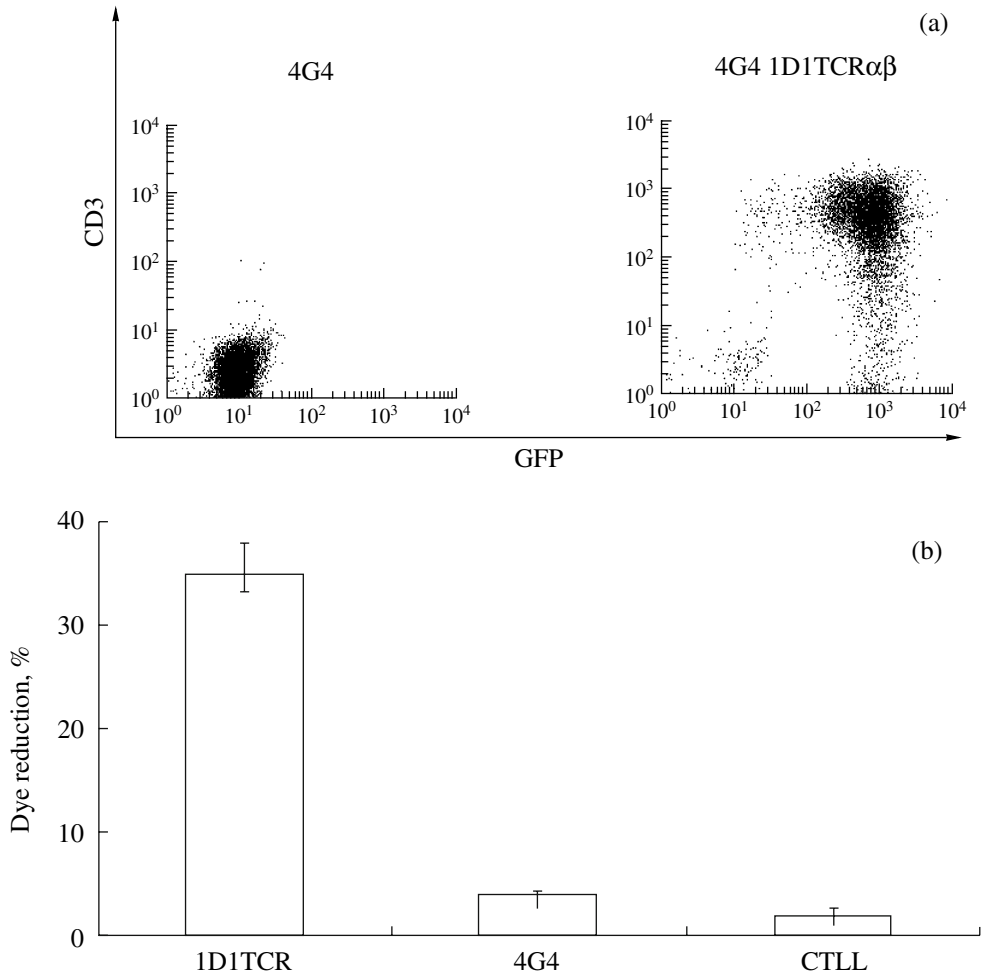

Fig. 1. Expression of the TCR/CD3 complex on the membrane of 4G4 thymoma cells after retroviral transduction of genetic constructs coding for the α and β chains of 1D1 TCR. (a) Flow cytometric detection of the intracellular expression of the GFP transgene and the membrane expression of CD3 in 4G4 thymoma cells (control) and the 4G4 1D1TCRα/β transfectant. Abscissa, GFP expression, which results from transduction of a vector containing the genes for the TCR α chain and IRES-GFP. Ordinate, intensity of cell staining with antibodies against CD3, which is exposed on the cell membrane as a result of a correct folding and joining of the α and β chains of TCR. (b) IL-2 production (by dye reduction) by transfectants after a contact with anti-CD3 antibodies sorbed on plastic. Abscissa, cell line. Ordinate, percent reduction of Alamar Blue by CTLL-2 cells.

with H-2K^{bml} and H-2K^{bm3}, the mutant forms of H-2K^{b}.

TCR was cloned from hybridoma 1D1, which responded to the wild-type MHC molecule H-2K^{b} and its mutant form H-2K^{bm3}, containing point amino acid substitutions in positions 77 and 89 of the heavy chain. The nucleotide sequences of the mRNAs coding for the α and β chains of 1D1 TCR are available from GenBank under accession numbers DQ983579 and DQ983580; the accession numbers of the protein sequences are ABJ88843 and ABJ88844.

To determine whether the resulting constructs were functional, 4G4 thymoma cells were used for retroviral transduction, and transfectants with stable transgene expression were selected and cloned. Detection of the CD3ε subunit of the TCR/CD3 receptor complex by flow cytometry suggested a correct folding, joining, and expression on the cell surface of the transgenic TCR chains, since CD3ε was not exposed on the membrane in their absence (Fig. 1a). Transfectants were capable of responding to anti-CD3 antibodies sorbed on plastic (Fig. 1b), suggesting the intact signaling function of the TCR/CD3 complex. In the absence of the coreceptors, the transfectants did not respond to the MHC molecules (data not shown). In this work, we used Chervonsky's idea that an exogenous coreceptor (CD4) introduced in 4G4 cells would make it possible to evaluate the true specificity of 1D1 TCR at the moment of selection in the thymus. Hence, genetic constructs coding for the CD4 and CD8 coreceptors were additionally introduced in the transfectants.

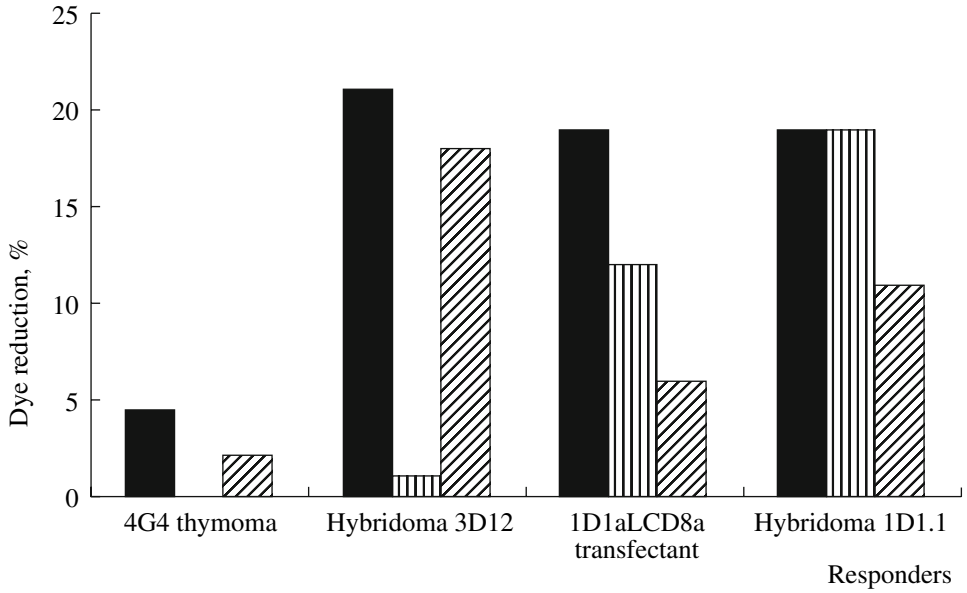

Fig. 2. Antibodies against CD4 and CD8 suppress the IL-2 production induced in response to C57BL/10 splenocytes. As responders, we used 4G4 thymoma; T-cell hybridoma 3D12, which expressed CD4 and TCR specific for the A^b molecule; 4G4 transfectant, which expressed CD8 and 1D1 TCR, specific for the H-$2K^b$ molecule; T-cell hybridoma 1D1, which expressed CD8 and 1D1 TCR. IL-2 production was assayed in the absence (control, black columns) and presence of antibodies against CD4 (vertical hatching) or CD8 (diagonal hatching).

Signal transduction from 1D1 TCR depended on CD8, which was evident from experiments with CD8 blocking with antibodies. The responses of the transfectants and the T-cell hybridoma used to clone 1D1 TCR were suppressed by antibodies against CD8 but not CD4 (Fig. 2). Under the same conditions, antibodies against CD4, but not CD8, suppressed the response of our T-cell hybridoma 3D12, specific for the A^b MHC class II molecule (positive control). We estimated the activation of the transfectants by splenocytes of C57BL/6 mice and TAP knockouts (Fig. 3). The transfectants almost did not respond to mouse splenocytes devoid of MHC class I molecules, as was expected from the specificity of 1D1 TCR for the H-$2K^b$ MHC class I molecule. Under the same conditions, 3D12 hybridoma cells, specific for the A^b MHC class II molecule, were activated in response to the stimulators of knockout mice. This confirmed the selective defect in expression of MHC class I molecules and the preserved capability of stimulating a response to MHC class II molecules and testified to the adequacy of our experiments. It is noteworthy that, although 1D1 TCR was specific for the H-$2K^b$ MHC class I molecule, CD4 was as efficient as CD8 in facilitating the activation of the transfectants. These findings indicate that T cells specific for MHC class I molecules are capable of activation in the presence of a coreceptor differing in specificity from TCR.

Interesting results were obtained with transfectants expressing both of the coreceptors. These transfectants were activated in response to H-$2K^b$ (Figs. 3, 4). While, as expected, the response of the transfectant expressing only the CD8 transgene was suppressed by anti-CD8 antibodies, the response of $CD4^+$ $CD8^+$ double-positive cells was blocked by antibodies to CD4 or the A^b MHC class II molecule but not to CD8 (Figs. 4a, 4b). In other words, although TCR of double-positive transfectants was specific for the MHC class I molecule, these transfectants were similar in behavior in the presence of anti-coreceptor antibodies to 3D12 hybridoma cells, which expressed CD4 and TCR specific for the A^b class II MHC molecule. These findings indicate that transgenic CD4 retained its specificity and interacted with MHC class II molecules during activation of double-positive transfectants. Moreover, not only did CD4 perform the function of CD8 in the response to the MHC class I molecule, but it also functionally dominated over CD8 when coexpressed in the same T cell. A similar pattern was observed for the transfectant response to the mutant K^{bm3} molecule, indicating that cloned 1D1 TCR cross-reacted indeed with K^b and K^{bm3} and that the ability of the original hybridoma to respond to the K^{bm3} mutant was not due to the expression of an undetected other allele of the TCR α chain.

To check whether the CD4 domination was associated with the signaling functions of the coreceptors or their role in stabilizing the TCR–MHC interactions, we constructed the transfectants with a truncated CD4 form devoid of the binding site for $p56^{lck}$ tyrosine kinase. The transfectants expressing the mutant CD4 coreceptors retained the capability of activation in

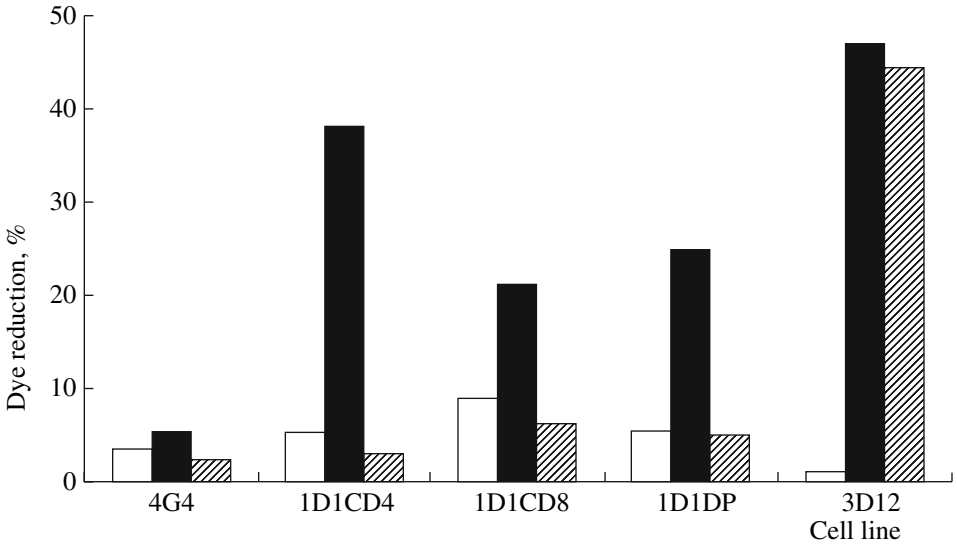

Fig. 3. IL-2 production in 4G4 transfectants expressing 1D1 TCR with CD4, CD8, or both CD4 and CD8 (double-positive cells) after stimulation with splenocytes of R101 (control, open columns), C57BL/10 (black columns), or *Tap* knockout (hatched columns) mice. Nontransfected 4G4 thymoma cells were used as a negative control. T-cell hybridoma 3D12, specific for the A^b MHC class II molecule, was used as a positive control.

response to the MHC class I molecule, suggesting that activation of 4G4 transfectants was independent of the signaling function of CD4. Hence, the domination was most likely due to a competition between CD4 and CD8 during the formation of the immunological synapse.

DISCUSSION

Our key findings are that CD4 is able to functionally substitute CD8 during the activation of T cells with TCR specific for the MHC class I molecule and that CD4 functionally dominates over CD8 during the activation of T cells coexpressing the two coreceptors. The first finding is important, indicating that the match of TCR and coreceptor specificities, which normally results from the commitment to particular T-cell lineages, does not limit the antigen-specific activation of $CD4^+$ T cells. In addition, the finding is of applied significance in the context of transgenesis and cancer immunotherapy, suggesting the possibility of simultaneous activation of T-killers and T-helpers after T-cell transfection with TCRs specific for MHC class I molecules in cancer patients.

Functional domination of CD4 over CD8 is important for understanding the mechanisms of T-cell selection and differentiation in the thymus, the only organ housing the T-cell developmental stage with coexpression of both of the coreceptors. Long-term discussion of the mechanisms of T-cell commitment has yielded the consensus hypothesis that the key role in the commitment is played by the duration of signaling that the developing T cell receives as its TCR and CD4 interact with endogenous intrathymic MHC–peptide complexes. Strong prolonged signaling triggers differentiation of $CD4^+$ T cells, while weak intermittent signaling results in default differentiation of $CD8^+$ T cells. It remained unclear, however, how the T cell identifies the MHC molecule interacting with its TCR and selects the coreceptor to stabilize this interaction and why the commitment to the CD4 lineage does not work with T cells whose TCRs are specific for MHC class I molecules. In fact, all hypotheses explaining the commitment proceed from the idea that the two coreceptors of double-positive thymic cells are both functional and independent of each other. Our experiments with transfectants demonstrate that this is not always the case and that CD8 does not work when expressed together with CD4. This suggests that CD4 plays a leading role in early positive and negative selection in the thymus, while CD8 starts functioning substantially later, at the stage of single-positive $CD8^+$ T cells. Such a lag would allow the cell to distinguish between the signals resulting from coreceptor interactions with MHC molecules and to switch on the corresponding differentiation program.

Our hypothesis implies that the central role in T-cell commitment is played by CD4. Its interaction with the MHC class II molecule results in prolonged activation signaling, inhibits the expression of the CD8 gene, and leads to differentiation of $CD4^+$ T cells when TCR is specific for the same MHC molecule. When TCR interacts with MHC class I molecule, i.e., the TCR and CD4 specificities are mismatched, signaling events are discoupled and the T cell receives a weak intermittent signal, which inhibits the expres-

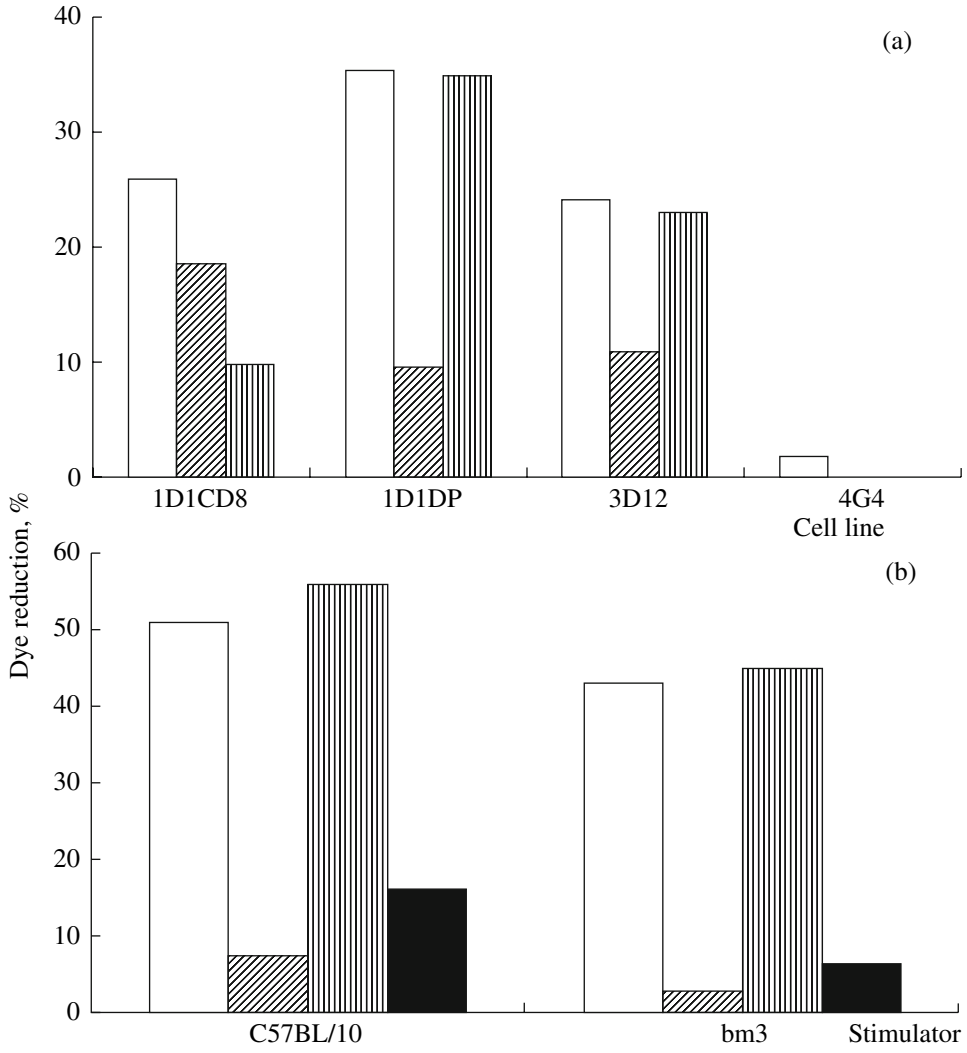

Fig. 4. Suppression of the transfectant response by antibodies against CD4, CD8, and the A^b MHC class II molecule. (a) Response of transfectants to C57BL/10 mouse splenocytes in the absence (open columns) and presence of antibodies against CD4 (diagonal hatching) or CD8 (vertical hatching). (b) Response of $CD4^+CD8^+$ transfectants to C57BL/10 mouse splenocytes and bm3 in the absence (open columns) and presence of antibodies against CD4 (diagonal hatching), CD8 (vertical hatching), or the A^b molecule (black columns). Nontransfected 4G4 thymoma cells were used as a negative control. T-cell hybridoma 3D12 was used as an analog of $CD4^+$ T cells specific for the MHC class II molecule.

sion of the CD4 gene and causes differentiation of the $CD8^+$ T-cell lineage.

This hypothesis makes it possible to explain several challenging issues related to the formation of the peripheral repertoire of mature T cells. It is unclear, for instance, why CD4 genetic knockouts produce $CD8^+$ T cells with a substantial proportion of T cells specific for MHC class II molecules [13]. Following our hypothesis, the lack of CD4 leads to weak short-term interactions of TCR with MHC class II molecules, and such interactions trigger aberrant differentiation of the $CD8^+$ T-cell lineage. Another explainable issue is why T cells with mismatched TCR and coreceptor specificities do not develop in CD8 knockouts. Antigen-specific killers (CTLs) and memory cells of such knockouts express the $CD4^-CD8^-$ double-negative phenotype but do not acquire the $CD4^+$ phenotype. In this case, CD4 ensures the normal commitment of T cells specific for MHC class II molecules to the $CD4^+$ T-cell lineage. T cells specific for MHC class I molecules receive an activation signal as TCR interacts with the MHC class I molecule and CD4 interacts with the MHC class II molecule, which allows the cells to pass through this developmental checkpoint. Although lacking CD8, T cells acquire other functional properties of killer precursors, including expression of the Runx3 and perforin genes, and become CTLs and $CD4^-CD8^-$ memory cells after stim-

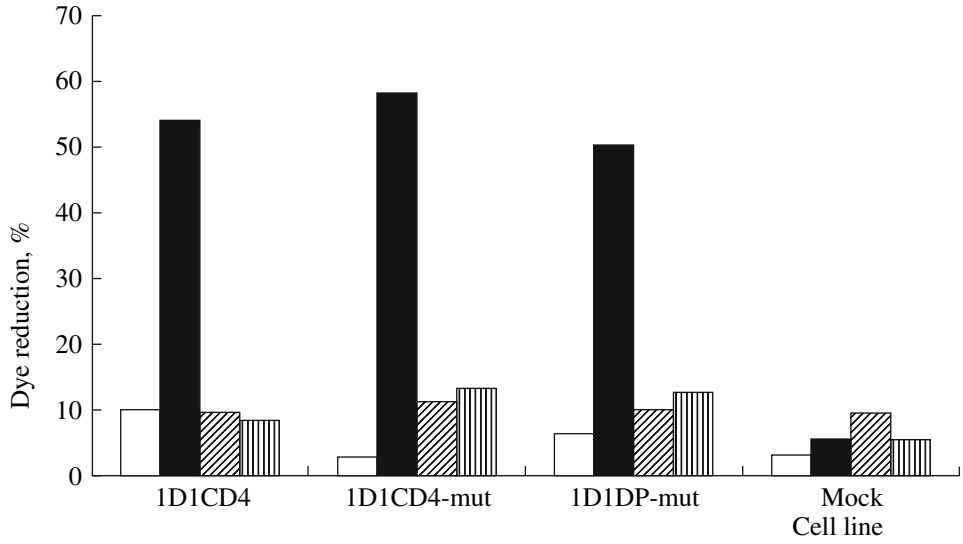

Fig. 5. Production of IL-2 by 4G4 thymoma variants expressing 1D1 TCR together with wild-type CD4 or its mutant variant with the cytoplasmic domain devoid of the binding site for $p56^{lck}$ tyrosine kinase in response to stimulators of R101 (syngenic control, open columns), C57BL/10 (black columns), β-microglobulin knockout (diagonal hatching), and TAP knockout (vertical hatching) mice. Transfectants carrying empty vectors (Mock) were used as a negative control.

ulation with allogenic MHC class I molecules (data not shown).

If our hypothesis is true, an interesting new explanation is possible for the mechanism of negative selection of T cells. According to the current views, negative selection affects autoreactive T cells that carry high-affinity TCRs to cognate MHC molecules and, consequently, receive an extremely strong activation signal, which triggers apoptosis. This mechanism plays a key role in generating and maintaining central tolerance of cognate molecules and preventing autoimmune reactions. If CD4 indeed dominates, negative selection would efficiently eliminate only autoreactive T cells that are committed to the $CD4^+$ cell lineage and recognize cognate MHC class II molecules. The population of T cells whose TCRs are specific for cognate MHC class I molecules would not receive a strong activation signal because their TCRs and CD4 are differently specific for MHC class I and class II molecules, respectively. Thus, the $CD8^+$ T-cell population would have a greater portion of clones with autoreactive, cross-reactive, or promiscuous TCRs as compared with the $CD4^+$ T-cell population. It is possibly incomplete elimination of autoreactive cells that is responsible for the fact that an appreciable portion of $CD8^+$ T cells have TCRs specific for syngeneic MHC class II molecules, as we have observed previously [14]. The hypothesis is supported by the well-known in vitro induction of lymphokine-activated killer cells that are able to nonspecifically destroy syngenic and alien target cells.

The mechanism sustaining functional domination of CD4 over CD8 remains unclear. The role of the coreceptors in T-cell activation is usually discussed in terms of their signaling function, which is based on their association with $p56^{lck}$ tyrosine kinase and interaction with the conserved epitopes of MHC molecules. The first function is important for the amplification of the activation signal via TCR, while the second one stabilizes the TCR interaction with MHC molecules and leads to the formation of the immunological synapse. The signaling function of the coreceptors seems to play no significant role in our experimental system used to assess the activation of T cells. We observed that truncated CD4, whose cytoplasmic domain is devoid of the binding site for $p56^{lck}$ tyrosine kinase, remains functional when expressed in malignant 4G4 thymoma cells, although the association of this kinase with the coreceptors is critical for activation of normal T cells [15, 16]. Hence, we cannot determine the extent of the domination due to a competition between CD4 and CD8 for $p56^{lck}$ binding in view of the 18-fold difference in their efficiency [17]. Yet the role of competitive physical interactions of the coreceptors with MHC molecules during the formation of the immune synapsis seems most likely.

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