Deficiency in the Anti-Apoptotic Protein A1-a Results in a Diminished Acute Inflammatory Response

Amos Orlofsky,2* Louis M. Weiss,*† Nicole Kawachi,* and Michael B. Prystowsky* A1 is an anti-apoptotic member of the Bcl-2 family that is up-regulated in inflammatory myeloid cells. In the present study, we investigated the role of A1 in the maintenance of acute inflammation in mice. Mice possess three genes encoding highly related isoforms of A1. A1-a isoform mRNA was minimally expressed in resident peritoneal macrophages, but was present at a 300-fold higher level in inflammatory macrophages elicited by i.p. infection with Toxoplasma gondii. In comparison, A1-b and A1-d levels were 3- and 10-fold higher, respectively. Peritoneal leukocytosis was decreased in infected A1-a-deficient mice compared with wild-type, and this reduction was associated with a small but reproducible enhancement of survival. These effects could not be explained by an alteration in peritoneal parasite load, nor by increased apoptosis of infected inflammatory cells, which were protected from cell death by an A1-a-independent mechanism. Increased apoptosis in inflammatory neutrophils was observed sporadically in A1-a-deficient mice. Regulation of apoptosis by A1-a may be an important proinflammatory event in acute host responses. The Journal of Immunology, 2002, 168: 1840–1846.

A growing body of evidence indicates that programmed cell death (apoptosis) is an important mechanism for the clearance of inflammatory cells, and that the entry of these cells into apoptosis is regulated during the inflammatory process. For neutrophils in particular, apoptosis appears to be the major mechanism of clearance (1), and both cell culture and in vivo studies have indicated that the lifespan of these cells is altered following extravasation to the inflammatory site and exposure to cytokines and other stimuli (2–6). A natural hypothesis is that modulation of leukocyte apoptosis by inflammatory mediators is a key factor in the regulation of the inflammatory response. In support of this idea, a recent study of mice deficient in the lectin galectin-3 showed deficient inflammation in this strain associated with enhanced apoptosis of cultured macrophages (7). Conversely, mice deficient in the integrin CD11b responded to i.p. thioglycollate with enhanced apoptosis of cultured macrophages (7). These studies examined genes that have important functions unrelated to apoptosis. To definitively address the hypothesis requires the identification and targeting of molecules that function more narrowly to regulate cell death in inflammatory cells.

The Bcl-2 family consists exclusively of proteins that either promote or inhibit apoptosis (8). Three of the anti-apoptotic family members have been observed in myeloid leukocytes: A1 and Mcl-1 occur in neutrophils (9–13), while A1 and Bcl-xL can both be up-regulated in macrophages by proinflammatory stimuli in vitro (14, 15). Hamasaki et al. (16) have shown that A1 deficiency accelerates spontaneous neutrophil apoptosis and abolishes the anti-apoptotic effects of either LPS treatment in vitro or elicitation in vivo with protease peptone. We have shown that A1 is strongly expressed in inflammatory neutrophils and macrophages in Toxoplasma gondii-infected mice (12). Therefore, we have focused our efforts on determining the role of this protein in inflammation. It has recently been demonstrated that mice contain at least three genes encoding isoforms of A1 that are 97% identical in amino acid sequence (13). Only one of these isoforms, A1-a, has been used in previous functional studies. In the current study, we show that A1-a is regulated more tightly than the other isoforms, such that its mRNA expression level in inflammatory macrophages is several orders of magnitude greater than that in normal macrophages. Furthermore, gene targeting of this isoform results in attenuation of inflammation and prolonged survival in T. gondii-infected mice.

Materials and Methods

Mice

Female BALB/c mice (6–8 wk) were obtained from The Jackson Laboratory (Bar Harbor, ME). To generate A1-a gene-targeted mice, an A1-a genomic clone was isolated from a 129 Ola phage library (a kind gift from Dr. W. Edelmann, Albert Einstein College of Medicine, Bronx, NY). A 6.5-kb EcoRI fragment, containing the intron flanking sequence and terminating in exon 1, was subcloned and inserted in the EcoRI site of pPNT (17), with transcriptional orientation opposed to that of the neoR gene. This construct was digested with BbrPI, which cuts at –387 in the 5′ flanking region with EcoRI to selectively cleave the site in exon 1, blunted and ligated to generate pA15pr. A 4.9-kb EcoRI fragment, containing the intron and exon 2, was subcloned from the A1-a phase. A 2.7-kb NsiI-PstI fragment from this subclone was inserted in the Sse8387I site of pA15pr. The resulting construct was linearized with NotI and electroporated into the embryonic stem cell line WW6 (18). Hemizygous G418R clones were identified by Southern blot detection of the novel NotI band described in Results and Discussion and injected into C57BL/6 blastocysts by the Albert Einstein College of Medicine Transgenic and Gene Targeting Core Facility (Bronx, NY). The resulting chimeras were mated with C57BL/6 mice. The resulting chimeras were mated with C57BL/6 mice. The embryonic stem cell-derived progeny of a single mating were characterized as hemizygous or wild-type by PCR detection of the neoR gene. These hemizygous and wild-type mice were self-mated to generate homozygous A1-a+/- and A1-a-/- stocks, respectively. Homozygosity was confirmed.
by Southern blot and A1-a-specific PCR as described in Results and Discussion. The genetic background of the resulting strains is ~60% C57BL/6, 38% 129/Sv, and 2% SJL.

Preparation of inflammatory exudates

The RH strain (Sabin of T. gondii) was maintained on human fibroblasts as described previously (19). Supernatants containing 107 tachyzoites/ml were diluted in calcium- and magnesium-free PBS. A volume of 0.4 ml containing 1–2 × 105 tachyzoites was injected i.p. For initiation of TG peritonitis, BALB/c mice were injected i.p. with 1.0 ml of sterile 3% TG broth (Difco, Detroit, MI) aged several months. Mice were sacrificed by cervical dislocation and the peritoneal cavity washed with 4 ml of ice-cold PBS containing 0.2% BSA (Sigma Aldrich, St. Louis, MO). Cellularity and extracellular tachyzoites were assessed with a hemacytometer. A portion of each sample was cytocentrifuged (700 rpm, 5 min). Slides were either air-dried, fixed in methanol and stained with Diff-Quik (Dade, Miami, FL) for differential counting and assessment of intracellular parasitization, or fixed in cold paraformaldehyde (4% in PBS) for 20 min, washed several times with PBS, and stored in PBS at 5°C. The remainder of the sample was centrifuged (at 150 × g for 10 min), lysed with TRIzol (Roche Molecular Biochemicals, Indianapolis, IN), and RNA extracted according to the manufacturer’s protocol.

Macrophage cell culture

Marrow expressed from femurs was spun over Histopaque, 1.077 g/ml (Sigma Aldrich), at 400 × g for 20 min. The interface was washed and cultured for 24 h at 37°C in MEM containing 20% heat-inactivated FCS, 1000 U/ml M-CSF (a gift from Chiron, Emeryville, CA; Ref. 20), and 0.1 mM Na selenite. Nonadherent cells were collected, diluted 3-fold, and cultured in tissue-culture dishes (BD Biosciences, Bedford, MA). M-CSF (1000 U/ml) was added on day 6. Each bar or lane represents an individual mouse. The genetic content of the PCR product, as previously described (13), the reaction was then digested with a combination of NsiI and BglII (Roche Molecular Biochemicals) using the manufacturer’s Buffer H. The relative proportions of the specific bands representing A1-a, A1-b, and A1-d were quantitated in agarose gels stained with Sybr Gold (Molecular Probes, Eugene, OR) using the Superscript preamplification system (Life Technologies) with an oligo(dT) primer. PCR was performed on 10% of the cDNA product using the primers described by Hatekayama et al. (Ref. 13; forward primer = AATTTCCAAACAGCCTCCAGATATG; reverse primer = GAAACAAAATATCTGCAACTCTGG), Taq polymerase (Life Technologies), and 1.5 mM MgCl2. Primers (200 nM) were added after the reaction mix was brought to 85°C. A total of 35 cycles were performed using an annealing temperature of 55°C. To analyze the isoform content of the PCR product, as previously described (13), the reaction was digested with NsiI and BglII (Roche Molecular Biochemicals) using the manufacturer’s Buffer H. The relative proportions of the specific bands representing A1-a, A1-b, and A1-d were quantitated in agarose gels stained with Sybr Gold (Molecular Probes, Eugene, OR) using a fluorimeter (Molecular Dynamics). Similar digestions of RT-PCR products from COS cells transfected with amplifiable constructs expressing either A1-a, A1-b, or A1-d cDNAs were used to verify that digestion went to completion under these conditions (data not shown). To compare the expression of each isoform between samples, each normalized Northern blot or RPA signal was multiplied by the relative fraction for each isoform.

RNA analysis

Total A1 expression was assessed either by Northern blot or ribonuclease protection assay (RPA). As previously described (12), Northern blots were sequentially probed with an A1-a cDNA clone and a 28S rRNA oligonucleotide, followed by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). For RPA, RNA (10 μg) was hybridized overnight at 56°C to the mAPO-2 Riboquant probe set (BD PharMingen, San Diego, CA) labeled with [32P]UTP and analyzed on a 5% polyacrylamide gel according to the manufacturer’s protocol. Bands were analyzed by PhosphorImager, using GADPH for normalization. For RT-PCR analysis, cDNA was generated from RNA samples (1 μg) using the Superscript premultiplication system (Life Technologies) with an oligo(dT) primer. PCR was performed on 10% of the cDNA product using the primers described by Hatekayama et al. (Ref. 13; forward primer = AATTTCCAAACAGCCTCCAGATATG; reverse primer = GAAACAAAATATCTGCAACTCTGG), Taq polymerase (Life Technologies), and 1.5 mM MgCl2. Primers (200 nM) were added after the reaction mix was brought to 85°C. A total of 35 cycles were performed using an annealing temperature of 55°C. To analyze the isoform content of the PCR product, as previously described (13), the reaction was digested with NsiI and BglII (Roche Molecular Biochemicals) using the manufacturer’s Buffer H. The relative proportions of the specific bands representing A1-a, A1-b, and A1-d were quantitated in agarose gels stained with Sybr Gold (Molecular Probes, Eugene, OR) using a fluorimeter (Molecular Dynamics). Similar digestions of RT-PCR products from COS cells transfected with amplifiable constructs expressing either A1-a, A1-b, or A1-d cDNAs were used to verify that digestion went to completion under these conditions (data not shown). To compare the expression of each isoform between samples, each normalized Northern blot or RPA signal was multiplied by the relative fraction for each isoform.

FIGURE 1. Pathogen-induced A1-a expression. BALB/c mice were inoculated with either 1000 T. gondii tachyzoites, or TG and exudates collected after 6 days. Each bar or lane represents an individual mouse. A, Northern blot analysis of total A1 mRNA expression. B, RT-PCR analysis of the relative proportion of three A1 isoforms within each sample. C, Quantitation of isoform expression by combining the data of A and B. Values represent the mean ± SE for three mice. The value for A1-a in control mice is set to one. The data are representative of two similar experiments.
as determined by the intensities of the RT-PCR bands after digestion, according to the formula: (A1-x isoform expression) = (normalized A1 PhosphorImager signal × A1-x fluorimager signal)/(sum of fluorimager signals for A1-a, A1-b, and A1-d).

Assessment of cell death
Paraformaldehyde-fixed cytoplasmic preparations were stained by fluorescein-labeled TUNEL (Roche Molecular Biochemicals), using the manufacturer’s protocol. Slides were counterstained with propidium iodide (50 μg/ml) for 5 min to permit distinction of granulocytes by nuclear morphology. Slides were counted blind (500 cells), and fields were systematically chosen for microscopic viewing by Nomarski optics before epifluorescent illumination. To assess the infection rate in apoptotic macrophage-like cells, TUNEL-positive cells were first identified under green fluorescence and then examined for organisms under red fluorescence.

Results and Discussion
Disproportionate up-regulation of the A1-a isoform by selective inflammatory stimuli
To assess the relative potential contributions of three A1 isoforms (A1-a, A1-b, and A1-d) to the inflammatory response, we measured the mRNA levels of these isoforms in inflammatory exudates from T. gondii-infected mice in comparison with resident peritoneal cells from uninfected mice. As previously described (12), total A1 mRNA was elevated in infected mice compared with either control or TG-treated animals (Fig. 1A). Analysis of the same samples by RT-PCR revealed a preferential relative enhancement of A1-a expression in infected mice, but not in TG-induced inflammation (Fig. 1B). The RT-PCR and Northern signals were combined as described in Materials and Methods to compare isoform expression among samples (Fig. 1C). Basal expression of A1-b and A1-d was evident in resident peritoneal cells from untreated mice, whereas A1-a expression was near the limit of detection (<0.5% of total A1 mRNA). In contrast, A1-a expression in T. gondii-elicted exudate was comparable to that of the other two isoforms, increasing to 24% of total A1 mRNA. Thus, while A1-b and A1-d levels increased 3- and 11-fold, respectively, A1-a mRNA increased ~300-fold. Corresponding induction of A1-a protein in T. gondii-elicted macrophages was previously demonstrated using an A1-a-specific Ab (12). Similarly, A1-a is weakly expressed in untreated bone marrow-derived macrophages, but is preferentially increased by treatment with LPS (not shown). Thus, A1-a is tightly regulated in normal macrophages, but is dramatically induced by certain proinflammatory stimuli.

Production of gene-targeted mice selectively deficient in A1-a
The progeny from a single chimera were intercrossed to generate A1-a−/− and A1-a+/+ homozygous lines. Southern blot analysis indicated the specific alteration of a single A1 gene (Fig. 2B), and PCR analysis confirmed that the affected gene was A1-a (Fig. 2C). The A1-a−/− mice had normal viability and did not display any abnormality by gross or histopathology. A previously reported targeting of the A1-a gene by Hamasaki et al. (16) resulted in a severe repression (>10-fold) of the expression of A1-b and A1-d in addition to the A1-a deficiency. These authors replaced ~1.8 kb of the A1-a 5′ flank, whereas we replaced only 386 bp. To determine whether our knockout strain was selectively deficient in A1-a, we performed a combined RPA + RT-PCR analysis on RNA derived from normal and GM-CSF-stimulated bone marrow-derived macrophages from A1-a−/− and A1-a+/+ mice. As shown in Fig. 3, A1-b and A1-d expression were unaffected in the knockout mice, whereas A1-a expression was completely eliminated.

Hamasaki et al. (16) reported no alteration in circulating neutrophil levels in their A1-deficient mice. Similarly, our A1-a−/− strain was comparable to wild-type with respect to both absolute neutrophil count (790 ± 140/μl vs 880 ± 250/μl, respectively) and total white blood cell count (7400 ± 800/μl vs 8100 ± 1100/μl; n = 5).
Attenuation of toxoplasmic peritonitis in A1-a-deficient mice

A1-a-targeted mice are selectively deficient in A1-a gene expression. A, RPA analysis of total A1 expression. RNA from control or GM-CSF-treated bone marrow-derived macrophages was analyzed. B, RT-PCR analysis of isoform expression in targeted mice. RNA from control (lanes 1, 3, and 5) or GM-CSF-treated (lanes 2, 4, and 6) bone marrow-derived macrophages from A1-a+/+ (lanes 1 and 2) or A1-a-/- (lanes 3–6) mice was analyzed. Lanes 5 and 6 are an overexposure of lanes 3 and 4 to show the absence of A1-a mRNA. C, Specificity of the isoform expression deficiency in targeted mice. The RT-PCR data from B were combined with the RPA signals from A to derive quantified isoform expression in GM-CSF-treated macrophages.

Attenuation of toxoplasmic peritonitis in A1-a-deficient mice

i.p. infection with tachyzoites of the virulent RH strain of T. gondii induces a vigorous acute peritoneal leukocytosis concomitant with a progressive systemic infection culminating in death of the host at ~1 wk postinfection (p.i.). At a dose of 2000 tachyzoites, the peritoneal inflammation manifests as a mild neutrophilia for ~2 days followed by a rapid influx of neutrophils and macrophages leading to increased cellularity by 4 days p.i. (12). When the course of inflammation was compared in A1-a+/+ and A1-a-/- mice, we found that while a 2-fold increase in peritoneal cellularity was observed as expected in the wild-type strain, no such increase occurred in A1-a-deficient mice (Fig. 4A). Nevertheless, the two strains were equivalent with respect to the shift in cellular composition accompanying inflammation, as evidenced by the similarity in the proportion of granulocytes present at all time points (Fig. 4B). Therefore, the effect of the removal of A1-a was to dampen rather than to retard or prevent the inflammatory response. A more detailed time-course confirmed that this dampening did not reflect any deficit in cellularity before the onset of massive leukocytic infiltration at 4 days p.i. (Fig. 4C). A decline in cellularity occurred before day 4 in both strains; this finding is similar to early-stage observations in other models of peritoneal inflammation (22, 23).

These findings of dampened inflammation are highly reproducible. Over the course of four experiments, we have analyzed 25
mice at day 4 p.i., and 27 mice at day 5 p.i. The aggregate mean peritoneal cellularity at day 4 (×10^6) is 14.7 ± 1.51 in the wild-type, compared with 9.69 ± 1.15 in the mutant (p = 0.013), while at day 5 p.i. the mean is 20.5 ± 1.83 in the wild-type, compared with 11.35 ± 0.94 in the mutant strain (p = 0.00008).

A second difference observed between the two strains was that the A1-a-/- mice survived a day longer (p < 0.05 by log-rank test; Fig. 5). This effect was reproducible and suggests that mortality is to some extent related to the progression of inflammation. This notion is consistent with a report identifying pneumonia as the principal cause of death in RH-infected animals (24). Our findings are also reminiscent of a recent study in which mice deficient in CCR1 were orally infected with RH. In this case, the mutant animals showed both a transitory decline in tissue neutrophil levels and a 1- to 2-day delay in the onset of death relative to controls (25).

An anti-apoptotic mechanism is retained in A1-a-deficient inflammatory cells

Several recent studies have linked intracellular protozoa with the modulation of apoptosis in host cells. One group has reported induction of apoptosis by RH in cultured peritoneal macrophages (26), while others have observed that apoptosis was prevented by RH infection of HL-60 cells (27) or lymphoid cells (28), as well as by Leishmania major infection of macrophages (29). Protection of HL-60 cells was especially pronounced in cells that harbored parasites (27). We asked whether such a mechanism might operate in vivo and might be A1-a dependent, thus providing a potential explanation for the dampened inflammation in our mutant mice. We examined exudate cells that had been stained for TUNEL reactivity and counterstained with propidium iodide to distinguish mononuclear and granulocytic cells by nuclear morphology as well as to determine parasite content. Total mononuclear cell apoptosis increased progressively in infected mice (Fig. 6A), although in a sporadic fashion that made it difficult to detect a genotype-dependent effect. To assess the association of apoptosis with intracellular parasitization, we focused on “macrophage-like” cells (mononuclear cells with extensive cytoplasm) in which parasitization could be readily determined. As shown in Table I, although most macrophages were infected at 5–6 days p.i., <20% of apoptotic macrophage-like cells harbored parasites. The number of parasites per infected cell was also substantially less in the apoptotic population (data not shown). Furthermore, the protective effect of intracellular infection was equivalent in wild-type and A1-a-deficient mice. An example of infected TUNEL-negative and uninfected TUNEL-positive macrophage-like cells is shown in Fig. 6B. These results

FIGURE 5. Enhanced survival of infected A1-a-deficient mice. Five A1-a+/+ and six A1-a-/- mice were assessed daily for survival. The data are representative of two similar experiments.

FIGURE 6. Apoptosis and parasite growth in A1-a-deficient mice. Mice were infected with 2000 tachyzoites and exudates collected for counting of free tachyzoites (C) and for staining for TUNEL reactivity, with a propidium iodide counterstain (A, B, and D). Each bar represents an individual mouse. B. The same field (representing a day-6 A1-a-/- animal) is viewed for either orange (propidium iodide) or green (TUNEL) emission. Arrows indicate intracellular RH organisms in macrophage-like cells, and an arrowhead indicates the TUNEL-positive nucleus of an uninfected cell.
argue strongly for a pathogen-mediated anti-apoptotic mechanism (that is demonstrated in vivo for the first time in this study), and indicate that this mechanism is A1-a-independent.

It is possible that even a modest increase in apoptosis of infected cells could significantly affect parasite growth, which might in turn result in dampened inflammation. Some variation was observed in parasite growth patterns from experiment to experiment. However, in the experiment shown in Fig. 4, there was no evidence of a decreased parasite growth rate in A1-a-deficient mice (Fig. 6C). Therefore, such a mechanism is unlikely to account for the observed phenotype.

Finally, it is possible that reduced inflammation is the consequence of accelerated apoptosis in uninfected “bystander” infiltrating leukocytes, or alternatively in myeloid precursors during reactive hematopoiesis. We have not obtained data that conclusively address this question; however, we observed the sporadic occurrence of greatly elevated frequencies of TUNEL-positive granulocytes in A1-a/-infected mice (Fig. 6D), consistent with such a model. Alternatively, the critical effect of A1-a may reside in very early infiltrating or even resident peritoneal cells. We have found that at least for cultured resident peritoneal macrophages, RH infection does not elicit A1-a-sensitive apoptosis, and in fact, results in A1-a-independent protection from spontaneous apoptosis in bystander cells (data not shown). Future experiments will address these various models.

It is still unclear why mice have three potentially functional forms of A1, while humans have one. It is possible that the three isoforms are functionally redundant, and that the phenotype we observe reflects a simple quantitative change in total A1 expression. Hamesaki et al. (16), using what was in effect a “total A1”-deficient strain, found that even heterozygote neutrophils were deficient in inflammation-mediated protection from spontaneous apoptosis, indicating that a 50% reduction in total A1 could produce an observable defect. A1-a mRNA was reported to represent about one-third of total neutrophil A1 mRNA (13), and the proportion is conceivably greater in the neutrophils generated in our system. Therefore, a total absence of A1-a may be quantitatively similar to a 50% loss of total A1. In contrast, the dramatic pathogen-elicited increase in the A1-a/total A1 ratio that we have observed both in this study and with mycobacterial infection (30) suggests a specific function for this isoform. Ultimately, isoform-specific gain-of-function or loss-of-function mutations will be necessary to resolve this issue.

The findings reported here represent the first demonstration that a genetic modification specifically targeted to the regulation of apoptosis can alter the acute inflammatory response to a pathogen. The implication of this result is that, at least for some period of the inflammatory response, the rate at which cells enter apoptosis is a critical limiting factor for the intensity of the response. It follows that appropriate regulation of this rate represents a crucial decision by the host, which must balance defense against the negative impact of inflammation. That negative impact is manifested here as reduced time-to-death, but in many clinically relevant settings is likely to involve autoimmunity or tissue damage. A key unresolved question is under what circumstances, if any, the host makes this decision incorrectly, and how can the outcome be corrected by intervention in such cases. The fact that the decision is made differently in different settings is indicated by the fact that the survival rate of peritoneal neutrophils can be either greater or less than that of circulating cells depending on the inflammatory stimulus used (2, 3). Our results suggest that the regulation of A1 expression may be an essential feature in the formation of host defense strategy during acute responses.

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