NK cell functions restrain T cell responses during viral infections

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NK cell functions for regulation of T cell responses were evaluated during acute viral infections. In vivo depletion studies established that the presence of NK cells in murine cytomegalovirus (MCMV)-infected immunocompetent mice negatively affected CD4 and CD8 T cell IFN-γ expression, bromodeoxyuridine (BrdU) incorporation, and expansion. To evaluate NK cell effects, under conditions when NK cells do not control viral replication, experiments were performed using lymphocytic choriomeningitis virus (LCMV). Depletion of NK cells did not affect LCMV-elicited T cell responses in immunocompetent mice; however, the presence of NK cells did inhibit CD4 T cell IFN-γ production, BrdU incorporation, and expansion in infected MHC class I- and CD8 T cell-deficient β2M−/− mice. Together, the results reveal a previously unappreciated immunoregulatory role of NK cells for downstream T cell responses.

Key words: NK cell / T lymphocyte / Viral infection / Immunoregulation / Cell-to-cell interaction

1 Introduction

Through their production of cytokines and acquisition of cytotoxic activity, NK cells have the potential to function in defense against viral infections by exerting either direct antiviral and/or immunomodulatory effects [1]. NK cell production of particular cytokines, such as IFN-γ and TNF, and/or killing of virus-infected cells may deliver direct antiviral functions. Alternatively or additionally, NK cells may mediate immunoregulatory effects, to promote or regulate particular adaptive immune response, by producing cytokines and/or by destroying cells such as APC. Although NK cell-mediated antiviral effects resulting from IFN-γ production [2] or perforin-dependent action [3] has been documented during MCMV infections, little has been done to elucidate NK cell immunoregulatory effects and the mechanisms for these during viral infections. It is remarkable that high NK cell cytotoxicity is induced during LCMV infections, but that the response, in contrast to that induced during MCMV infections, has no detectable role in controlling viral replication [1, 4, 5]. The issue is timely because of the recent association of perforin deficiencies with a genetic immune dysregulation disease [6, 7].

2 Results

2.1 NK cell regulation of T cell responses during MCMV infections

To determine whether NK cells had effects on later T cell responses, immunocompetent C57BL/6 mice were infected with 1×10⁴ pfu of MCMV and control-treated or...
depleted of NK cells by administration of specific antibodies in vivo (n=12). Splenic leukocytes at day 9 after infection were evaluated by flow cytometry for CD4 and CD8 T cell numbers as well as intracellular IFN-γ protein expression. Although proportions of CD4 T cells decreased from 18±0% to 14±1% as a result of NK cell depletion (p<0.01), total numbers of CD4 T cells were not significantly different (p=0.5). CD8 T cell proportions were elevated from 16±1% to 23±1% with NK cell depletion (p<0.01) (Fig. 1A), and CD8 T cell numbers increased from 12(±1)×10^6 to 21(±3)×10^6 (p<0.05). Interestingly, however, in vivo depletion of NK cells did result in increased proportions of CD4 T cells primed to express IFN-γ. Of the day 9 CD4 T cells in the NK cell-replete, infected mice, 7±1% were IFN-γ+. In contrast, 22±5% of the CD4 T cells were IFN-γ+ in NK cell-depleted, infected mice (p<0.01) (Fig. 1B). Absolute numbers of CD4 T cells expressing IFN-γ also were increased in the NK cell-depleted samples to 2(±0.3) as compared to 0.9(±0)×10^6 per spleen (p<0.01) (Fig. 1C). Similarly, of the day 9 CD8 T cells, 17±4% were IFN-γ+ and depletion of NK cells resulted in increased proportions with 37±8% of CD8 T cells being IFN-γ+ (p<0.05) (Fig. 1B). Absolute numbers of CD8 T cells expressing IFN-γ also were increased in the NK cell-depleted group to 6±0.8, compared to 2(±0.3)×10^6 per spleen (p<0.01) (Fig. 1D). Thus, the presence of NK cells resulted in limiting the proportions and numbers of both CD4 and CD8 T cell primed to express IFN-γ.

To determine whether the early NK cell response also affected T cell proliferative responses, mice were administered BrdU in drinking water during the course of infection. Splenic leukocytes (n=7) at day 9 after infection were evaluated by flow cytometric methods for BrdU incorporation into DNA. Of the ex vivo control day 9 CD4 T cells, 27±4% were BrdU+ (Fig. 1E). In vivo depletion of NK cells resulted in increased proportions of CD4 T cells incorporating BrdU, with 34±6% of CD4 T cells being BrdU+ (p=0.2); however, absolute numbers per spleen of CD4 T cells incorporating BrdU did not change, i.e. 2.7(±0.5)×10^6 in the NK cell-depleted group compared to 3(±0.5)×10^6 in the control-treated group (p=0.9) (Fig. 1F). Of the ex vivo control day 9 CD8 T cells, 54±6%...
were BrdU+ (Fig. 1E). *In vivo* depletion of NK cells resulted in increased proportions of CD8 T cells expressing BrdU, with 70±6% of CD8 T cells being BrdU+ (*p*<0.05). Absolute numbers per spleen of CD8 T cells incorporating BrdU, at this later time point, however, were increased, with 9.1±1×10^6 in the NK cell-depleted group compared to 6±0.6×10^6 in the control-treated group (*p*<0.05) (Fig. 1G). As demonstrated by immunohistochemical studies on splenic sections, following NK cell depletions of day 9 MCMV-infected C57BL/6 mice, BrdU-incorporating cells were increased, with 792±61 BrdU+ nuclei enumerated per area as compared to 166±58 in control-treated mice (*p*<0.05). BrdU+ nuclei were distributed throughout T cell-containing regions of the spleen but were particularly concentrated at red and white pulp borders (Fig. 2A, B). Thus, during MCMV infections the presence of NK cells limits both T cell IFN-γ expression and T cell proliferation.

### 2.2 NK cell regulation of T cell responses during LCMV infections

The above data suggested that NK cell responses exerted immunoregulatory functions on downstream T cell responses. Although the virus was cleared in both the control and NK cell-depleted mice by day 9 under the conditions of infection used (data not shown), it was possible that the presence of NK cells, by decreasing early viral burdens, secondarily limited T cell responses. To circumvent this potentially confounding factor, experiments were performed using an infection with no demonstrable role for NK cells in regulation of viral burden, LCMV [1, 4, 5]. Depletion of NK cells in LCMV-infected immunocompetent mice did not alter CD4 or CD8 T cell IFN-γ expression, BrdU incorporation, or cell expansion (data not shown). Previous studies have shown that LCMV infections of MHC class I- and CD8 T cell-deficient β2M<sup>−/−</sup> mice induce NK cell blastogenesis, proliferation, and cytotoxicity to prominent levels, and that similar but lesser NK cell responses are observed in CD8 T cell-depleted C57BL/6 mice [10–12]. To de-

![Fig. 2. Immunohistochemical localization of BrdU+ cells. Day 9 MCMV-infected C57BL/6 mice (A, B) or day 7 LCMV-infected β2M<sup>−/−</sup> mice (C, D), treated with either control Ig (A, C) or PK136 mAb (B, D) to deplete NK cells, were given BrdU in drinking water from the day of infection onwards. Immunohistochemical staining of splenic sections for BrdU-incorporating cells was performed. Shown are representative fields at a magnification of ×400. Arrows in (B) and (D) point to positive cells.](image-url)
termine whether conditions of augmented NK cell responses limit T cell responses during LCMV infections, CD4 T cell expansion was evaluated in CD8 T cell-deficient mice. Numbers of CD4 T cells were not elevated in NK cell-depleted compared to control-treated CD8−/− C57BL/6 mice (data not shown). However, depletion of NK cells in β2M−/− mice did result in increased CD4 T cell responses similar to those observed during MCMV infections (see below).

During LCMV infections of β2M−/− mice, peak levels of IFN-γ are produced in the spleen on day 7 after infection [12]. Depletion of NK cells resulted in a >17-fold increase by day 7 splenic leukocytes, with minimal changes in plasma IFN-γ levels (Table 1). In contrast, CD4 T cell depletion resulted in a >95% decrease in IFN-γ production by day 7 ex vivo splenic leukocytes, as well as an approximately 40% decrease in plasma IFN-γ levels (Table 1). Similar trends were observed later on day 9, with depletion of NK cells resulting in increased IFN-γ production by splenic leukocytes and systemically in plasma, and depletion of CD4 T cells resulting in decreased IFN-γ production in both compartments (Table 1). Intracellular flow cytometric analyses, performed to evaluate IFN-γ expression, revealed low proportions of CD4 T cells expressing IFN-γ protein in β2M−/− mice. Of the ex vivo control day 9 CD4 T cells (n=7), 8±1% were IFN-γ+ (Fig. 3A). In contrast, no detectable IFN-γ was observed in NK cells even after short-term incubation using immobilized anti-NK1.1 to boost cytokine signal (data not shown). In vivo depletion of NK

<table>
<thead>
<tr>
<th>Treatment Conditioned media IFN-γ (pg/10⁷ cells)</th>
<th>Plasma IFN-γ (pg/ml)</th>
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<tr>
<td>Day 0</td>
<td>&lt; 10</td>
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<tr>
<td>Day 7</td>
<td>4110 ± 2106</td>
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<tr>
<td>Day 7 control Ig</td>
<td>2401 ± 1248</td>
</tr>
<tr>
<td>Day 7 anti-NK1.1</td>
<td>41668 ± 17897**</td>
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<tr>
<td>Day 7 anti-CD4</td>
<td>76 ± 27**</td>
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<td>Day 9</td>
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<tr>
<td>Day 9 control Ig</td>
<td>631 ± 163</td>
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<tr>
<td>Day 9 anti-NK1.1</td>
<td>6205 ± 422*</td>
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<td>Day 9 anti-CD4</td>
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a) Antibody treatments, sample preparation, and ELISA quantitation were performed as described in Sect. 4. Values shown are mean ± SE, with one to two mice for untreated groups and three to eight individual mice per antibody-treated group. Differences were significant compared to control Ig, * 0.05<p<0.1, ** p<0.05. Results shown are representative of two independent experiments, with additional two to four mice per group.

cells resulted in increased proportions of CD4 T cells expressing IFN-γ, with 19±2% of CD4 T cells being IFN-γ+ (n=8) (p<0.01). Absolute numbers per spleen of

Fig. 3. Effects of NK cell depletion on T cell responses to LCMV infection in β2M−/− mice. Day 7 or day 9 LCMV-infected β2M−/− mice, untreated, control Ig-treated or given PK136 mAb to deplete NK cells, were either not treated or given BrdU in drinking water from the day of infection onwards. Flow cytometric analyses for CD4 (log x-axes) versus intracellular IFN-γ (log y-axes) were performed to evaluate proportions and absolute numbers of cells per spleen primed to express IFN-γ (A and B). Similarly, flow cytometric evaluation of CD4 (log x-axes) versus incorporation of BrdU (log y-axes) were performed to quantitate proportions (C) and absolute numbers (D) of BrdU+ cells. Representative contour plots from day 9 (A) and day 7 (C) studies, and mean results ± SE are presented. Results from uninfected mice, day 0, are given (B, D) for comparison. In these experiments, there were six to eight mice per group, except for untreated mice (n=3).
CD4 T cells expressing IFN-γ also were increased, with \(3(\pm1)\times10^6\) in the NK cell-depleted group, compared to \(0.5(\pm0)\times10^6\) in the control-treated group (\(p<0.01\)) (Fig. 3B). Similar trends were observed in analogous experiments using day 7 LCMV-infected mice. Hence, in \(\beta 2 M^{-/-}\) mice, IFN-γ is predominantly due to CD4 T cell synthesis, and the presence of NK cells negatively regulates this response.

To determine whether the NK cells in \(\beta 2 M^{-/-}\) mice inhibited T cell expansion, BrdU incorporation was evaluated. Of the ex vivo control day 7 CD4 T cells (\(n=7\), 9±2% had incorporated BrdU into DNA (Fig. 3C). In vivo depletion of NK cells resulted in increased proportions of CD4 T cells incorporating BrdU, with 23±3% of CD4 T cells NK cells resulted in increased proportions of CD4 T cells incorporating BrdU, with 23±3% of CD4 T cells NK cell-depleted (\(n=6\)) (\(p<0.01\)). Absolute numbers per spleen of CD4 T cells expressing IFN-γ+ were \(9(\pm0.7)\times10^6\) in the NK cell-depleted group compared to 0.4(±0.1)\times10^6 in the control-treated group (\(p<0.01\)) (Fig. 3D). As demonstrated by immuno-histochemical studies, the number of BrdU-incorporating cells was increased following depletions of LCMV-infected \(\beta 2 M^{-/-}\) mice, with 683±127 BrdU+ nuclei enumerated over eight ×400 fields, as compared to 135±50 in control Ig-treated mice (\(p<0.05\)) (Fig. 2C, D). These cells were also distributed throughout T cell-containing regions of the spleen but appeared to be at higher proportions at red and white pulp borders. Both relative proportions and absolute numbers of splenic CD4 T cells on either day 7 or 9 after infection were elevated in NK cell-depleted compared to control-treated \(\beta 2 M^{-/-}\) mice, respectively with 18(±2)\times10^6 versus 6(±1)\times10^6 (\(p<0.0001\)) and 18(±2)\times10^6 versus 7(±0)\times10^6 total CD4 T cells per spleen (\(p=0.001\)). Similar increases in CD4 T cell numbers were observed in inguinal and mesenteric lymph node compartments (data not shown). The augmented CD4 T cell responses in the absence of NK cells did not result from increased viral replication, as kidney viral titers, expressed as log (pfu/g), were 4.3±0.1 in control day 7 infected mice compared to 0.4(±0.1)\times10^6 in the NK cell-depleted group (\(n=4\) on day 7 after infection, and 5.3±0.1 in the control-treated group (\(n=5\) and 5.4±0.1 in the NK cell-depleted group (\(n=6\)) on day 9 after infection. These data indicate that the presence of NK cells results in negative regulation of CD4 T cell proliferation as well as cytokine production responses during LCMV infections of \(\beta 2 M^{-/-}\) mice.

### 3 Discussion

The studies presented here reveal a previously unappreciated immunoregulatory function for NK cell responses. During MCMV infections of immunocompetent mice, the absence of NK cells resulted in augmented CD4 and CD8 T cell IFN-γ production, and in the case of CD8 T cells, increased BrdU incorporation and cell expansion. NK cell depletions in LCMV-infected immunocompetent mice did not alter T cell responses. However, NK cell depletions in MHC class I- and CD8 T cell-deficient \(\beta 2 M^{-/-}\) mice resulted in augmented CD4 T cell IFN-γ production and proliferative responses. Together, the studies indicate that NK cells can function to restrain or shape T cell responses during viral infections.

Interestingly, our results are consistent with the report that in common cytokine receptor \(\gamma\)-chain-/- mice, lacking both NK and CD8 T cells, CD4 T cells producing IFN-γ protect against Toxoplasma gondii infections [13]. In total, the studies suggest that NK cells may act to provide conditions regulating CD4 T cells, but that these effects are only demonstrable or critical in the absence of dramatic CD8 T cell responses. NK and CD8 T cells mediate many of the same types of effects including IFN-γ production and target cell lysis. Although both MCMV and LCMV dramatically increase NK cell cytotoxicity, CD8 T cell responses to MCMV infection are more modest than those to LCMV infection. Thus, the identification of a role for NK cells in T cell regulation during infections of immunocompetent mice with MCMV may be possible because CD8 T cells do not readily substitute the function. In contrast, the ability to only demonstrate an NK cell-mediated effect on CD4 T cell responses requires the common \(\gamma\)-chain-deficient mouse may in part be a consequence of substitution of the functions by activated CD8 T cells. Likewise, the revelation of an important CD4 T cell IFN-γ response to T. gondii infections in the common \(\gamma\)-chain-deficient mouse might be possible because both NK and CD8 T cell responses are missing.

The mechanism or mechanisms by which NK cells regulate the T cell responses are unknown. NK cells express perforin and have been shown to kill a variety of hematopoietic cells including T and B cell progenitors and APC such as members of monocyte/macrophage and dendritic cell lineages [14, 15]. NK cell-mediated lysis of APC is an attractive potential mechanism because reductions in T cell responses are likely to result from decreases in APC available for their stimulation. Interestingly, we have previously demonstrated that under the conditions of infection, there is an NK-dependent cell trafficking to splenic marginal zone areas rich in APC [16]. Such a trafficking pattern may get NK cells to sites to down-regulate the numbers and/or functions of these populations. Complementary studies at the later times during LCMV infections associated with profound CD8 T cell cytotoxic responses have demonstrated that CD8 T cells can be found at similar sites [17, 18], and that there is perforin-dependent negative regulation of the magnitude of CD8 T cell expansion [19–21]. As NK and CD8 T cells...
can mediate overlapping functions, there is a potential for a perforin-dependent regulation from either or both populations. However, we have only been able to reveal a partial role for perforin in regulation of CD4 T cells during LCMV infections of β2M−/−×perforin−/− as compared to β2M−/−×perforin+/+ mice (unpublished data). NK cells are armed with several mechanisms capable of decreasing numbers of other immune cell subsets; they have been reported to kill by secreting TNF, as well as through cognate interactions using Fas ligand [1, 22, 23].

The inability to reveal NK cell-mediated inhibition of CD4 T cell responses in CD8 T cell-deficient only as compared to CD8 T cell-deficient β2M−/− mice suggests that additional variables are altered in the latter case. One possibility is that NKT cells, present in immunocompetent and CD8 T cell-deficient but not in β2M−/− mice, also contribute. This is not likely because the conditions used to deplete NK cells, i.e. anti-NK1.1 antibody treatments, also should have depleted NKT cells. A more likely possibility is that the presence of MHC class I molecules inhibited the immunoregulatory function of NK cells. Although in vitro studies have demonstrated that MHC class I molecules inhibit the immunoregulatory function of NK cells, the presence of MHC class I molecules cannot inhibit NK cell responses, the ability of developing NK cells to adapt to differing basal levels of MHC class I to set a threshold for reactivity has not been demonstrated in β2M−/− mice [11].

The results presented here suggest that an endogenous function of NK cells is to negatively regulate T cell responses to microbial challenges. The unique requirement for NK cells may occur only under conditions where there is little stimulation of CD8 T cell responses because antigens are not readily processed and presented by class I MHC pathway. These would include infections with certain viruses, in particular the herpes group viruses including MCMV, which have evolved evasion mechanisms to down-regulate or sequester MHC class I molecules, thereby preventing development of efficient CD8 T cell responses. The effects may act to shape the nature of the adaptive responses. Their major importance, however, is likely to be to limit potential immunopathology resulting from deregulated immune functions. Continuous stimulation of T cells by APC and the feedback stimulation of APC by T cells could result in escalating production of cytokines, such as TNF and IFN-γ, which mediate detrimental and even life-threatening effects at high concentrations. Such a function is consistent with the apparent role for perforin in protecting against the immune dysregulation disease, familial hemophagocytic lymphohistiocytosis [6, 7]. Taken together, these observations indicate that immune functions mediated by NK cell responses may act to protect the host against both the infectious agent and the immune response to the agent.

4 Materials and methods

4.1 Mice

Specific pathogen-free male C57BL/6J-β2mmtm1Unc, C57BL/6-CD8αmtm1Mak, and C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, ME. In some cases, C57BL/6J-β2mmtm1Unc mice were maintained by breeding at Brown University. Mice were used at 5–28 weeks of age and were handled in accordance with institutional guidelines for animal care and use. Mice were inoculated i.p. on day 0 with 1×10⁶ pfu MCMV, Smith strain, or 2×10⁶ pfu LCMV, Armstrong strain, clone E350. For in vivo depletion of NK or CD4 T cell subsets, mice were, respectively, given partially purified PK136 or GK1.5.6 i.p. 1 day prior to infection, and again at 2 and 5 days after infection. Control treatments were with equivalent amounts of mouse or rat Ig (Sigma Chemical Co., St. Louis, MO). The protocols, respectively, resulted in <1.7% or <0.4% detectable remaining NK or CD4 T cells in spleens. BrdU (Sigma) was administered ad libitum in drinking water, at a concentration of 0.4 mg/ml, starting on day 0. Plasma were collected as described [9, 12].

4.2 Preparation of cells and conditioned media samples

Splenic leukocytes were obtained, quantitated, and cultured in the absence of additional stimuli for 24 h as previously described [12]. Cell-free conditioned media were harvested and stored at −20°C.

4.3 Flow cytometric analyses

Detection of cell surface NK and/or T cell markers and intracellular IFN-γ expression was performed as reported [10, 12]. Antibodies (BD Pharmingen, San Diego, CA) were anti-NK1.1 R-PE-conjugated mouse mAb PK136, anti-CD4 R-PE-conjugated rat mAb RM4–5, anti-CD8α chain FITC- or allophycocyanin-conjugated rat mAb 53–6.7, anti-CD8β chain FITC-conjugated rat mAb 53–5.8, anti-CD3ε FITC- or cyChrome-conjugated hamster mAb 145–2C11. FITC-conjugated streptavidin was used to detect biotinylated reagents. Controls were antibodies lacking specificities for murine determinants and/or absence of primary antibody. For intracellular flow cytometric analyses, cells were stimulated with immobilized anti-CD3 mAb 145–2C11 or anti-NK1.1 mAb PK136 for 6 h at 37°C, with brefeldin A added during the last 2 h for anti-CD3 or the last 4 h for anti-NK1.1
stainings. Cells were labeled with surface makers, fixed, permeabilized, and treated with anti-mouse-IFN-γ R-PE-conjugated rat mAb XMG1.2. Cytokine specificity was proven by preincubation of antibodies with rmIFN-γ (Phar-Mingen). For identification of BrdU-incorporating cells, surface stained cells were fixed in 75% ethanol at 4°C for 30 min, and permeabilized with 0.01% Tween 20 in 1% paraformaldehyde at room temperature (RT) for 30 min. Samples were treated with 50 units of DNase I (Sigma) in 0.15 M NaCl/0.04 M MgCl2, for 15 min at RT, then incubated for 30 min at RT with FITC- or PE-conjugated mouse anti-BrdU mAb clone 3D4 or control mouse IgG1 clone MOPC. Results were acquired on a FACSCalibur, using CellQuest versions 1.2.2, 3.0, or 3.1 software package. Argon laser output was 15 mW at 488 nm, and red diode laser 15 mW at 635 nm.

4.4 ELISA
Quantitation of IFN-γ proteins by sandwich ELISA was performed as previously described [2, 12]. Limit of detection was 9.8–19.7 pg/ml.

4.5 Viral titers
LCMV and MCMV titers were quantitated in homogenates of previously frozen tissues by standard plaque assays on Vero or NIH 3T3 monolayers, respectively, as previously described [2, 12]. Viral titers are expressed as log pfu/g tissue.

4.6 Immunohistochemistry
Spleens were formalin-fixed, paraffin-embedded, and sectioned as described [16]. Slides were deparaffinized by warming at 56°C for 15 min, clearing in two changes of xylene, washing in graded alcohol series, and rehydrated with PBS. Endogenous peroxidase activity was blocked in 3% H2O2 for 10 min at 37°C. DNA was denatured by placing slides in 2 N HCl for 30 min at 37°C. Unmasking was performed by incubating tissues with 0.1% trypsin solution for 30 min at 37°C. Tritrated amounts of anti-BrdU mAb clone BU-33 (Sigma) were added for 2 h at 37°C, and blocked and detected using avidin/biotin blocking and mouse on mouse immunodetection kits as per manufacturer’s instructions (Vector Laboratories, Burlingame, CA). Nova-Red peroxidase substrate solution was applied for approximately 2–5 min. Tissues were counterstained with hematoxylin for 10 s. Numbers of BrdU+ nuclei were enumerated over eight fields at a magnification of ×400.

4.7 Statistical analysis
Unless otherwise indicated, mean ± SE are shown. Two-tailed Mann-Whitney U tests were performed using STATVIEW 4.5 (Abacus Concepts, Inc., Berkeley, CA).

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