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Differences in the Immune Response During the Acute Phase of E-55+ Murine Leukemia Virus Infection in Progressor BALB and Long Term Nonprogressor C57BL Mice

Vily Panoutsakopoulou, C. Scott Little, Thomas G. Sieck, Elizabeth P. Blankenhorn, and Kenneth J. Blank

E-55+ murine leukemia virus infection of both progressor (BALB) and long term nonprogressor (C57BL) mouse strains is characterized by an acute and a persistent phase of infection. During the acute phase, progressor strains require CD8+ T cells to decrease virus burden, whereas the long term nonprogressor strains do not. In the present studies the immune response in BALB and C57BL mice during the acute phase of E-55+ murine leukemia virus infection was examined. The results demonstrate that BALB mice produce both IL-4 and IFN-γ, in contrast to C57BL mice, which produce only IFN-γ. In BALB mice, IL-4 production results in the absolute requirement for CD8+ T cells to reduce the virus burden during the acute phase of infection. The anti-virus immune response in these mice is IFN-γ dependent. On the other hand, C57BL mice do not produce IL-4 and, in the absence of both CD8+ T cells and IFN-γ, still generate an effective anti-virus immune response. Genetic studies suggest that these distinct immune responses are regulated by more than one non-MHC-linked gene. Two candidate regions that may encode this gene(s), located on chromosomes 7 and 19, respectively, were identified by recombinant inbred strain linkage analysis.

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Abbreviations used in this paper: MuLV, murine leukemia virus; BALB, BALB.K and BALB/c mice; H-2, MHC of the mouse; C57BL, B10.BR and C57BL/6 mice; FV, Friend murine leukemia virus LCMV, lymphocytic choriomeningitis virus; RI, recombinant inbred; B-4 KO, interleukin-4 knockout; IL-6 KO, interferon-γ knockout; FFA, fluorescent focus assay; SDP, strain distribution pattern; LOD, logarithm of the odds; CM, centimorgans.
responses against viruses such as lymphocytic choriomeningitis virus (LCMV) (7), hepatitis (8), vaccinia (9), and herpes simplex virus (10). IFN-γ has also been shown to regulate the generation of Th subsets, which are best studied in the Leishmania major infection (11). Production of IFN-γ, a type 1 cytokine, restricts the proliferation of Th2 clones, while IL-4, a type 2 cytokine, has been demonstrated to promote type 2 responses in vitro and in vivo and consequent suppression of IFN-γ production (12). IL-4 also plays an important role in the regulation of the immune response during LP-BM5 virus infection (13).

In the present studies IL-4 and IFN-γ production were examined to determine whether regulation of the BALB.K and B10.BR phenotypes might be related to differential expression of these molecules early in the immune response. Mice with targeted disruption (knockout) of their IFN-γ or IL-4 genes were used to examine the roles of these cytokines in regulating the anti-virus immune response in these strains. The results demonstrate that BALB mice produce both IL-4 and IFN-γ, in contrast to C57BL mice, which produce only IFN-γ. In BALB mice, IL-4 production results in the absolute requirement for CD8+$^+$ T cells to reduce the virus burden during the acute phase of E-55 +MuLV infection. The production of IFN-γ in these mice is necessary for the development of the anti-virus CD8+$^+$ T cell response. On the other hand, C57BL mice do not produce IL-4 and do not require IFN-γ (or CD8+$^+$ T cells) to generate an effective anti-virus immune response during the acute phase of infection.

In addition, the CBX recombinant inbred (RI) strains (14, 15) that derive from the cross between BALB/c (progressors, similar to BALB.K) and C57BL/6 (LTPN, similar to B10.BR) mice were used to map genes responsible for the immune response against E-55 +MuLV. Backcross analysis of this phenotype suggests that more than one non-MHC gene is responsible for the differences in the anti-virus immune response in these two strains of mice; at least one of these genes may regulate IL-4 production. Two candidate regions that may encode this gene(s), located on chromosomes 7 and 19, respectively, were found based on RI strain linkage analysis.

**Materials and Methods**

**Mice**

Adult C57BL/6 (B6), BALB/c, C57BL/10-H-2$^d$ (B10.BR), CB6F$_1$ (BALB/c × C57BL/6), BALB/c-12-4 KO, BALB/c-IgF KO, C57BL/6-IgF KO, and the 13 CBX RI strains were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c-H-2$^d$ (BALB.K) mice were bred at the Research Animal Facility at Allegheny University of the Health Sciences. BALB.K mice are congenic to BALB/c mice, which express the H-2$^d$ haplotype. B10.BR mice (H-2$^b$) are congenic to C57BL/10 (B10) mice with respect to the H-2 haplotype. B10 mice are closely related to B6, and both strains express the H-2 haplotype.

**Virus**

E-55 +MuLV was isolated from a BALB.K leukemic spleen that was infected with cell-free culture supernatant from a T cell line derived from a leukemic mouse (16). The virus used in these studies was passaged in vivo by i.p. injections of immunosuppressed BALB.K. For the present experiments, each mouse was i.p. injected with 2$x^{}^{10^3}$ fluorescent focus-forming units of E-55 +MuLV.

**Antibodies**

Hybridoma cells producing mAb 2.43 (anti-CD8) and GK1.5 (anti-CD4) used for the in vivo depletion were obtained from Dr. David Weiss and American Type Culture Collection (Rockville, MD), respectively. Hybridoma cells producing the mAb m34, specific for p15-gag (17) used for the fluorescent focus assay (FFA) were a gift from Dr. Bruce Chesebro. mAb 145.2C11, used for spleen cell stimulation, is specific for CD3 e and was obtained from Boehringer Mannheim (Indianapolis, IN). Anti-cytokine Abs used for the ELISA were obtained from PharMingen (San Diego, CA).

**Cells**

The 663B tumor cell line (class I$^+$ and K$^+$ positive, class II negative) was established in our laboratory from the leukemic spleen of an immunosuppressed BALB/c mouse inoculated in vivo with E-55 +MuLV. The P815 cell line is a mastocytoma from a DBA/2 (H-2$^d$) mouse obtained from American Type Collection (TIB 64). The Ti-6 cell line is an x-ray-induced thymoma from a C57BL/6 mouse and was a gift from Dr. Paul Jolicoeur. The 663B, P815, and Ti-6 cell lines were maintained in RPMI 1640 (10% FCS, 100 U penicillin, 0.1 mg streptomycin, 2 mM glutamine, at 5$x^{}^{10^4}$ M Spleen) Mus dunni fibroblasts were a gift from Dr. Harvey Schlesinger and were maintained in DMEM (10% FCS, 2 mM L-glutamine, 100 U penicillin, and 0.1 mg streptomycin).

**In vivo CD8$^+$ or CD4$^+$ T cell depletion**

The depletions were performed as previously described (3). Mice were inoculated i.p. three times with 25$^{}^{µg}$ of anti-CD8 (2.43) or 100$^{}^{µg}$ of anti-CD4 (GK1.5) on days −2, 0, and 2, where day 0 was the day on which the mice were inoculated with E-55 +MuLV (2$x^{}^{10^3}$ focus-forming units). Control mice were injected with the Ab alone. The percentages of target cells (CD8$^+$ or CD4$^+$) in in vivo depleted mice were determined by flow cytometry (FACS) and were reduced to background levels in the first 4 to 5 wk (data not shown). Mice were euthanized, and their spleens were harvested 8 wk after virus inoculation. Ten percent spleen homogenates were chilled and prepared in cold DMEM containing protease inhibitors (Kontes, Vineland, NJ) and centrifuged, and virus titers were determined by FFA. For each experiment, a group of mice was immunosuppressed by a sublethal dose of irradiation (550 rad) (18).

**Fluorescent focus assay**

The FFA assay was performed as described in Sibton et al. (19). On day 1, Mus dunni fibroblasts were plated at a concentration of 6000 cells/well in a 24-well plate (DMEM, 10% FCS, 2 mM L-glutamine, 100 U penicillin, and 0.1 mg streptomycin) with 10$^5$ µg polybrene/ml. On day 2, the Mus. dunni cells were infected with 100 µl/well of 10-fold serial dilutions of spleen homogenate, ranging from 10$^{-1}$ to 10$^{-5}$. One hour after infection, supernatant was aspirated, the wells were washed with PBS, and fresh medium was added. On day 5, when cells had grown to confluence, the medium was aspirated, the wells were washed with PBS and 2% FCS, incubated with Ab m34 for 1 h at 4°C, washed again with PBS and 2% FCS, and incubated with FITC-conjugated anti-mouse Ig Ab (Southern Biotechnology, Birmingham, AL) for 1 h at 4°C. Finally, the cells were washed three times with PBS and 2% FCS, and fluorescent foci were counted on an inverted fluorescent microscope. Virus titers were expressed as fluorescent focus-forming units per milliliter of spleen homogenate.

**Cell-mediated cytotoxicity (CTC) assay**

Spleen cells from uninfected or E-55 +MuLV-infected B6 mice were cultured in 24-well plates at a concentration of 1$x^{}^{10^4}$ cells/ml of RPMI 1640 (10% FCS, 100 U penicillin, 0.1 mg streptomycin, 2 mM glutamine, and 5$x^{}^{10^3}$ M 2-ME) in the presence of stimulator cells. The stimulators were either the H-2$^d$ 663B or the H-2$^d$ P815 tumor cells (the cell line used as target for the CTC assay) and was used at 1$x^{}^{10^4}$ cells/ml. Target cells were cultured for 5 days at 37°C in 5% CO$_2$. Responder cells were then washed, counted, and resuspended in RPMI for the JAM test used to measure cytotoxicity (20). Briefly, the target cells (663B tumor cell line for anti-virus response, P815 for allogeneic response, and uninfected Ti-6 cells as control) were pulsed with 3 to 4 µCi of $[^3^H]$Hthymidine/ml for 6 h before the assay, washed twice, and plated at 1$x^{}^{10^4}$ cells/well in round bottom 96-well plates. In some cases, responder cells were depleted in vitro of CD8$^+$ T cells before the JAM test. Responder cells were added in different concentrations, resulting in varying E:T cell ratios. Target and effector cells were incubated for 2 to 4 h and harvested using a PHD cell harvester (Cambridge Technologies, Cambridge, MA), and retained radioactivity was counted on a beta scintillation counter. The percent specific lysis was determined as: [(S − E) / S] x 100, where S is spontaneous release, retained DNA in the absence of effector cells, and E is experimentally retained DNA in the presence of effector cells. The allogeneic response against P815 was intact for the undepleted infected and uninfected spleen cells (30–50% specific lysis at 50:1 ratio). The depletion of CD8$^+$ T cells resulted in almost complete ablation (4% specific lysis) of allogeneic cytotoxicity. The B6 tumor cell line Ti-6 was used as uninfected target cell control for all the CTC assays performed, and at every E:T cell ratio the percentage of specific lysis was ≤4%.
The Journal of Immunology

Table I. BALB/c mice require CD8+ T cells during the acute phase of E-55 + MuLV infectiona

<table>
<thead>
<tr>
<th></th>
<th>E-55 + MuLV (flu/ml)</th>
<th>550R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-CD8</td>
<td>No Ab treatment</td>
</tr>
<tr>
<td></td>
<td>Pos./total</td>
<td>Range (mean ± SD/10^5)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>4/4</td>
<td>(4–6) × 10^3 (4.75 ± 0.82)^b</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>0/4</td>
<td>0 (0)</td>
</tr>
<tr>
<td>BALB/c × C57BL/6</td>
<td>3/4</td>
<td>0–20 (12.5 ± 8.29) × 10^-5c</td>
</tr>
</tbody>
</table>

a Adult BALB/c, C57BL/6, and (BALB/c × C57BL/6)F1 mice were depleted of CD8+ T cells (denoted as anti-CD8) and infected with E-55 + MuLV as described in Materials and Methods. CD8+ T cells are not essential for C57BL/6 and the (BALB/c × C57BL/6)F1 control groups included mice that were not CD8+ T cell-depleted (denoted as no Ab treatment) and mice immunosuppressed by sublethal irradiation (denoted as 550R) 24 h prior to injection. Mice were euthanized 8 wk after infection, and the level of infectious virus in the spleen was quantitated by FFA as described in Materials and Methods. Pos./total. Number of mice positive for virus presence per total of mice tested. The range is expressed as flu/µl. b p = 0.001 in comparison to CD8-depleted C57BL/6. c p = 0.001 in comparison to CD8-depleted BALB/c.

In vitro CD8+ T cell depletion

Spleen cells from 2-wk E-55 + MuLV infected B6 mice were cultured in the presence of stimulator cells as described in the CTL assay (above). After 5 days, spleen cells were resuspended at 2 × 10^5 cells/ml in RPMI 1640 containing 10% FCS, 100 U penicillin, 0.1 mg streptomycin, 2 mM glutamine, and 1% HEPES in the presence or the absence of immobilized anti-CD3 mAb (Boehringer Mannheim, Indianapolis, IN). The percentage of CD8+ cells in the depleted samples was reduced to background levels (1%).

ELISA for cytokines

BALB.K, B10.BR (BALB.K × B10.BR)F1, BALB/c, and C57BL/6 mice were injected with E-55 + MuLV and challenged with the same virus after a 4-wk interval. Five days after challenge the mice were euthanized, the spleens were removed, and a single cell suspension was prepared. Splenocytes were cultured at a concentration of 3.3 × 10^5 cells/ml in RPMI 1640 containing 10% FCS, 100 U penicillin, 0.1 mg streptomycin, 2 mM glutamine, and 1% HEPES in the presence or the absence of immobilized anti-CD3 mAb (145.2C11). The cells were incubated at 37°C in 5% CO2, and culture supernatant was removed after 48 h. The culture supernatant was stored at −20°C until tested for the presence of IFN-γ or IL-4 using a sandwich ELISA specific for each cytokine. (Anti-cytokine Abs for the ELISA were obtained from Pharmingen and the assay was performed following the recommended protocol provided by this vendor.)

Linkage analysis

Each RI strain was designated C (if its phenotype was similar to BALB/c) or B (if similar to C57BL/6). The strain distribution pattern (SDP) of the RI phenotypes was compared with that of pre-existing SDP for markers typed extensively by our group (21) and other groups (22) that are available in the Mouse Genome Database (Jackson Laboratory web site, http://www.jax.org). The 153 markers typed by our group are distributed in an average distance of 8.6 centimorgan and span >81% of the mouse genome. The computer software used for the qualitative linkage analysis is Map Manager (23), and that used for quantitative linkage analysis is Map Manager QTb11. For the quantitative analysis, the RI strains were given isogenic values (except those for the linkage analysis) were calculated using Student’s t test. Bayesian statistics (24) were used for the qualitative linkage analysis, and simple regression (25) was used for the quantitative analysis.

Results

Genetic differences between BALB and C57BL strains determine the T cell subsets required to generate an effective immune response during the acute phase of E-55 + MuLV infection

Previous studies in this laboratory have demonstrated that BALB.K and B10.BR mice infected with E-55 + MuLV show an initial increase in virus burden in the acute phase of infection followed by dramatic decrease in virus burden that is mediated by T cells (2). In BALB.K mice, in vivo depletion of either CD4+ or CD8+ cells before infection results in the failure of these mice to decrease their virus burden (3). On the other hand, depletion of CD4+, but not CD8+, cells from B10.BR mice results in the failure to reduce the virus burden in these mice (3).

Studies to determine the immunologic basis for differences between these strains that could account for the variation with respect to the T cell subsets that mediate the anti-virus immune response during the acute phase of infection required the use of mice in which certain immunologically relevant genes had been disrupted (knocked out). In addition, analysis of the genetic basis for this variation required the use of RI strains of mice to map genes that regulate this early anti-virus immune response and identify potential candidate genes. However, because mice with appropriate disrupted genes were available only on the C57BL/6 (B6) and BALB/c backgrounds, and the RI strains needed for this study were produced from BALB/c and B6 parents, it was first necessary to determine that BALB/c and B6 mice (which express the H-2^b and H-2^d haplotypes, respectively) demonstrated the same phenotype with respect to the immune response in the acute phase of infection as BALB.K and B10.BR mice (which both express the H-2^d haplotype). This comparison demonstrated that the anti-virus immune responses in BALB/c and B6 mice were identical with the immune responses that occurred in BALB.K and B10.BR mice, respectively (Tables I and II).

For this analysis, BALB/c and B6 mice were depleted of CD8+ T cells by in vivo administration of anti-CD4 or anti-CD8 mAbs as previously described (3). These depleted mice were inoculated with E-55 + MuLV, and 8 wk later splenic virus titers were determined by FFA. The results demonstrated that untreated control mice from both strains have undetectable levels of virus by FFA in a manner identical with that of untreated BALB.K and B10.BR mice (3). In vivo CD4+ T cell depletion demonstrated that both B6 and BALB/c parental and F1 mice require CD4+ T cells during the acute phase of infection to decrease the virus burden to undetectable levels (Table II) in the same manner as B10.BR and BALB.K mice (3). BALB/c mice depleted of CD8+ T cells failed to develop an effective anti-virus immune response, as determined by the presence of detectable virus titers in the spleens of these mice (Table I), and thus behaved like H-2^b congenic BALB.K mice (3). B6 mice depleted of CD8+ T cells, like H-2 congenic B10.BR mice (3), demonstrated the ability to generate an effective anti-virus immune response in the same manner as immunologically intact B6 control mice. (BALB/c × C57BL/6)F1 mice were also tested to determine whether they display a phenotype similar to B6 or BALB/c. Three of
four F₁ mice had virus barely above undetectable levels and significantly lower (p = 0.001) than the virus levels in the CD8-depleted BALB/c mice. Because the F₁ mice, like B6, do not require CD8

Poss/total, Number of mice positive for virus presence per total of mice tested. The range is given in ffu/ml of 10% spleen homogenate. ND, experiment not done.

### Materials and Methods

Prior to virus injection, mice were euthanized 8 wk after infection, and the level of infectious virus in the spleen was quantitated by FFA as described in Materials and Methods. Pos/total. Number of mice positive for virus presence per total of mice tested. The range is given in ffu/ml of 10% spleen homogenate. ND, experiment not done.

#### FIGURE 1.

Virus-specific CD8

CTLs are generated in C57BL/6 mice during the acute phase of E-55+MuLV infection. Spleen cells from 2-wk infected, 4-wk infected, or uninfected adult C57BL/6 mice were used as effectors in a CTL assay (as described in Materials and Methods) against 663B tumor cells (H-2b) that express E-55+MuLV epitopes and class I molecules but are negative for class II (A). Spleen cells from 2-wk infected C57BL/6 mice were depleted in vitro of CD8

T cells (2wk, anti-CD8) and were used as effectors in a CTL assay against 663B tumor cells (B). The C57BL/6 tumor cell line Ti-6 was used as uninfected target cells for all the CTL assays performed, and the percentage of specific lysis was insignificant (≤5%).

#### TABLE II.

<table>
<thead>
<tr>
<th>Anti-CD4</th>
<th>No Ab treatment</th>
<th>550R</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-55+MuLV (fu/ml)</td>
<td>Pos./total</td>
<td>Range (mean ± SD/10²)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>3/3</td>
<td>(5–7) × 10³</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>3/3</td>
<td>(6–9) × 10³</td>
</tr>
<tr>
<td>BALB/c × C57BL/6</td>
<td>4/4</td>
<td>(3–10) × 10³</td>
</tr>
</tbody>
</table>

*Adult BALB/c, C57BL/6, and (BALB/c × C57BL/6)/F₁ mice were depleted of CD4³ T cells and infected with E-55+MuLV (denoted as anti-CD4) as described in Materials and Methods. Control groups included mice that were not CD4³ T cell-depleted (denoted as no Ab treatment) and mice immunosuppressed by sublethal irradiation (550R) 24 h prior to virus injection. Mice were euthanized 8 wk after infection, and the level of infectious virus in the spleen was quantitated by FFA as described in Materials and Methods. Pos/total. Number of mice positive for virus presence per total of mice tested. The range is given in ffu/ml of 10% spleen homogenate. ND, experiment not done.

* p = 0.005 in comparison to untreated BALB/c.

* p = 0.006 in comparison to untreated C57BL/6.

* p = 0.012 in comparison to untreated F₁.

The difference between BALB (BALB/c and BALB.K) and C57BL (B6 and B10.BR) mice with respect to their requirement for CD8

T cells to mediate an effective anti-virus immune response demonstrates that there is a difference in the anti-virus T cell response generated in the acute phase of infection between these two strains. Previous studies have demonstrated the role of cytokines in regulating differences between inbred strains of mice in the generation of an effective immune response against Leishmania major, and it is likely that differential cytokine production might play a role in the differences observed in the early anti-E-55+MuLV immune responses as well. To examine this possibility, spleen cells from B10.BR, B6, BALB.K, and BALB/c E-55+MuLV-infected mice (4 wk after infection) were stimulated to produce cytokines by incubation with anti-CD3 Ab. This non-specific stimulation of spleen cells from mice infected with various other retroviruses and pathogenic organisms as well as from mice with autoimmune disease has been used extensively by other groups to measure cytokine production (26–28). Subsequently, IFN-γ and IL-4 production were measured in the culture supernatants by ELISA. The results demonstrated that infected BALB and C57BL mice both produce IFN-γ (Table III). However, BALB mice also produce IL-4, whereas this cytokine is not produced by C57BL mice (Table IV). F₁ mice produce IFN-γ but not IL-4, indicating that the trait defined by IL-4 production is recessive. Interestingly, levels of IFN-γ produced by infected BALB and C57BL mice are lower in infected mice than those in uninfected control mice. In contrast, levels of IL-4 in infected BALB are higher than those in uninfected control mice from this strain.

### Differences between BALB/c and C57BL/6 mice in the requirement for IFN-γ and the regulation and effect of IL-4 production during the acute phase of infection

Because the previous experiment demonstrated that BALB, but not C57BL, mice infected with E-55+MuLV produced IL-4, the role of this cytokine during the acute phase of the immune response was investigated using BALB/c-Il4 KO mice (BALB/c-Il4tm3Nnt).
### Table III. IFN-γ production during the acute phase of E-55+MuLV infection

<table>
<thead>
<tr>
<th></th>
<th>BALB/K</th>
<th>B10.BR</th>
<th>F1</th>
<th>BALB/c</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mice</td>
<td>Range (pg/ml)</td>
<td>(mean ± SD/10⁶)</td>
<td>No. of mice</td>
<td>Range (pg/ml)</td>
</tr>
<tr>
<td>Uninfected</td>
<td>6</td>
<td>(1–10) × 10⁵</td>
<td>(5.5 ± 3.4)ᵇ</td>
<td>5</td>
<td>(5–10) × 10⁵</td>
</tr>
<tr>
<td>E-55+ infected</td>
<td>5</td>
<td>(0.8–4) × 10⁵</td>
<td>(0.22 ± 0.17)</td>
<td>7</td>
<td>(3–50) × 10⁴</td>
</tr>
</tbody>
</table>

ᵃ Splenocytes from adult uninfected and 4-wk E-55+MuLV-infected BALB/K, B10.BR, (BALB.K × B10.BR)F₁, BALB/c, and C57BL/6 mice were stimulated with plate bound anti-CD3 Ab. Culture supernatants were harvested at 48 h and assayed by sandwich ELISA for IFN-γ as described in Materials and Methods. The level of detection for IFN-γ was 10 pg/ml. BD, below the level of detection for the assay. Data are representative of four independent experiments.

ᵇ p = 0.009 in comparison to infected BALB/K; p = 0.198 in comparison to uninfected B10.BR.

d p = 0.004 in comparison to infected BALB/K; p = 0.075 in comparison to uninfected B10.BR.

d p = 0.017 in comparison to infected BALB/K; p = 0.106 in comparison to uninfected F₁.

### Table IV. IL-4 production during the acute phase of E-55+MuLV infection

<table>
<thead>
<tr>
<th></th>
<th>BALB/K</th>
<th>B10.BR</th>
<th>F₁</th>
<th>BALB/c</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mice</td>
<td>Range (U/ml)</td>
<td>(mean ± SD)</td>
<td>No. of mice</td>
<td>Range (U/ml)</td>
</tr>
<tr>
<td>Uninfected</td>
<td>6</td>
<td>1.0–3.0</td>
<td>(2.0 ± 0.8)ᵇ</td>
<td>5</td>
<td>BD</td>
</tr>
<tr>
<td>E-55+ infected</td>
<td>5</td>
<td>6–14</td>
<td>(10.4 ± 2.7)ᶜ</td>
<td>7</td>
<td>BD</td>
</tr>
</tbody>
</table>

ᵃ Splenocytes from adult uninfected and 4-wk E-55+MuLV-infected BALB/K, B10.BR, (BALB.K × B10.BR)F₁, BALB/c, and C57BL/6 mice were stimulated with plate bound anti-CD3 Ab. Culture supernatants were harvested at 48 h and assayed by sandwich ELISA for IL-4 as described in Materials and Methods. The level of detection for IL-4 was 0.16 U/ml. BD, below the level of detection for the assay; NA, not available. Data is representative of four independent experiments.

ᵇ p = 0.001 in comparison to uninfected B10.BR.

c p = 0.001 in comparison to uninfected BALB/K.

d p = 0.003 in comparison to infected BALB/c.
in which the IL-4 gene has been disrupted by homologous recombination (29). E-55+MuLV-infected BALB/c-Il-4 KO mice, like normal BALB/c mice, demonstrated the ability to decrease the virus burden to undetectable levels during the acute phase of infection (Table V). However, in contrast to normal BALB/c mice, BALB/c-Il-4 KO mice depleted of CD8+ T cells still demonstrated an effective anti-virus immune response that reduced virus titers to undetectable levels. Thus, these BALB/c-Il-4 KO mice appear to express the same phenotype with respect to the generation of an anti-virus immune response as normal B6 and B10.BR mice. This result strongly indicates that IL-4 plays an important role in the anti-virus immune response generated in BALB/c mice and specifically appears to regulate the anti-virus activity of immune cells in CD8-depleted mice.

The role of IFN-γ, which is produced by both BALB.K and B10.BR in the anti-virus immune response during the acute phase of infection, was investigated using BALB/c-Ifg KO (BALB/c-Ifg tm1Ts ) and C57BL/6-Ifg KO (C57BL/6-Ifg tm1Ts ) in which the IFN-gamma gene has been disrupted (30). BALB/c-Ifg KO mice failed to generate an effective anti-virus immune response during the acute phase of infection, as measured by the presence of virus 8 wk after infection in the Ifg KO mice but not in normal BALB/c mice (Table VI). In contrast, B6-Ifg KO mice did not require IFN-γ to generate an effective anti-virus immune response, because neither B6-Ifg KO mice nor CD8-depleted B6-Ifg KO mice expressed detectable virus 8 wk after infection. Interestingly, E-55+MuLV-infected B6-Ifg KO mice differed from normal B6 mice with respect to the production of IL-4 and IFN-γ. In contrast to normal B6 mice, B6-Ifg KO mice produced no IFN-γ (as expected), but detectable levels of IL-4 were produced (2–3 U/ml). Thus, although B6-Ifg KO mice appear to produce IL-4, these mice are still capable of generating an effective anti-virus immune response even when CD8+ T cells have been depleted.

### Table V. BALB/c-IL-4 KO mice do not require CD8+ T cells during the acute phase of E-55+MuLV infection

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<th>Anti-CD8</th>
<th>No Ab treatment</th>
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<tr>
<td></td>
<td>Pos./total</td>
<td>Range</td>
</tr>
<tr>
<td>BALB/c</td>
<td>4/4</td>
<td>0.1–4</td>
</tr>
<tr>
<td>BALB/c-IL-4 KO</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>0/4</td>
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As described above, the difference in phenotype between BALB/c and B6 mice with respect to the requirement for CD8+ T cells to generate an effective anti-virus immune response appears to be regulated by non-MHC-linked loci. To map the gene(s) that regulates the expression of this phenotype, CXB RI strains (derived from the intercross of BALB/c and B6) (14, 15) were depleted of CD8+ T cells following the same protocol as that described for the parental strains. Only 2 (CXB N and CXB O) of the 13 CXB RI strains demonstrated the BALB phenotype characterized by high virus titers 8 wk after infection (Fig. 2). E-55+MuLV-infected (BALB.K × B10.BR) × BALB.K backcross mice were also CD8 depleted in vivo before infection and were observed for virus expression 8 wk after infection. In 5 of the 16 backcross mice, virus was detectable at this time point (data not shown). These results indicate that at least one and perhaps two genes are responsible for the expression of the resistant phenotype. To perform linkage analysis using the RI strains, each RI strain was typed as B when its phenotype resembled the B6 parent (i.e., resistance was still observed after CD8+ T cell depletion) and C when it resembled BALB/c (Fig. 2). The distribution of parental phenotypes among the RI strains gave an SDP that was compared with the Mouse Genome Database, which contains SDPs of known genetic markers that scan the whole mouse genome. Linkage analysis for candidate chromosomal regions that control the requirement for CD8+ T cells during the acute phase of infection was performed using Bayesian statistics (24) on Map Manager. This

### Table VI. C57BL/6 mice do not require IFN-γ during the acute phase of infection in contrast to the BALB/c for which the IFN-γ is essential

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<td></td>
<td>Pos./total</td>
<td>Range</td>
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<tr>
<td>BALB/c</td>
<td>6/6</td>
<td>(0.01–0.4) × 10^3</td>
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<tr>
<td>BALB/c-Ifg KO</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C57BL/6-Ifg KO</td>
<td>0/8</td>
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As described above, the difference in phenotype between BALB/c and B6 mice with respect to the requirement for CD8+ T cells to generate an effective anti-virus immune response appears to be regulated by non-MHC-linked loci. To map the gene(s) that regulates the expression of this phenotype, CXB RI strains (derived from the intercross of BALB/c and B6) (14, 15) were depleted of CD8+ T cells following the same protocol as that described for the parental strains. Only 2 (CXB N and CXB O) of the 13 CXB RI strains demonstrated the BALB phenotype characterized by high virus titers 8 wk after infection (Fig. 2). E-55+MuLV-infected (BALB.K × B10.BR) × BALB.K backcross mice were also CD8 depleted in vivo before infection and were observed for virus expression 8 wk after infection. In 5 of the 16 backcross mice, virus was detectable at this time point (data not shown). These results indicate that at least one and perhaps two genes are responsible for the expression of the resistant phenotype. To perform linkage analysis using the RI strains, each RI strain was typed as B when its phenotype resembled the B6 parent (i.e., resistance was still observed after CD8+ T cell depletion) and C when it resembled BALB/c (Fig. 2). The distribution of parental phenotypes among the RI strains gave an SDP that was compared with the Mouse Genome Database, which contains SDPs of known genetic markers that scan the whole mouse genome. Linkage analysis for candidate chromosomal regions that control the requirement for CD8+ T cells during the acute phase of infection was performed using Bayesian statistics (24) on Map Manager. This

### Table VII. C57BL/6 mice do not require IFN-γ during the acute phase of infection in contrast to the BALB/c for which the IFN-γ is essential

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analysis revealed linkage to both a region of chromosome 7 defined by the D7 Mit223 microsatellite marker (Panoutsakopoulou, MGD-INUM43753; i.e., Mouse Genome Database: the mouse genome database accession number) and a region of chromosome 19 defined by the D19 Mit59 (Taylor, MGI28086) and D19 Mit109 (21) (Panoutsakopoulou, MGI86969) microsatellite markers (Fig. 3) with the highest LOD score of 1.5 (p < 0.05). Quantitative analysis (RI strains were given scores that reflected virus levels instead of C or B designations) revealed the same regions as the qualitative analysis, with a maximum LOD score of 1.48 (p < 0.01) for D7 Mit223 (at 72.4 cM), D19 Mit59 (at 0.5 cM), and D19 Mit109 (at 4 cM). These markers were associated with the C genotype for the two strains (CXB L and CXB O) that produce virus after CD8 T cell depletion (i.e., these two strains resemble the BALB/c parent with respect to this phenotype). Thus, regions of chromosomes 7 and 19 linked to these microsatellite markers may harbor genes that contribute to the regulation of the anti-virus immune response during the acute phase of E-55+MuLV infection.

### Discussion

In these studies, the immune responses of progressor BALB/c and long term nonprogressor B6 mice against E-55+MuLV during the acute phase of infection were examined. These two strains, which differ with respect to their genetic backgrounds and specifically their H-2 haplotypes (BALB/c and B6 mice express the H-2^d and H-2^k haplotypes, respectively) were shown to generate anti-virus immune responses identical with those of their closely related counterparts, BALB/c-H-2^d (BALB.K) and C57BL/10-H-2^k (B10.BR) mice, which both express the same H-2 haplotype. BALB/c and BALB.K mice require both CD4+ and CD8+ T cells to accomplish this decrease in virus burden. Based on the results of this study indicating that the anti-virus immune response of these pairs of mice were identical, it was possible to use genetically altered B6 mice in which genes encoding particular cytokines were disrupted (knockout mice) to examine the roles of these cytokines in regulating the immune response in B6 and BALB/c mice. In addition, CXB RI strains of mice (derived from B6 and BALB/c mice) could be used to map genes.

**FIGURE 2.** Virus titers in the spleens of the CXB RI strains 8 wk after in vivo CD8+ T cell depletion. Adult BALB/c, C57BL/6, and CXB RI strains of mice were CD8+ T cell depleted at the time of E-55+MuLV injection as described in Materials and Methods. The control groups were BALB/c and C57BL/6 mice that were depleted of CD8+ T cells and injected with E-55+MuLV. Mice were euthanized 8 wk postinfection, and the level of infectious virus in the spleen was quantitated by FFA as described in Materials and Methods. Each point represents virus titers from a single mouse. SDP is the strain distribution pattern of the CXB RI strains. The phenotype is presence or the absence of infectious E-55+MuLV in the spleen 8 wk postinfection when depleted of CD8+ T cells. C designates the BALB/c parent phenotype (high levels of virus), and B designates the C57BL/6 parent phenotype (undetectable levels of virus). For CXBL, p = 0.002 compared with C57BL/6, and p = 0.204 compared with BALB/c. For CXBO, p = 0.04 compared with C57BL/6, p = 0.076 compared with BALB/c, and p = 0.29 for CXBL compared with CXBO.

**FIGURE 3.** Chromosome 7 and 19 LOD scores for the phenotype of the acute phase of E-55+MuLV infection based on linkage analysis using the CXB strains. Each CXB RI strain was typed as B when its phenotype was like that of C57BL/6 (no requirement for CD8+ T cells, undetectable virus) and as C when its phenotype was like that of BALB/c (requirement for CD8+ T cells, high levels of virus). The distribution of parental phenotypes among the RI strains resulted in a SDP (see Fig. 2) that was compared with a pre-existing database (Mouse Genome Database, described in Materials and Methods). cM is the distance in centiMorgans as estimated by the Mouse Genome Database. D – Mit – are microsatellite markers. References for the markers (Mouse Genome Database, author name, accession numbers): Gα2 (M.F. Colombo, MGI2945); D19 Mit59, D19 Mit61, D19 Mit41, D19 Mit40, D19 Mit19, D19 Mit34, D19 Mit6 (B. A. Taylor, MGI28086); D7 Mit117, D7Nds1, D7 Mit281, D7 Mit100, D7 Mit68, D19 Mit109, D19 Mit85, D19 Mit19, D19 Mit1, D19 Mit6 (V. Panoutsakopoulou, MGI86969); and D7 Mit223 (MGD-INUM43753).
responsible for the immunologic phenotypes of these mice with respect to their abilities to generate effective anti-virus responses.

In the present studies, IL-4 and IFN-γ production were examined to determine whether regulation of the BALB/c and B6 phenotypes might be related to differential expression of these molecules early in the immune response. This possibility was suggested by previous studies that demonstrated that these cytokines regulate the type of immune response generated against other infectious organisms particularly Leishmania major. The results demonstrate that BALB spleen cells from E-55+MuLV-infected mice produce both IFN-γ and IL-4. In contrast, spleen cells from infected C57BL mice produce high levels of IFN-γ but no detectable levels of IL-4.

The observation that BALB mice generate an effective immune response that is ablated when mice are CD8 depleted demonstrates that an anti-virus CD8+ T cell response is necessary to reduce virus burden during the acute phase of infection. This type of immune response mediated by CD8+ T cells has previously been demonstrated to depend on the production of type 1 cytokines and generally does not occur in mice that produce type 2 cytokines (31). The role of IFN-γ, a type 1 cytokine, in the generation of the anti-E-55+MuLV immune response in BALB mice was confirmed by the observation that infected BALB/c-Ifg KO mice do not generate an effective immune response that results in a decrease in virus burden. Thus, in this virus infection, the production of IL-4 in BALB/c mice does not interfere with their ability to generate CD8+ T cells that mediate anti-E-55+MuLV immunity, whereas the absence of IFN-γ does.

In contrast to BALB mice, both normal and CD8+ T cell-depleted C57BL mice demonstrated the ability to reduce virus burden during the acute phase of infection. Thus, in CD8-depleted animals from this strain, CD4+ T cells appear to mediate the reduction in virus burden. However, normal C57BL mice generate anti-virus cytotoxic CD8+ T cells (Fig. 1), suggesting that cytotoxic CD8+ T cells are probably involved in reducing virus burden during the acute phase of virus infection in nondepleted mice. This observation is similar to that of previous studies in which Sendai virus-infected mice that lacked CD8+ T cells were still able to eliminate virus as the result of CD4+ T cell activity (32). The manner in which the CD4+ T cells reduce the virus burden in CD8− T cell-depleted E-55+MuLV-infected C57BL mice is presently unclear. However, three possible roles for CD4+ effector cells can be envisaged: direct, CD4+ T cell-mediated cytotoxicity (32, 33); CD4+ T cell production of cytokines that stimulate other effector cells; and CD4+ T cell production of cytokines that act directly to inhibit virus production (34). The regulation of these CD4+ T cells that mediate the effective anti-virus immune response in C57BL mice is also unclear. The present studies using C57BL/6-Ifg KO mice depleted of CD8− T cells demonstrate that the CD4+ effector T cells generated in these mice are not dependent on the production of IFN-γ. In addition, differences between BALB and C57BL mice with respect to the generation of these CD4+ effector T cells indicates that the process is regulated by a non-H-2-linked gene(s).

Similar to C57BL mice, but in contrast to BALB mice, BALB/c-Ifg KO mice are able to generate an effective immune response in the absence of CD8+ T cells (Table V). This result demonstrated the important role of IL-4 production in influencing the generation of CD4+ effector cells during the acute phase of E-55+MuLV infection. Interestingly, uninfected BALB spleen cells, like infected BALB, exhibited elevated levels of IL-4, indicating that these mice have a predisposition to generate IL-4-producing cells. These cells could be Th2 CD4+ T cells, non-T cells (eosinophils, basophils, mast cells), or NK1.1 cells, and their early IL-4 production may strongly influence the immune response to a pathogen (12). Other groups have also reported that BALB/c mice may have a predisposition to early IL-4 production. For example, it has been demonstrated that T cells from TCR transgenic BALB/c mice produce significantly higher levels of IL-4 compared with the same TCR transgenic B10.D2 T cells in vitro and in vivo (35, 36). Therefore, differences in the genetic backgrounds of BALB and C57BL mice appear to influence early IL-4 expression, which, in turn, may suppress the generation of anti-virus CD4+ effectors.

Infected B6 mice produce high IFN-γ (Table III) but, in contrast to BALB mice, no detectable IL-4 (Table IV), which is consistent with a type 1 response. IFN-γ is known to be a very important and usually required participant in the induction of anti-virus immune responses (7, 37–40). However, in a manner similar to E-55+MuLV infection, the generation of effective immune responses against gammaherpes virus (41), Sendai virus (42), rotavirus (43), and LCMV (when high affinity anti-LCMV CD8+ T cells are generated) (44) have been demonstrated to be IFN-γ independent, although the anti-virus immune response is mediated by CD8+ T cells. In the present studies, B6-Ifg KO mice (Table VI) are capable of decreasing the virus burden even in the absence of both IFN-γ and CD8+ T cells, indicating that the generation and function of the CD4+ effectors in this infection is IFN-γ independent. It appears that this is the first study to observe that neither IFN-γ nor generation of CD8+ T cells is required for an effective anti-MuLV immune response.

Although infected B6-Ifg KO mice produce low levels of IL-4 (2–3 U/ml), this IL-4 production does not reduce the ability of these mice to generate CD4+ effectors in the absence of CD8+ T cells. This lack of effect of IL-4 production in mice with a B6 background was in contrast to the pronounced effect of IL-4 production on the ability to generate CD4+ effector cells in mice with a BALB background. This difference in the effect of IL-4 could indicate either that the level of IL-4 production in B6 mice is too low to influence the response in these mice or that there is a difference in responsiveness to IL-4 between BALB and C57BL mice. Another plausible explanation is related to the fact that timing of IL-4 production appears to be very important in the generation of immune responses to L major (6). Thus, differences in the timing of IL-4 production in BALB/c and B6-Il-4 KO mice may be responsible for the phenotypic differences between these strains with respect to CD4+ T cell activity in CD8-depleted mice.

The studies described in this report were designed to examine the role of the T cell response during the acute phase of E-55+MuLV infection. It is clear from previous studies with other viruses that Ab production also plays an important role in resistance to virus infection. Generation of virus-neutralizing Ab appears to be essential for recovery from certain virus infections, such as FV infection (45). During FV infection (FV is an acute transforming retrovirus, in contrast to E-55+MuLV, which is a chronic transforming retrovirus), BALB/c mice produce low titers of virus-neutralizing Ab and, therefore, develop erythroleukemia. In contrast, B6 mice that are resistant to FV-induced erythroleukemia generate high titers of neutralizing Ab (5). However, both BALB.K and B10.BR mice generate similar levels of E-55+MuLV-neutralizing and cytotoxic Ab (2). Therefore, there is no evidence that a difference in the humoral immune response between BALB and C57BL mice contributes to the differences between these strains with respect to their requirement for CD8+ T cells to decrease virus titers during the acute phase of E-55+MuLV infection. However, preliminary studies in this laboratory indicate that Ab production is essential for an effective
anti-E-55+ MuLV immune response during the acute phase of infection. The observation that B6 μMT (B cell-deficient) mice were unable to decrease virus burden during the acute phase of E-55+MuLV infection (our unpublished observations) indicates that Ab production is necessary to mediate the decrease in virus titer that occurs during the acute phase of infection in normal mice.

The present study confirms previous findings (3) that show that the immune response to a certain stimulus (E-55+MuLV infection) is genetically controlled by non-MHC-linked genetic loci. Elimination of CD8+ T cells in BALB and C57BL strains reveals a difference in phenotype between the strains studied; BALB mice are unable to lower the virus-infected cells during the acute phase of infection, whereas C57BL mice retain this ability. Phenotypic analysis of the RI (CXB) strains and backcross mice depleted of CD8+ T cells indicate that more than one genetic locus influences this phenotype. Genes that control this phenotype could regulate the ability to employ an alternative mechanism that evolves in the absence of CD8+ T cells, and that may reflect differences in the type of response under normal conditions. Since IL-4 appears to be a modulator of this immune response, these loci (or one of them) may regulate early IL-4 production and/or responsiveness to IL-4 via other molecules. Linkage analysis based on the CXB strains resulted in two candidate regions: one on distal chromosome 7 and another at the centromeric end of chromosome 19.

In the candidate region of chromosome 19 there are several genes (cd98, cd20, ptpcap) that regulate immune-related functions. Ptpcap (or cd45-ap) is a candidate gene located at the same position as D19Mit23 (0.5 cM) that encodes the CD45-associated protein (46, 47). CD45 is a receptor phosphatase that mediates signals that induce production of several cytokines, including IL-4. CD45-associated protein is expressed in T cells (48) and acts as an adaptor that may bind to lck and allow CD45 to deactivate lck (49). Deactivation of lck, activates, via the PLCg phospholipase, a pathway that results in activation of the transcription factor NF-AT, which induces transcription of several cytokines, including IL-4. NF-AT knockout mice exhibit a striking defect in early production of IL-4 (50).

Linkage analysis also pointed to the D7Mit223 marker, which is at the same position as the fibrolast growth factor-3 and -4 (51). However, the possible influence of these molecules in the immune response during the acute phase of E-55+MuLV infection is currently unclear. Other genes not yet identified in the same region may play a role in this infection. The chromosomal candidate regions described above will be further investigated with the use of a large number of backcross mice. It is interesting that the regions of chromosomes 7 and 19 associated with the CD8+ requirement during the acute phase of E-55+MuLV infection have no correlation with the genetic loci that regulate cytokine profile or resistance to L. major in mice (52, 53).

The 13 CXB RI strains were also observed for progression to lymphoma or long-term survival after infection with E-55+MuLV (our unpublished data). The SDP obtained for this phenotype was not concordant with the SDP for the phenotype defined by the requirement for CD8+ T cells to reduce virus burden during the acute phase of the E-55+MuLV infection (Fig. 4). The results showed that the CXB L and the CXB O strains that had demonstrated inability to generate an effective immune response in the absence of CD8+ T cells during the acute phase of infection (similar to the parental BALB/c) did not progress to lymphomas as did the parental BALB/c mice. Therefore, there is no apparent correlation between the differential requirement of CD8+ T cells during the acute phase and the progression to E-55+MuLV-induced disease. This conclusion is also indicated by the observation that all (eight progressors of a total of eight mice) BALB/c-IL-4 KO mice progress to lymphomas similar to normal BALB/c mice (our unpublished data). Thus, although the BALB/c-IL-4 KO mice are able to generate an effective anti-virus immune response in the absence of CD8+ T cells, these mice still progress to disease. These observations do not exclude the possibility that one of the genes that control the immune response during the acute phase of infection plays a role, in combination with other genes, in the regulation of the progression to E-55+MuLV-induced disease during the persistent phase of infection.

In most cases, different immune responses result in different outcomes. However, in the case of E-55+MuLV infection of BALB and C57BL mice described in these studies, the same outcome (decline of virus burden during the acute phase of infection) is accomplished in mice that display differences in their anti-virus immune responses. This observation highlights the complexity as well as the flexibility of the immune system. Differences in the mechanism of the immune response during the acute phase of infection may contribute to different outcomes during the persistent phase of the same infection. This possibility and the fact that the response is genetically regulated may contribute to the understanding of differences during the persistent phase of HIV infection that distinguish individuals who progress to AIDS and those who remain long term nonprogressors (54, 55).

Acknowledgments

We thank Kathryn Hunter for technical assistance, and Dr. John Dzuris for establishing and characterizing the 663B Tumor cell line.

References

MuLV IMMUNE RESPONSE DURING ACUTE PHASE OF INFECTION


