

Altered Proliferative Response by T Lymphocytes of Ly-6A (Sca-1) Null Mice

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Summary

Ly-6A is a murine antigen which is implicated in lymphocyte activation and may be involved in activation of hematopoietic stem cells. Antibody cross-linking studies and antisense experiments have suggested that Ly-6A is a lymphocyte coactivation molecule. To better understand the function of Ly-6A, we used gene targeting to produce Ly-6A null mice which are healthy and have normal numbers and percentages of hematopoietic lineages. However, T lymphocytes from Ly-6A-deficient animals proliferate at a significantly higher rate in response to antigens and mitogens than wild-type littermates. In addition, Ly-6A mutant splenocytes generate more cytotoxic T lymphocytes compared to wild-type splenocytes when cocultured with alloantigen. This enhanced proliferation is not due to alterations in kinetics of response, sensitivity to stimulant concentration, or cytokine production by the T cell population, and is manifest in both in vivo and in vitro T cell responses. Moreover, T cells from Ly-6A-deficient animals exhibit a prolonged proliferative response to antigen stimulation, thereby suggesting that Ly-6A acts to downmodulate lymphocyte responses.

Ly-6A (a.k.a., TAP or Sca-1) is a glycosyl phosphatidylinositol (GPI)¹-anchored molecule (1–3) expressed on most peripheral lymphocytes, thymocytes, and hematopoietic precursors including stem cells, as well as on nonhematopoietic fibroblasts, kidney epithelial cells, and osteoblasts from the bone marrow (4–10). In the peripheral lymphoid organs, Ly-6A expression is upregulated on activated lymphocytes (4). Although a ligand of Ly-6A has not yet been determined, cross-linking Ly-6A by mAbs activates T and B lymphocytes in the presence of appropriate secondary signals. For example, Ly-6A-specific mAbs induce B cells to proliferate in the presence of IFN- γ and IL-4 (11). Cross-linking Ly-6A molecules on T cells leads to an influx of intracellular calcium and IL-2 production in the presence of accessory cells. IL-2 production leads to an upregulation of IL-2R expression and subsequent proliferation via an IL-2-driven autocrine pathway (12, 13). Cross-linking of Ly-6A can also activate T cells to proliferate in the presence of PMA (14). Several studies suggest that T cell activation by Ly-6A-specific antibodies is directly interrelated with the TCR signaling pathway. When Ly-6A expression is either down-

regulated by antisense DNA (15, 16) or ablated by mutation (17), T cell lines cannot be activated via the TCR. Correlatively, loss of TCR expression leads to an inability to activate T cells by anti-Ly-6A crosslinking (18, 19). In addition, downregulation of Ly-6A expression by antisense also results in downregulation of TCR β chain transcription and p59^{lck} activity (16). In contrast, costimulation of T cells with anti-Ly-6A and anti-CD3 cross-linking can induce downregulation of IL-2 production (20–22). Thus, the role of Ly-6A in T lymphocyte activation is complex and unclear.

The likelihood that Ly-6A plays a critical role in thymocyte differentiation is suggested by its regulated expression during thymocyte development. Ly-6A is expressed on bone marrow-derived prothymocytes which seed the thymic cortex and are phenotypically differentiated from hematopoietic stem cells by Sca-2 expression (23, 24), but expression is turned off at an early stage of CD3⁺CD4⁺CD8⁺ thymocyte differentiation (5, 25). Ly-6A is reexpressed by mature single-positive medullary thymocytes and peripheral T cells (23, 25). When Bamezai et al. used a human CD2 enhancer-driven transgene to constitutively express Ly-6A at high levels during all stages of thymocyte development (26), thymocyte development was arrested at the CD3⁺CD4⁺CD8⁺ stage, the stage at which Ly-6A expression is normally terminated. However, despite the expression analysis and evidence for a

¹Abbreviations used in this paper: Con A, Concanavalin A; ES, embryonic stem; GANC, gancyclovir; GPI, glycosyl phosphatidylinositol; SSLP, simple sequence length polymorphism.

functional role in lymphocyte activation, the biological role of Ly-6A is largely unknown.

To better understand the role of Ly-6A in hematopoietic development and lymphocyte activation, we have employed the strategy of gene targeting in ES (embryonic stem) cells to produce mice lacking Ly-6A expression. Ly-6A null mice are apparently normal and contain all hematopoietic lineages. Although the response by thymocytes to Concanavalin A (Con A) stimulation is not significantly altered between wild-type and mutant littermates, the response by peripheral T cells to antigens and mitogens which act through the TCR is significantly different. In contrast to published Ly-6A antisense experiments, including those from our laboratory, splenic T cells derived from Ly-6A^{-/-} mice proliferate more vigorously to antigen and mitogens than wild-type littermates. Ly-6A mutant splenocytes proliferate at significantly higher levels to stimulation with Con A, allogenic antigen, and anti-CD3 mAb, but not when stimulated with PMA plus ionomycin when compared to wild-type splenocytes. Furthermore, T cells from mutant mice challenged in vivo with KLH antigen proliferate at significantly higher levels in response to rechallenge with KLH in vitro compared to T cells from similarly challenged wild-type littermates. In contrast, antibody levels to KLH in primed Ly-6A mutant mice are significantly lower than antibody levels to KLH in KLH-primed wild-type littermates.

Materials and Methods

Construction of Targeting Plasmid. The p193⁺ plasmid containing a 4.5-kb EcoRI fragment encoding the *Ly-6A.2* chromosomal gene has been described previously (27). The 1.7-kb fragment containing exons 1–3 was removed using methylation-sensitive BclI after cycling the plasmid through the dam⁻ dcm⁻ GM2163 *Escherichia coli* strain (*E. coli*; Genetic Stock Center, Department of Biology, Yale University, New Haven, CT). Oligonucleotide adapters (XSB-L1, 5'-TACACCCTCCCTTCATAGGAGCT-3'; XSB-U, 5'-CCTATGAAGGGAGGGTGTACTAG-3') were used to anneal XhoI/SalI and BclI ends. XSB-L1 was kinased, annealed to XSB-U, and ligated to the BclI digested p193⁺. To construct the p193neo⁺ plasmid the 1.1-kb XhoI/SalI fragment of pMC1neo (Stratagene, La Jolla, CA), containing the neo^R gene driven by the HSV-tk promoter and a polyoma enhancer, was inserted into the adapter-generated XhoI/SalI overhang of p193⁺. In p193neo⁺ the neo^R transcriptional orientation is the same direction as *Ly-6A*. The HSV-tk cassette, obtained from Dr. M. Capecchi (Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT; reference 28), was inserted into the SalI site in the multiple cloning site of p193neo⁺ to generate the targeting plasmid pLy6ASDI-1 (Fig. 1).

Production of Targeted ES cells. The 129/Ola ES cell line E14TG2a (29) was cultured on irradiated (3000R) primary embryonic fibroblast feeder layers in DMEM supplemented with 15% (vol/vol) fetal bovine serum (lot tested) and 0.1 mM 2-mercaptoethanol. The fibroblast feeder cells were derived from d14.5 (129/Ola × C57Bl/6J)F₂ embryos carrying a copy of a neo^R gene in the β₂-microglobulin locus (30) and were able to grow in media containing G418. Rapidly growing cells were trypsinized and washed in ES media. Approximately 2 × 10⁷ cells were re-

suspended in 0.4 ml of ES media and incubated for 10 min on ice with linearized pLy6ASDI-1 at a concentration of 3 nM. Cells were resuspended again immediately before electroporations. Electroporations were carried out using an electro cell manipulator (600; BTX, Inc., San Diego, CA) at 160 V, 50 μF, and 360 Ω. Fresh media were added to electroporated samples which were allowed to recover on ice for 10 min. After electroporation, ES cells were plated onto feeder layers in 100-mm tissue culture dishes. After 24 h, the media were replaced with media containing 200 μg/ml G418 (Sigma Chemical Co., St. Louis, MO) in one dish or media containing 200 μg/ml G418 and 1 μM gancyclovir (GANC) (a gift from Syntex, Palo Alto, CA) in the remaining dishes. After 10–14 d, colonies were picked and transferred to individual wells in 24-well plates seeded with feeder cells.

PCR Screening and Southern Blot Analysis. After 48 h, colonies growing in 24-well plates were trypsinized and half the cells were removed for PCR analysis. ES cells were pelleted and resuspended in 50 μl lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 9, 2 mM MgCl₂, 0.45% Triton X-100, 0.35% Tween 20). The samples were boiled for 10 min, cooled, digested with 250 μg/ml proteinase K for 1 h at 37°C, then boiled for 10 min to inactivate proteinase K. The forward primer, NeoF (5'-ATGGCCTTCT-TGACGAGTTCTTCTG-3'), is specific for the 3' end of the pMC1neo gene of the targeting construct and the reverse primer, Ly6Aonpa (5'-GGGAGAACAAGGGTTTATTGGAC-3'), is specific for the 3' end of the *Ly-6A.2* gene and is not contained in the targeting construct (Fig. 1 A). PCR amplifications were carried out using 10 μl of lysates with 50 pmol of each primer and 2 U *Taq* polymerase (GIBCO BRL, Gaithersburg, MD) in standard PCR buffer and standard amplification programs in a Cetus thermocycler. An aliquot of each PCR reaction was fractionated on agarose gels. All PCR screening experiments used E14TG2a cell lysates as a negative control, and all cell lysates were tested for β-actin amplification to check for the quality of DNA template.

PCR-positive clones were transferred and expanded on feeder layers in 60-mm and later in 100-mm tissue culture dishes. Southern blot analysis was used to confirm PCR-positive clones and determine the genotype of mice. The procedure for genomic DNA isolation was adapted from Miller et al. (31). DNA was digested with either EcoRV, BamHI, BglII, or HindIII, fractionated on 0.8% agarose gels (1 × TAE), transferred to Hybond-N nylon membrane (Amersham, Arlington Heights, IL), and hybridized with neo^R (probe B) or *Ly-6A* probes (probes G or H). Probes G and H were generated by PCR amplification of DNA from an *Ly-6A.2* genomic γ phage clone and subsequent cloning into the pCRII plasmid (Invitrogen, San Diego, CA). The forward primer, 5'-GCACTGTGTGGACCATGGTT-3', and the reverse primer, 5'-GTATAGGCAGGATCATCACGTG-3', were used to amplify the 199-bp probe G. The forward primer, 5'-GCTATCACCATCCACACATAAG-3', and the reverse primer, 5'-GGAGAGGTAATGTGGGTGGCT-3', were used to amplify the 195-bp probe H. Probes were prepared using the random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Blots were prehybridized and hybridized at 68°C in Quikhyb (Stratagene, Inc., La Jolla, CA) for 20 min and 1 h, respectively. Blots were washed twice (15 min each) in 2 × SSC and 0.1% SDS at 65°C, then once for 15 min in 0.2 × SSC and 0.1% SDS at 65°C. Blots were exposed to XAR-2 autoradiographic film (Eastman Kodak Co., Rochester, NY) at -80°C with intensifying screens.

Generation of Mutant Mice. C57Bl/6J blastocysts were obtained from superovulated females. Uteri were flushed with M2 medium

(32) at day 3.5 after conception. Blastocysts were collected and placed in a droplet of M2 medium under paraffin oil. ES cells were trypsinized, washed once with fresh DMEM-H, and diluted to 2×10^6 cells/ml. Cells (5 μ l) were added to the droplet containing the blastocysts. Between 8 and 12 cells were injected into the inner cell mass of the blastocysts. After injection, six to nine blastocysts were implanted into each uterine horn of pseudopregnant females B6D2 which had been mated 2.5 d before with vasectomized males. Chimeric progeny were identified by coat color and were bred with C57Bl/6J mice. F₁ mice were screened for germ-line transmission of the ES cell line by the presence of agouti coat color. Agouti-positive F₁ mice were genotyped by Southern blot analysis as described above from tail biopsies. Mice heterozygous for the Ly-6A mutation were crossed with heterozygous siblings. F₂ mice were similarly genotyped.

Simple Sequence Length Polymorphism (SSLP) Analysis and Mapping Linked Mutation. PCR primers for SSLP markers *D15Mit3*, *D15Mit13*, *D15Mit14*, *D15Mit17*, *D15Mit29*, *D15Mit33*, *D15Mit34*, *D15Mit35*, *D15Mit39*, and *D15Nds1* were obtained from Research Genetics, Inc. (Huntsville, AL). The distances between loci cited in this manuscript are from the latest maps by National Institutes of Health (Bethesda, MD) and Massachusetts Institute of Technology (reference 33 and www.wi.mit.edu), respectively. PCR was performed using 25 ng tail DNA and 300 pM primer sets in 25 μ l reactions with Boehringer Mannheim *Taq* polymerase and buffers. Using 129 and C57Bl/6 wild-type DNAs, it was determined that all but *D15Mit34* and *D15Mit39* are polymorphic between 129 and C57Bl/6. PCRs were performed on tail DNAs from all mice which were related to viable Ly-6A null mice and ~25% of the mice which did not generate Ly-6A null mice. The consensus difference between lines of mice was the presence of C57Bl/6 *D15Mit33* marker in the mice which could generate Ly-6A null mice.

Antibodies and Flow Cytometry. The live cells were separated from dead cells and RBCs by Ficoll isolation (density 1.077g/ml; Organon Teknika, Durham, NC) using standard procedures. Viable cells were washed twice with RPMI-1640 medium with 5% FCS, followed by two washes in PBS with 2% FCS, and resuspended in supernatant containing anti-FcR mAb (2.4 G2) (34) and incubated at 4°C for 30 min to block FcR and thus prevent nonspecific binding of antibodies. Cells were washed twice in PBS with 2% FCS and incubated with antibodies at optimally determined concentrations at 4°C for 30 min. Cells were then washed three times in PBS with 2% FCS and either resuspended in secondary antibodies or in 1% paraformaldehyde in PBS. Purified anti-Ly-6A.2 (SRT7) was purchased from Serotec Ltd. (Kidlington, Oxford, UK). FITC- and PE-conjugated streptavidin and biotinylated FITC- and PE-conjugated antibodies against isotype controls (rat IgG), mouse IgG_{2a/2b} (R2-40), mouse Ig (polyclonal), B220 (RA3-6B2), CD3 ϵ (145-2C11), CD4 (RM4-5), CD8 (53-6.7), Ly-6A (D7 and E13-161.7), Ly-6C (AL-21), Ly-6G (RB6-8C5), MAC-1 (M1/70), SCA-2 (MTS 35), TCR- α/β (H57-597), TCR- γ/δ (GL3), and ThB (49-H4) were purchased from PharMingen (San Diego, CA). Flow cytometry was performed using a FACScan® (Becton Dickinson, San Jose, CA) and Lysis II analysis software.

In Vitro Colony Assay. The final concentrations of methylcellulose media are as follows: 1.5% methylcellulose (Sigma Chemical Co.), 15% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 1% BSA (Sigma Chemical Co.), 9 μ M monothioglycerol (Sigma Chemical Co.), 50 ng/ml rmlL-3 (R & D Systems, Inc., Minneapolis, MN), 5 U/ml rmEPO (Boehringer Mannheim), and 10 ng/ml rmGM-CSF (Genzyme Corp., Cambridge, MA) in

IMDM. Multiple aliquots of single cell suspensions were counted using a hemacytometer to insure identical amounts of input cells. Premixed methylcellulose medium was added to the cells and transferred to 30 \times 15 mm dishes (Nunc, Inc., Naperville, IL), and grown at 5–6% CO₂, 37°C. Colonies were counted between days 8 and 11 after the initiation of cultures. Cells were stained for hemoglobin expression with 0.1% benzidine (Sigma Chemical Co.) and plates were recounted to confirm erythroid and mixed colonies.

Lymphocyte Activation Assays. In all experiments, sex-matched wild-type littermates were used as controls. Multiple aliquots of single cell suspensions were counted using a hemacytometer to insure identical amounts of input cells. Some experiments used whole splenocyte preparations; however, most experiments used enriched splenic T cell preparations or lymph node cells. Splenic T cells were prepared for proliferation assays by Ficoll isolation followed by B cell depletion using T Collect columns (BioTex Laboratories, Edmonton, Canada). The enriched T cells were plated in triplicate wells in 96-well plates at 2×10^5 cells/well at various concentrations of ConA (0.25–2.0 μ g/ml; see figure legends) or 1.0 μ g/ml of PMA and 10 mg/ml of ionomycin in RPMI-1640 media and 10% FCS (Dutchland Laboratories, Denver, PA) at 37°C, 5% CO₂ for 48 h. Cell proliferation was assayed by the addition of 1.0 μ Ci of [³H]thymidine (ICN Pharmaceuticals, Costa Mesa, CA) during the last 18 h of culture. Cells were then harvested and measured for radioactivity in liquid scintillation cocktail. Ficoll separated splenocytes were incubated (in triplicate wells) in 50 μ g/ml LPS in RPMI-1640 media and 10% FCS at 37°C, 5% CO₂ for 48 h, and cell proliferation was measured as described above. To measure activation of T cells to antibodies, enriched T cells were incubated in supernatant from either C363.29B (anti-CD3; reference 35) or D7 (anti-Ly-6A; reference 12) hybridomas at 10^6 cells/ml at 4°C for 1 h. The cells were washed and plated in triplicate wells in 96-well plates at 2×10^5 cells/well in 0.1 ml RPMI-1640 media and 10% FCS. Then, 0.1 ml of goat anti-rat serum (diluted in media) was added to the cells and incubated for 72 h (unless otherwise indicated), and cell proliferation was measured as described above. Enriched T cells (2×10^5 /well) were stimulated (in triplicate wells) with irradiated (2000R) CBA (H-2^k) and DBA (H-2^d) splenocytes for 5 d at a 2:1 responder/stimulator ratio for MLR assays, and cell proliferation was measured as described above.

C3H (H-2^k), Ly-6A^{+/+} (H-2^b), and Ly-6A^{-/-} (H-2^b) Ficoll-separated splenocytes were used as both stimulator and responder cells in CTL assays. Nine CTL cultures were established by culturing each group of responder cells with each group of stimulator cells. CTL cultures contained 2×10^6 cells/well in 24-well plates (6 wells/culture) with irradiated (2000R) stimulators at a 2:1 responder/stimulator ratio. After 5 d, the cells were harvested and tested at various effector/target ratios for cytotoxic activity in standard 4-h ⁵¹Cr-release assays using both 6130 (H-2^k) and EL-4 (H-2^b) tumor cells as targets.

Priming and Response to KLH. 0.1 ml of 1 mg/ml KLH in Freund's adjuvant was injected into each footpad of each mouse. Primary immunizations were performed on day 0 in complete Freund's adjuvant, while secondary and tertiary immunizations were performed on days 10 and 20, respectively, in incomplete Freund's adjuvant. Draining lymph nodes were removed on day 24. Single cell suspensions were prepared, washed, and plated in triplicate wells in 96-well plates at 10^5 cells/well in RPMI-1640 media, 10% FCS, and 1 μ g/ml ConA or 50 μ g/ml LPS, or various concentrations of KLH (100, 50, or 10 μ g/ml). Mice were bled on day 20 and relative serum concentrations of anti-KLH

antibodies were determined by ELISA analysis. ELISA plates were coated with 500 ng (50 μ l) of KLH antigen at 37°C for 75 min. 150 μ l of 2% BSA in PBS was added and stored overnight at 4°C. The plates were washed three times with 50 mM Tris, pH 7.5, 0.2% Tween 20 (wash buffer). 50 μ l of serum (in triplicate wells) was then incubated in plates for 2 h at 37°C. The plates were then washed three times, and 100 μ l of biotinylated anti-mouse Ig was added to wells for 1 h at 37°C. The plates were then washed three times, and 100 μ l of horseradish peroxidase conjugated-streptavidin (1:8,000 dilution in 2% BSA; Zymed Laboratories, Inc., South San Francisco, CA) was added to wells for 30 min at room temperature. The plates were then washed three times, and 100 μ l of tetra-medhyl-benzadione substrate (DAKO Corp., Carpinteria, CA) was added and incubated (in the dark) for 30 min at room temperature. The reactions were stopped with 100 μ l of 0.18 M H₂SO₄. ELISA plates were read using an ELISA plate reader.

Results

Gene Targeting of Ly-6A and the Generation of Ly-6A Mutant Mice. The Ly-6A gene is comprised of four exons, with the sequence encoding the mature protein beginning in the third exon (27). A targeting plasmid was constructed by replacing exons 1–3 with the neo^R gene in the 5'–3' orientation (Fig. 1 A). The targeting construct did not contain a polyadenylation site; which meant that cells containing the targeting construct would not express the neo^R gene unless the targeting construct integrated 5' of a usable polyadenylation site. Using this strategy, fewer random integration events should be G418^R, thereby enriching for homologous recombinants among the G418^R colonies. In addition, the HSV-tk cassette (28) was inserted outside the homologous sequence of the targeting vector to generate a positive/negative selection targeting plasmid, pLy6ASDI-1 (Fig. 1). After electroporation with pLy6ASDI-1, E14TG2a ES cells were selected with G418 and GANC to enrich for homologous recombinants. In each experiment, between 20 and 100 colonies survived, an ~10-fold decrease in total colonies compared to electroporated cells selected with G418 alone. G418^R, GANC^R colonies were analyzed for homologous recombinants by a PCR-based assay (36). From seven electroporation experiments, five colonies were PCR-positive for homologous recombination. The positive clones were analyzed by Southern blots to verify Ly-6A gene targeting and determine whether there were any anomalous rearrangements. These are considerable concerns because Ly-6A is a member of a large gene family (18 genes encoded by a single locus on murine chromosome 15; (reference 37) and it is conceivable that other members of the gene family could be targeted by this construct. Three clones contained the expected RFLP patterns (data not shown and Fig. 1 B and C). Southern analysis of EcoRV digested DNA from a litter derived from heterozygous matings is depicted in Fig. 1 B and C. The endogenous Ly-6A gene is 5.2 kb and the mutated allele is 4.5 kb. Probes derived upstream and downstream as well as a neo^R gene probe were used to determine that the recombination event occurred as expected. The clones were karyotyped

and found to contain the normal 40 chromosomes. Subsequently, all three clones were injected into C57Bl/6J blastocysts and reimplanted into pseudopregnant females. Chimeras were backcrossed with C57Bl/6J and germ-line transmission was determined in the F₁ litters. Only clone 106-60 produced germ-line transmission. Approximately 43% of the agouti F₁ mice were heterozygous for the Ly-6A targeting event.

Originally we did not detect any Ly-6A homozygous mutant mice born from heterozygous matings and we pursued the possibility that Ly-6A null mutation results in embryonic lethality. In fact, Ly-6A protein is expressed during preimplantation and Ly-6A null embryos died between embryonic days 3.5 and 6.5 (data not shown). However,

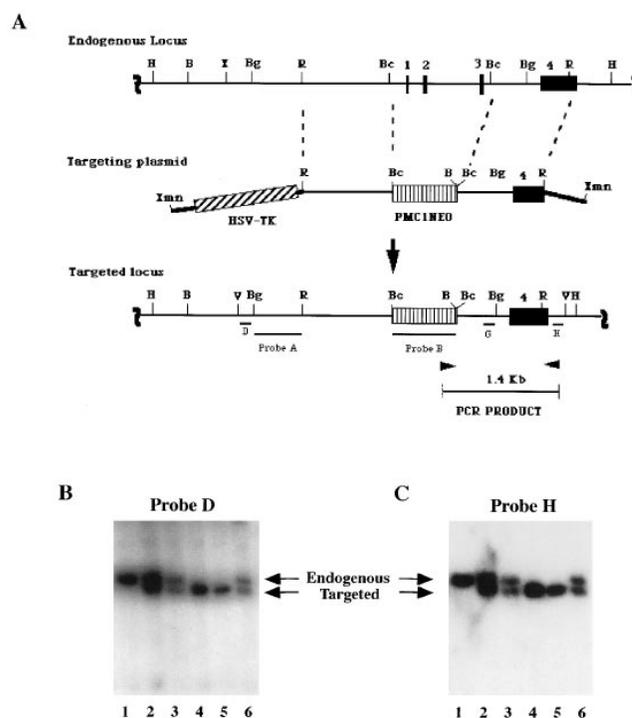


Figure 1. Gene-targeting of Ly-6A.2. (A) Strategy of Ly-6A gene targeting. A restriction map of the Ly-6A germ-line locus, the targeting construct, and the targeted locus are depicted. The four exons of Ly-6A are boxed and labeled. The Ly-6A targeting construct (pLy6ASDI-1) was prepared by cloning the 4.5-kb EcoRI fragment encoding the Ly-6A.2 gene into the EcoRI site of pBluescript. The BclI fragment encoding exons 1–3 was excised and replaced with the pMC1neo gene. The HSV-tk gene was cloned into the Sall site of the multiple cloning site of the targeting construct. Probes D, G, and H are PCR fragments using generated PCR primers corresponding to sequences upstream or downstream from the targeting construct, and probe B is the pMC1neo insert. The restriction enzyme sites are designated as follows: BamHI (B), BclI (Bc), BglII (Bg), EcoRI (R), EcoRV (V), HindIII (H), XbaI (X), and XmnI (Xmn). (B and C) Detection of targeted and endogenous Ly-6A alleles by genomic Southern blot analysis of EcoRV-digested DNA from a litter from a heterozygous cross using the 5' probe D (B) and the 3' probe H (C). The replacement of the 1.8-kb fragment encoding exons 1–3 with the Neo^R reduces the size of the endogenous EcoRV band by 700 bp. The blot was hybridized with probe H, stripped, and reprobed with probe D. The endogenous (5.2-kb) and targeted (4.5-kb) bands are marked with arrows.

eventually pups sired by multiple founders were born which when bred gave rise to viable $Ly-6A^{-/-}$ mice. To determine the genetic basis differences between the mice which gave rise to the homozygous mice versus the ones which did not give rise to viable homozygous embryos, analysis of chromosome 15 was employed (38). SSLP analysis demonstrated that the difference between the two lines of mice was that all mice which were able to give rise to viable null pups contained C57Bl/6 markers (*D15Mit33*) ~0.1 cM distal to the *Ly-6* locus (33) instead of 129 markers, suggesting that a crossover event occurred early in the line which restored function of a gene (or genes) distal to the *Ly-6* locus. All breeders which did not give rise to null mice contained the 129 *D15Mit33* marker distal to the targeted *Ly-6A* allele, suggesting that these mice have a lethal mutation distal to the *Ly-6* locus. The linked lethal mutation was further mapped proximal to *D15Mit14*, leaving ~20 cM in which the lethal mutation could have occurred. Additional evidence which suggests that the linked lethal mutation did not occur in the *Ly-6* locus is that the use of multiple probes which hybridize to all *Ly-6* gene family members did not show any RFLP differences between wild-type DNA or the 106-60 targeted ES cell line with the exception of the *Ly-6A* targeted locus (data not shown). All the breeders were tested for the C57Bl/6 *D15Mit33* marker, and the *Ly-6A* mouse line was derived from these mice which are currently at C57Bl/6 backcross eight. Genotypes of these mice do not deviate from the expected Mendelian frequencies.

Homozygous animals had no apparent health problems, including breeding, in a pathogen-free animal facility. Histological analysis was performed on tissues known to express *Ly-6A* in wild-type animals. Examination of hematoxylin and eosin stained sections of femur, kidney, liver, lymph node, spleen, and thymus revealed no obvious differences between homozygous mutant and wild-type littermates.

Analysis of Lymphoid and Myeloid Subpopulations. Flow cytometry was used to verify the absence of *Ly-6A* expression in homozygous mutant mice (Fig. 2). Approximately 60% of wild-type splenocytes express *Ly-6A*. Splenocytes from heterozygous animals show a slight decrease in *Ly-6A* expression intensity; however, splenocytes from homozy-

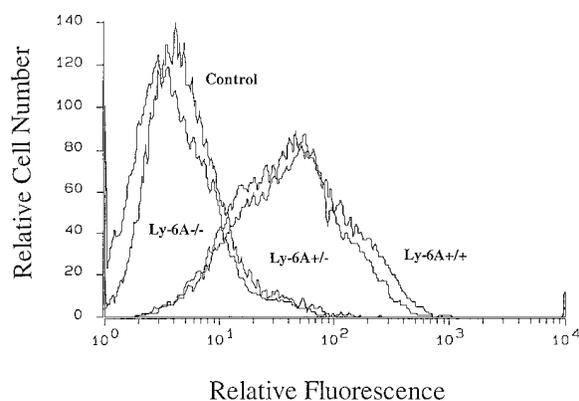


Figure 2. Absence of cell surface *Ly-6A* expression in $Ly-6A^{-/-}$ mice. Splenocytes were stained with the PE-conjugated anti-*Ly-6A* antibody D7 (12). Stainings of wild-type, heterozygous, and homozygous mutant splenocytes from littermates were determined. No expression of *Ly-6A* on mutant lymphocytes was detected using two other anti-*Ly-6A* antibodies (data not shown). 16,000 gated events were collected with the FACScan®. Negative control staining was determined with PE-conjugated anti-rat IgG.

gotes do not stain with any of three antibodies to *Ly-6A* (Fig. 2). In addition, phenotypic analysis was performed to determine if the absence of *Ly-6A* expression in homozygous mutant mice altered the differentiation of various cell populations. Although there are minor variations between littermates, as a population *Ly-6A* null animals (as old as 8 mo) contain normal percentages of B220, TCR- α/β , TCR- γ/δ , CD3, CD4, CD8, Mac-1, and *Ly-6G* (Gr-1) positive cells in the bone marrow, lymph nodes, spleen, and thymus (data not shown). In addition to *Ly-6G*, antibodies to other members of the *Ly-6* gene family were used to determine if the lack of phenotypic changes is due to compensation by other family members. *Ly-6C*, Sca-2 (TSA-1), and ThB do not appear to be overexpressed in any hematopoietic tissues (data not shown).

To analyze myeloid precursors, the *in vitro* colony-forming potential of bone marrow cells from $Ly-6A^{-/-}$ was compared to that of wild-type littermate bone marrow. Table 1 displays the combined total number of colonies from three experiments. As expected from the FACS® analysis, the $Ly-6A^{-/-}$ bone marrow contained a normal

Table 1. Colony Formation

Strain	Cell type*							Total
	Mac	Gran	Meg	Ery	GM	Mix		
$Ly-6^{-/-}$	926 [‡] (56.5 [§])	121 (7.4)	14 (0.1)	174 (10.6)	337 (20.6)	69 (4.2)		1641
$Ly-6^{+/+}$	997 (57.4)	167 (9.6)	15 (0.1)	178 (10.3)	292 (16.8)	88 (5.1)		1737

*Mac CFU-macrophage; *Gran*, CFU granulocyte; *Meg*, CFU-megakaryocyte; *Ery*, CFU-erythroid; *GM*, CFU-granulocyte/macrophage; *Mix*, CFU-granulocyte/macrophage/erythroid with or without megakaryocyte. [‡]Number of colonies from 750,000 input bone marrow cells; [§]Percentage of total colonies.

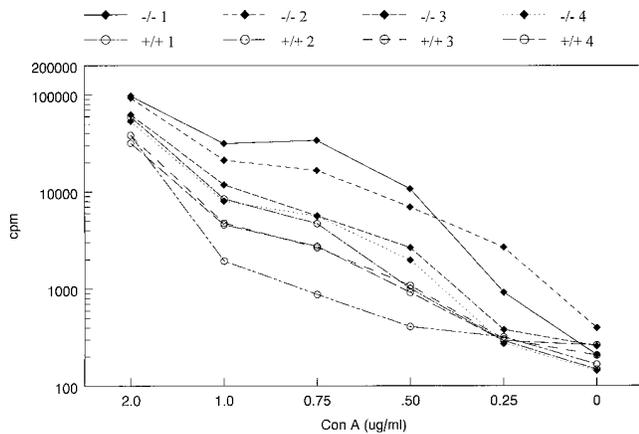


Figure 3. Ly-6A^{-/-} splenic T cells have an enhanced proliferative response to Con A in a blind assay. Splens from four wild-type and four Ly-6A null littermates were harvested and coded by one individual and a proliferation assay using various concentrations of Con A was performed by a second individual. 2×10^5 enriched T cells were added to 96-well plates (in triplicate wells) and incubated in RPMI-1640 with 10% FCS with various concentrations of Con A for 48 h. Incorporation of [³H]TdR was used to measure proliferation. As a population, Ly-6A^{-/-} splenic T cells proliferated at higher levels than T cells from wild-type littermates.

number and percentage of all CFU types. Although in each experiment the total number of colonies was lower, the total percentage of pure granulocyte colonies was lower, and the total percentage of CFU-granulocyte/macrophage colonies was higher in the Ly-6A^{-/-} cultures, none of these differences are statistically significant due to the large variation in CFUs within each group of mice.

Functional Analysis of Lymphocytes In Vitro. To determine whether the T lymphocytes from Ly-6A null animals are functionally normal, a Con A response experiment with two runs was performed in a blinded manner. For each run, the splens were removed and coated from four wild-type and four Ly-6A null littermates by one individual and given to a second individual to perform the experiment. B cell-depleted splenocytes were stimulated with various concentrations of Con A for a total of 48 h, with addition of 1 μ Ci tritiated thymidine deoxyribose ([³H]TdR) after 24 h, and triplicate cpm were obtained. Fig. 3 is a logarithmic plot of the [³H]TdR incorporation of T cells versus the concentration of Con A from one of the experimental runs. This experiment demonstrates that as a group the splenic T cells from Ly-6A null animals proliferate at a much higher rate in response to Con A stimulation than the T cells from wild-type littermates. For example, the average [³H]TdR incorporation of the T cells from Ly-6A null animals is 156% of the response of wild-type littermates when stimulated with 2.0 μ g/ml Con A ($P < 0.03$). However, Fig. 3 also demonstrates that there is a significant variation in response within each group of mice. Therefore, the results of both blind experiments were combined and independent statistical analysis performed for Con A levels between 1.0 and 0.5 μ g/ml. An ANOVA model was constructed to examine the separate effects of Con A con-

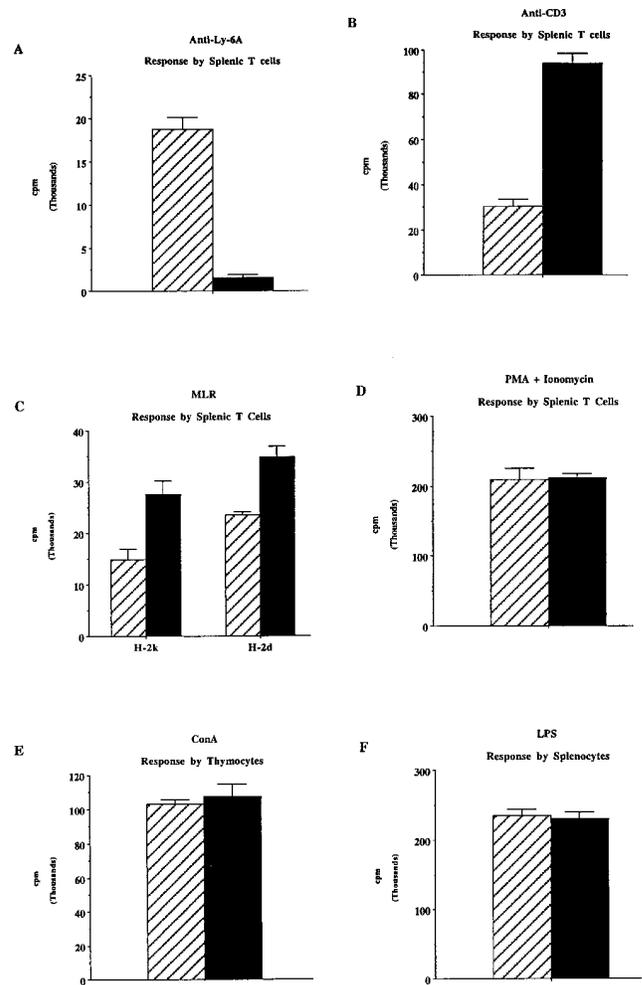


Figure 4. Analysis of proliferative response by lymphocytes of null (black columns) and wild-type (striped columns) littermates. Proliferation assays were performed using 2×10^5 lymphocytes (either whole splenocytes, splenic T cells, or thymocytes) plated in triplicate wells of a 96-well plate in incubated in RPMI-1640 with 10% FCS with various stimuli. Cell proliferation was measured by [³H]TdR incorporation. (A and B) Splenic T cells have an enhanced proliferative response to anti-CD3 (B) but not anti-Ly-6A (A). (A) The average cpm by wild-type T cells in response to anti-Ly-6A mAb differed from the Ly-6A^{-/-} T cells ($P < 0.01$). (B) The average cpm by wild-type T cells in response to anti-CD3 differed from the Ly-6A^{-/-} T cells ($P < 0.01$). (C) Ly-6A^{-/-} splenic T cells have an enhanced MLR proliferative response. Enriched T cells were stimulated with irradiated (2000R) CBA (H-2^k) and DBA (H-2^d) splenocytes for 5 d at a 2:1 responder/stimulator ratio for MLR assays. The average cpm by Ly-6A^{-/-} T cells was different from wild-type T cells for the anti-H-2^k response ($P < 0.01$) and for the anti-H-2^d response ($P < 0.02$). (D) There is no significant difference between Ly-6A^{-/-} splenic T cells and wild-type splenic T cells in response to PMA plus ionomycin. Enriched T cells were stimulated with PMA and ionomycin for 48 h. (E) Con A induced proliferation by Ly-6A^{-/-} and wild-type thymocytes. Thymocytes were stimulated with 2.0 μ g/ml Con A for 48 h. The average cpm by Ly-6A^{-/-} thymocytes compared to wild-type T cells is not statistically different ($P < 0.13$). (F) LPS-induced proliferation by Ly-6A^{-/-} and wild-type splenocytes. Splenocytes were stimulated with 100 μ g/ml LPS. The average difference in cpm by Ly-6A^{-/-} splenocytes compared to wild-type splenocytes is not significantly different ($P < 0.38$).

centration and genotype adjusted for the run and mouse effects, where mouse was nested within level and genotype. The effect of each factor was statistically significant. Of particular interest, Ly-6A null mice had higher incorporation of [³H]TdR ($P < 0.0001$) and a dose-response effect was found for Con A ($P < 0.0001$).

The response to cross-linked anti-Ly-6A and anti-CD3 by splenic T cells was also evaluated. A representative experiment is depicted in Fig. 4 A and B. As expected, T cells from Ly-6A null animals do not respond when exposed to anti-Ly-6A followed by goat anti-rat serum, whereas T cells from wild-type littermates show normal levels of proliferation (Fig. 4 A). In contrast, when cell surface CD3 was cross-linked by C363.29B mAb followed by goat anti-rat serum, Ly-6A null T cells showed a 211% increase ($P < 0.001$) in the level of proliferation when compared to T cells from wild-type littermates (Fig. 4 B). The average increase of [³H]TdR incorporation by the Ly-6A^{-/-} T cells compared to wild-type littermate T cells from four other experiments ranged from 61 to 619%.

T cells from Ly-6A deficient and wild-type littermates were also tested for their response to allogeneic antigen. A representative experiment is shown in Fig. 4 C, which demonstrates that splenic T cells from Ly-6A^{-/-} (H)2^b mice generated a significantly higher proliferative response than T cells from wild-type littermate mice. In the experiment shown, the response of Ly-6A^{-/-} T cells was 86% higher ($P < 0.01$) than the response of Ly-6A^{+/+} T cells when stimulated with irradiated spleen cells from CBA (H-2^k), and 48% higher ($P < 0.02$) when stimulated with irradiated cells from DBA (H)2^d mice. The average increase of [³H]TdR incorporation by the Ly-6A^{-/-} T cells compared to wild-type littermate T cells from other experiments ranged from 12 to 49% ($P < 0.02$) for anti-H-2^k response and from 46 to 79% ($P < 0.01$) for anti-H-2^d response in three independent experiments.

Unlike antigenic and ConA activation, which stimulate T cells through the TCR complex, the addition of PMA and ionomycin stimulates T cells by activating protein kinase C directly. PMA with ionomycin activates T cells from Ly-6A mutant and wild-type littermates at similar levels. The results from a representative experiment are displayed in Fig. 4 D. In other experiments, whole splenocytes were tested for response to the various stimuli and similar percentage differences between Ly-6A null and wild-type littermate splenocytes results were obtained compared to those results using enriched T cells, although the total [³H]TdR incorporation was lower than the results obtained with the enriched T cells (data not shown). In addition, Con A responses by heterozygous T cells were compared to those of wild-type and null littermates and found to be no different than wild-type responses (data not shown).

The stimulation of thymocytes in response to Con A was also examined. In contrast to stimulation of splenic T cells, stimulation of Ly-6A^{-/-} thymocytes did not show a statistically significant difference in their ability to respond to ConA when compared to thymocytes from wild-type littermates. In two experiments involving three mice in each

group, the Ly-6A^{-/-} thymocytes proliferated at a slightly higher but statistically insignificant rate when compared to thymocytes from wild-type littermates. The results shown in Fig. 4 E demonstrate that in the first experiment, the Con A response by Ly-6A^{-/-} thymocytes was an average 4% higher than wild-type thymocytes ($P < 0.13$), while in the second experiment Ly-6A^{-/-} thymocytes proliferated by an average of 16% greater than wild-type controls ($P < 0.28$). Neither experiment is statistically significant due to the wide variation of values for each group.

Splenocytes were treated with LPS to determine if there was a difference between Ly-6A^{-/-} and wild-type mitogen-induced B cell proliferation. The results in Fig. 4 F demonstrate that Ly-6A null splenocytes do not show a significant difference to LPS than splenocytes from wild-type littermates. Fig. 4 F shows the results of two independent experiments; the first shows a 2% decrease in proliferation for Ly-6A null splenocytes ($P < 0.38$) when independently testing three animals in each group, while the second experiment shows the Ly-6A^{-/-} splenocytes proliferated 6% more ($P < 0.08$) than wild-type littermate controls when four animals in each group were tested.

A kinetics experiment was performed to determine the rate of proliferation at three different time points by splenic T cells from 4-, 6-, and 8-mo-old Ly-6A mutant and wild-type littermates (three mice from each group). T cells were activated by cross-linking cell surface CD3 and their proliferation rates were measured at 48, 72, and 98 h by adding [³H]TdR to the cells 3 h before measuring [³H]TdR incorporation. Fig. 5 demonstrates that Ly-6A^{-/-} T cells proliferate

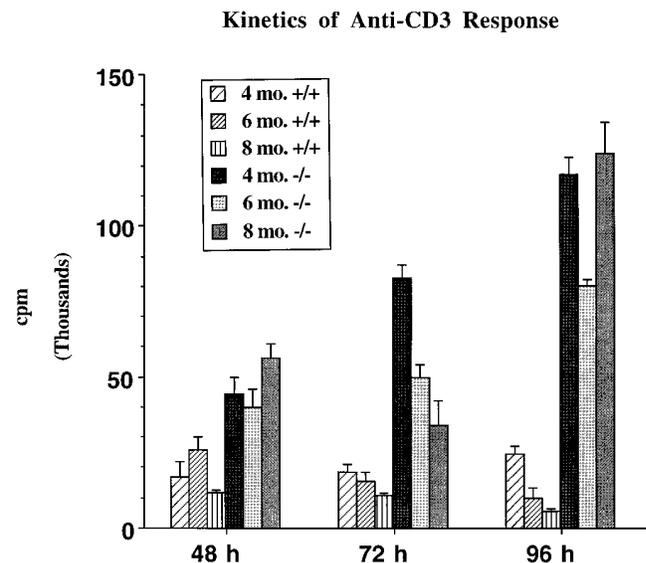


Figure 5. Ly-6A null T cells sustain their proliferative response longer than the wild-type T cells. Splenic T cells from 4-, 6-, and 8-mo old Ly-6A mutant and wild-type littermates were activated by cross-linking cell surface CD3 and their proliferation rates were measured at 48, 72, and 98 h by adding [³H]TdR to the cells 3 h before measuring [³H]TdR incorporation. Ly-6A^{-/-} T cells proliferate at higher rates than the age-matched wild-type T cells at all time points. The percent increase in [³H]TdR incorporation by mutant T cells over wild-type T cells was 159% at 48 h ($P < 0.01$), 272% at 72 h ($P < 0.06$), and 994% at 96h ($P < 0.01$).

erate at higher rates than the age-matched wild-type T cells at all time points. In fact, the Ly-6A null T cells appear to sustain the proliferative response longer than the wild-type T cells; in other words, the percent increase in [³H]TdR incorporation by mutant T cells over wild-type T cells was 159% at 48 h ($P < 0.01$), 272% at 72 h ($P < 0.06$), and 994% at 96 h ($P < 0.01$).

To determine if the enhanced proliferation activity of Ly-6A^{-/-} T cells was due to an upregulation of autocrine growth factor production, supernatants and cell lysates were harvested at 16, 24, 48, and 72 h time points during activation assays and used to determine IL-2, -4, and -6, TNF- α , and IFN- γ production by ELISA analysis. There were no consistent differences in cytokine production in T cell activation assays between splenocytes from Ly-6A mutant and wild-type littermates (data not shown). However, it is possible that differences in cytokine production were not detected because the cytokines were used as soon as they were produced. Therefore, RNA was isolated from Ly-6A null and wild-type T cells at various time points during activation assays and semiquantitative reverse transcription-PCR analysis on transcript levels was performed for the aforementioned cytokines. No differences in cytokine transcription between Ly-6A null and wild-type littermates were detected (data not shown).

In addition to measuring proliferation responses to antigen and mitogen, splenocytes from wild-type and Ly-6A mutant littermates were tested for their ability to mediate an allogenic CTL response. Splenocytes were incubated with irradiated C3H (H-2^k) and syngenic irradiated splenocytes as stimulators for 5 d. Splenocytes were harvested and counted. Consistent with the proliferation experiments, in each culture 5–11% more cells were recovered from the Ly-6A null cultures than the wild-type littermate cultures. Effector cells were tested for their ability to lyse ⁵¹Cr-loaded 6130 (H-2^k) and EL-4 (H-2^b) target cells at various effector/target ratios (Fig. 6). In all three experiments, Ly-6A null splenocytes efficiently lysed H-2^k targets at a slightly higher, but not statistically significant, rate than did wild-type splenocytes. Thus, although the total number of lytic units obtained from the stimulation cultures were higher for the Ly-6A^{-/-} splenocytes as compared to wild-type splenocytes, the lytic activity of these CTLs at a given effector/target cell ratio was unchanged. Neither wild-type nor Ly-6A mutant splenocytes lysed control H-2^b target cells. In addition, Ly-6A^{-/-} and wild-type littermate splenocytes were tested for activation of CTL response against either Ly-6A^{-/-} or wild-type irradiated splenocytes. Neither group of responders was activated by the syngenic stimulators, although C3H splenocytes were activated equally well by both Ly-6A^{-/-} and wild-type irradiated splenocytes to kill H-2^b target cells.

Functional Analysis of Lymphocytes In Vivo. Ly-6A null and wild-type littermates were challenged with KLH by three footpad injections of 0.1 mg KLH in Freund's adjuvant, and draining lymph nodes were harvested 4 d after the tertiary immunization. Lymph node cells were then activated in culture in the presence of LPS, ConA, or KLH, and

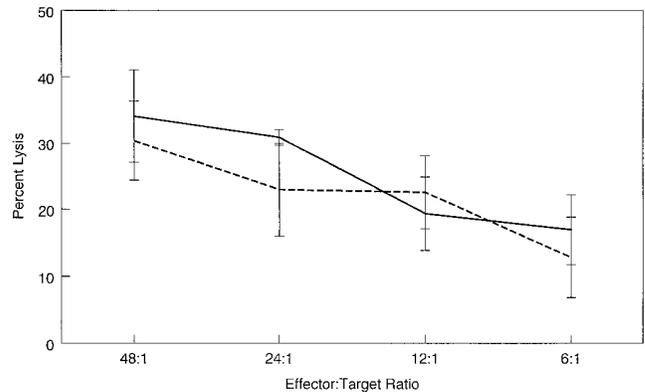


Figure 6. Cytotoxic T cell response by Ly-6A^{-/-} and wild-type splenocytes is not significantly different. In this representative experiment, effector cells were tested for their ability to lyse ⁵¹Cr-loaded 6130 (H-2^k) target cells at various effector/target ratios after stimulation with irradiated H-2^k splenocytes for 5 d. Although on average the lysis is slightly higher by Ly-6A^{-/-} (solid line) than wild-type (dashed line) effectors, these differences are not significantly different.

their proliferation rate measured by [³H]TdR incorporation. Ly-6A null cells proliferated at significantly higher rates to KLH and ConA than wild-type lymphocytes (Fig. 7 A). In fact, incorporation of [³H]TdR by Ly-6A^{-/-} cells was 150% higher at 1 μ g/ml KLH ($P < 0.03$), 168% higher at 5 μ g/ml KLH ($P < 0.02$), 191% higher at 10 μ g/ml KLH ($P < 0.01$), and 302% higher at 1 μ g/ml ConA ($P < 0.01$). Lymph node cells isolated from animals immunized with Freund's adjuvant alone did not respond to KLH antigen in vitro (data not shown). In contrast, LPS activation of cells did not demonstrate any significant differences ($P < 0.11$) in B cell proliferation between KLH immunized Ly-6A null and wild-type littermates (Fig. 7 A). However, the anti-KLH antibody response was significantly lower in Ly-6A null animals in comparison to wild-type animals (Fig. 7 B). Fig. 7 B summarizes the results from ELISA analysis of serum from 10 d post-secondary immunized littermates (four in each group) against four dilutions of KLH antigen. At all dilutions of KLH antigen, the ELISA absorbance was lower by the serum of the Ly-6A null mice than the wild-type littermates (ranging from 38 to 99% lower; $P < 0.03$).

Discussion

Our approach to determine whether Ly-6A protein expression was necessary for normal hematopoietic development or T cell activation was to produce Ly-6A null animals by gene targeting. Flow analysis demonstrated that all normal percentages of hematopoietic lineages were represented in the bone marrow, spleen, lymph node, and thymus, thereby demonstrating that Ly-6A was not necessary for normal hematopoietic development. In addition, colony forming assays were performed on bone marrow from Ly-6A null and wild-type littermates. In all three experiments, the total number of colonies was lower, the total percentage of pure granulocyte colonies was lower, and the

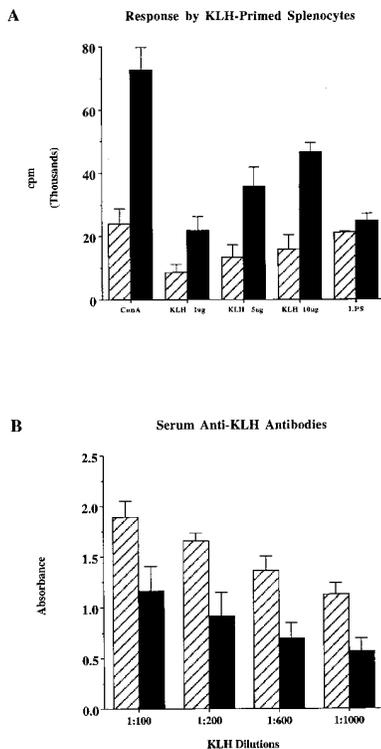


Figure 7. In vivo antigen responses are altered in Ly-6A^{-/-} mice. Ly-6A^{-/-} (black columns) and wild-type (striped columns) littermates were primed with KLH antigen at days 0, 10, and 20. Mice were bled at day 20 for analysis of serum anti-KLH antibodies levels (B) and killed at day 24 to measure proliferative responses by draining lymph node cells (A). (A) Ly-6A^{-/-} draining lymph node cells proliferate at significantly higher levels to Con A and various concentrations of KLH than wild-type littermates ($P < 0.01$ and $P < 0.03$, respectively) but show no significant differences in LPS response ($P < 0.11$). (B) Serum concentration of anti-KLH antibodies is lower in Ly-6A^{-/-} mice compared to wild-type littermates measured by absorption of antibodies to ELISA plates coated with various concentrations of KLH antigen ($P < 0.03$).

total percentage of CFU-GM colonies was higher in the Ly-6A^{-/-} cultures; however, none of these differences are statistically significant due to the large variation in CFUs within each group of mice. Proliferation assays were performed using a variety of mitogens and antigens and Ly-6A null splenic T cells proliferated at statistically higher levels compared to T cells of wild-type littermate controls in all cases except PMA plus ionomycin, which does not activate through the TCR complex. These results suggest that Ly-6A exerts its effects when T cells are signaled through the TCR complex. CTL killing activity was found to be similar at various effector/target ratios between Ly-6A null and wild-type splenocytes, although CTL cultures produced greater numbers of CTLs from Ly-6A null mice compared to wild-type littermates, which parallels the results of the MLR proliferation experiments. The enhanced proliferation is regulated because Ly-6A^{-/-} splenocytes did not respond with increased proliferation or CTL generation when stimulated with self-antigen. Kinetics experiments determined that the Ly-6A null T cells sustain their proliferation longer than T cells from wild-type littermates. In

contrast, when thymocytes were tested for mitogen-induced proliferation, Ly-6A mutant thymocytes incorporated between 4 and 16% more [³H]TdR in response to Con A stimulation than thymocytes from littermate controls in two experiments, and these differences are not statistically significant due to the wide variation of values for each group.

Perhaps more importantly, in vivo T cell responses recapitulate the in vitro proliferation data. Lymphocytes were primed in vivo by footpad injections of KLH antigen into Ly-6A null and wild-type littermates. Lymphocytes were harvested and tested in vitro for proliferation to KLH, Con A, or LPS. Ly-6A mutant lymphocytes demonstrated significant increases in proliferation to KLH and Con A compared to lymphocytes from primed wild-type littermates; in contrast, there were no significant differences in LPS response between the two groups, suggesting normal B cell proliferative responses. Interestingly, serum antibody levels against KLH were significantly lower in primed Ly-6A null mice than wild-type littermates, suggesting that the effects of Ly-6A deficiency on immune responses are highly complex.

Bamezai et al. suggest a negative role for Ly-6A in thymic selection (26). Normally, Ly-6A expression during thymocyte differentiation is strictly controlled. Ly-6A is expressed on the thymocyte progenitor cell which seeds the thymic cortex (23, 24). During the transition of CD3⁻4⁻8⁻ thymocytes into CD3⁺4⁺8⁺ thymocytes, Ly-6A, CD44, and CD25 expression are temporally regulated. Ly-6A is expressed on 49% of CD44⁺25⁻ (stage 1), 34% of CD44⁺25⁺ (stage 2), and <4% on CD44⁻5⁺ and CD44⁻25⁻ (stages 3 and 4, respectively) (26). After maturation, Ly-6A is reexpressed on mature, single-positive thymocytes and most CD4⁺ and ~40% CD8⁺ peripheral T cells (25, 26, 39). Thymocyte development is arrested in transgenic mice with constitutive lymphocyte expression of Ly-6A at stage 2, when Ly-6A expression is normally terminated (26). However, thymocyte development appears unaltered in Ly-6A null mice which have normal percentages of TCR- α/β , TCR- γ/δ , CD3, CD4, CD8, Sca-2, and ThB positive cells in the thymus and periphery (data not shown). This suggests that although overexpression of Ly-6A abrogates thymocyte maturation, Ly-6A expression is not necessary for normal thymocyte development.

Although a ligand of Ly-6A has not been identified, Ly-6A transgenic thymocytes spontaneously adhere to thymocytes, B cells, and T cells, suggesting that these cell types express a ligand for Ly-6A (40). In addition, there are many published experiments which suggest that Ly-6A is involved in T cell activation. Ly-6A expression on T cells is upregulated upon activation or stimulation with cytokines (41, 42), and cross-linking cell surface Ly-6A leads to IL-2 driven T cell proliferation (12, 43). Several groups have shown that Ly-6A activation is apparently interrelated with TCR signaling. For example, TCR mutant cell lines cannot be activated by Ly-6A cross-linking (14, 19), and Ly-6A antisense downregulates TCR-mediated activation of T

cells and T cell lines (15, 16). In fact, cell lines expressing essentially no Ly-6A due to high expression of antisense constructs have impaired transcription of TCR- β chain and impaired p59^{fyn} but not p56^{lck} phosphorylation activity (16). The effect on p59^{fyn} phosphorylation activity is consistent with the data by Stefanova et al. demonstrating that GPI proteins are weakly associated with protein tyrosine kinases (44). However, despite the absence of TCR or Ly-6A, PMA plus ionomycin activates these cells via a protein kinase C pathway. Our results demonstrating that Ly-6A null and wild-type T cells respond similarly to PMA plus ionomycin but significantly differently to ConA and antigens are consistent with the hypothesis that Ly-6A is involved in signaling via the TCR. However, based on previous antisense experiments, it was surprising that Ly-6A null T cells proliferated at much higher levels to antigen than wild-type cells instead of the reverse. The results from antisense experiments and those with Ly-6A knockout mice appear to give contradictory results; however, they illustrate the difference between a response generated by cells which never expressed Ly-6A (Ly-6A^{-/-}) and cells which expressed Ly-6A and then were altered to downregulate Ly-6A expression (antisense). A similar observation was also demonstrated by the CD2 knockout mice. Although cross-linking cell surface CD2 activates T cells (45), and mutant cell lines lacking CD2 expression have diminished activation through the TCR (46), the T cells from CD2-deficient animals do not have any overt altered function compared to wild-type T cells (47). However, finer analysis showed that CD2 regulates positive selection of CD4⁻CD8⁺ T cells (48).

Several approaches were taken to determine the mechanism driving the enhanced proliferation by the mutant T cells. One possible mechanism of increased proliferation in mutant cells is increased cytokine production. However, there were no consistent differences in cytokine production detected at either the RNA or protein level between splenocytes from Ly-6A mutant and wild-type littermates (data not shown). Another possible mechanism of enhanced proliferation activity by Ly-6A^{-/-} T cells may be that a subpopulation of cells is either present or absent in Ly-6A knockout animals which normally upregulates or downregulates the T cell response. Although this possibility cannot be ruled out at this point, the cursory analysis of subpopulations did not show any differences between wild-type and null thymocytes or T cells, and the T cell activation experiments are consistent with all stimuli which act through the TCR which were tested. Another possibility is that another member of the *Ly-6* gene family is overcompensating for the lack of Ly-6A; however, there is no difference in expression of the other cloned members of the *Ly-6* gene family (data not shown). Interestingly, there is growing evidence which suggests that GPI-anchored proteins associate

with Src kinases in caveolae, small invaginations of the plasma membrane lacking clathrin coats (for review see reference 49). It is possible that GPI-anchored proteins act as positive or negative regulators of activation by trapping and concentrating receptors and other signaling molecules. This model is supported by Romagnoli and Bron, who recently demonstrated that stimulation of the TCR in GPI-mutant T cell lines generated reduced activity by the *fyn* and *lck* kinases which resulted in failure to induce tyrosine phosphorylation of the TCR δ chain and ZAP-70 (50). The Ly-6A null mice and antigen-specific T cell lines generated from these mice (Alexander, R., and P.M. Flood, unpublished results) should be useful to test this model.

The role of Ly-6A on the functional maturation of hematopoietic precursors is also very complicated. In mice which express the Ly-6.2 allele, which includes C57Bl/6 and 129 (the two backgrounds of the Ly-6A null mice and their littermates), Ly-6A is expressed on all hematopoietic stem cells and a significant proportion of committed progenitors (7, 51); however, only 25% of the stem cell activity of the adult bone marrow expresses Ly-6A in Ly-6.1 allele mice (52). Thus, the differences in the expression pattern of the two alleles suggests that Ly-6A is not essential for development of hematopoietic stem cells. This is consistent with normal hematopoietic development in Ly-6A null mice. The CFU assay suggests that there may be some subtle alterations in myeloid precursor activity in Ly-6A mutant bone marrow compared to wild-type bone marrow, similar to CD34-deficient animals (53, 54).

In conclusion, although interactions governing the regulation of the immune responses and peripheral tolerance remain unclear, recent experiments have shed light upon several important regulators of T lymphocyte responses. For example, IL-2-deficient and IL-2R-deficient mice have demonstrated the critical role which cytokines and cytokine receptors may play in regulating T cell responses and self-tolerance (55–59). Other studies have shown that molecules such as CTLA-4 may provide an important negative signal to downregulate T lymphocyte activity (60, 61). In addition, members of the TNF/TNFR family also contribute to the activation and elimination of lymphocytes during an immune response (62–65). This report suggests that Ly-6A may play an important role in regulating T lymphocyte responses. Although activation and proliferation of Ly-6A null thymocytes is normal, *in vitro* and *in vivo* activation of peripheral T cells from Ly-6A-deficient mice generates higher and more sustained proliferative responses than T cells from wild-type littermates. These data suggest that Ly-6A acts to downmodulate the T lymphocyte response to antigen. Further analysis of the Ly-6A-deficient mice will provide valuable insight into the signaling pathway of Ly-6A and how molecules act in concert to maintain homeostasis and peripheral tolerance.

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