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ORIGINAL ARTICLE

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Melphalan-induced up-regulation of B7–1 surface expression on normal splenic B cells

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Abstract We have previously shown that exposure of MOPC-315 or P815 tumor cells to the widely used anticancer drug melphalan (L-PAM, L-phenylalanine mustard) leads to rapid up-regulation of B7–1 surface expression. Since B7–1-expressing tumor cells depend on B7-expressing host antigen presenting cells (APC) for the generation of $CD8^+$ T-cell-mediated antitumor immunity, and since L-PAM promotes the acquisition of tumor-eradicating immunity by $CD8^+$ T-cells from MOPC-315 tumor bearers, the current studies were undertaken to determine if L-PAM also up-regulates B7–1 expression on host APC. Here we show that exposure of normal spleen cells to L-PAM leads within 24 h to up-regulated B7–1 expression on $B220^+$ cells (B cells). Studies into the mechanism through which L-PAM leads to up-regulated B7–1 expression revealed that within 2 h after exposure of normal spleen cells to L-PAM, accumulation of B7–1 mRNA is evident and this accumulation requires de novo RNA synthesis, indicating that the regulation is at the transcriptional level. The L-PAM-induced accumulation of B7–1 mRNA was prevented with the antioxidant *N*-acetyl-L-cysteine (NAC), indicating that reactive oxygen species are important for the transcriptional regulation. Although AP-1 and NF-κB are considered redox-sensitive transcription factors, L-PAM led only to activation of NF-κB that bound specifically to a probe containing the corresponding binding site in the B7–1 gene. Moreover, selective inhibition of NF-κB activation prevented the L-PAM-induced B7–1 mRNA accumulation, indicating that NF-κB activation is essential for L-PAM-induced B7–1 gene expression in normal spleen cells. Finally, in vivo administration of an immunopotentiating dose of L-PAM to normal mice was found to up-regulate B7–1 mRNA expression in their spleens. Thus, the ability of L-PAM to up-regulate B7–1 expression not only on tumor cells but also on host cells may contribute to the potentiating activity of L-PAM for the acquisition of $CD8^+$ T-cell-mediated tumor-eradicating immunity in tumor bearers.

Keywords B cell · Costimulatory molecule · Gene regulation · Melphalan-induced gene expression

Abbreviations *ADM* adriamycin · *APC* · antigen presenting cell · EMSA electrophoretic mobility shift assay · IKK IκB-kinase · L-PAM L-phenylalanine mustard · *mAb* monoclonal antibody · NAC · *N*-acetyl-L-cysteine · *NEMO* NF-κB essential modifier · *NIgG* normal IgG

Introduction

T-cell activation requires at least two signals. The first signal is antigen specific and is delivered by interaction between the T-cell receptor and peptide presented in association with major histocompatibility complex (MHC). The second signal is antigen non-specific and can be delivered by interaction between costimulatory molecules on antigen-presenting cells (APC) and their receptors on T-cells [6, 24, 25]. Interaction between CD28 on T-cells and members of the B7 family on APC is widely recognized as a major costimulatory pathway in a wide array of T-cell responses including the acquisition of antitumor immunity [6], and inadequate costimulation via CD28 has been implicated in tumor progression in immunocompetent hosts [1, 8]. Therefore, in an attempt to elicit a more powerful antitumor immune response several groups of investigators have transfected the B7–1 or the B7–2 gene into tumor cells (that express tumor-associated antigens in the context of MHC) in order to turn the tumor cells into efficient APC [17, 27, 46, 50]. These studies revealed that tumor cells transfected with the B7–1 gene or the B7–2 gene can in

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some tumor systems trigger the development of sufficient T-cell-mediated tumor-eradicating immunity to lead to their rejection and provide immunoprotection against a challenge with unmodified (B7-negative) parental tumor cells. Given the ability of B7 expression on tumor cells to promote the acquisition of tumor-eradicating immunity, it is interesting to note that the widely used anticancer drug L-PAM, which was shown to bring about the acquisition of $CD8^+$ T-cell-mediated tumor-eradicating immunity in mice bearing a large MOPC-315 tumor [33, 45], was recently found to rapidly induce B7–1 (although not B7–2) expression on the surface of MOPC-315 and P815 tumor cells [11, 43].

However, although B7–1 expression on tumor cells is important for the acquisition of $CD8^+$ T-cell-mediated tumor-eradicating immunity, the vast majority of the $CD8^+$ T-cell-mediated antitumor activity elicited by B7–1-expressing tumor cells was shown in several systems to depend on B7-expressing host APC [23, 28, 44]. In fact, B7–1 expression on tumor cells is believed to promote the acquisition of $CD8^+$ T-cell-mediated antitumor activity by making tumor-associated antigens more readily available to host APC for antigen presentation [7, 20, 35, 49]. This may happen as a result of the ability of B7–1-expressing tumor cells to activate directly $CD8^+$ T-cells and NK cells as well as a result of increased susceptibility of B7–1-expressing tumor cells to lysis by cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells [7, 20, 35, 49].

The importance of B7 expression on host APC for the acquisition of $CD8^+$ T-cell-mediated antitumor activity against B7-expressing tumor cells suggests that it would be beneficial for the realization of the immunopotentiating effect of L-PAM for $CD8^+$ T-cell-mediated tumor-eradicating immunity if L-PAM would up-regulate B7–1 expression not only on tumor cells but also on host APC. The current studies were undertaken to determine if L-PAM can actually up-regulate B7–1 surface expression on host APC. As a prototype of host APC, we examined the effect of L-PAM on B7–1 expression on the surface of normal splenic B cells. Here we show that L-PAM rapidly induces the expression of B7–1 molecules on the surface of a substantial percentage of splenic B cells. In addition, we describe studies into the molecular mechanisms through which L-PAM up-regulates B7–1 expression on normal spleen cells. Finally, we provide data illustrating that in vivo administration of a low-dose of L-PAM, which was previously shown to exert an immunopotentiating effect of $CD8^+$ T-cell-mediated tumor-eradicating immunity in tumor bearers [33, 45], can lead to up-regulated expression of B7–1 mRNA in host cells.

Materials and methods

L-PAM treatment

A fresh stock solution of L-PAM (Sigma-Aldrich, St. Louis, Mo.) was prepared as previously described [6]. In experiments assessing the effect of in vitro exposure to L-PAM on B7–1 expression, spleen cells from normal 8–12-week old BALB/cAnNCrlBR mice (Charles Rivers Breeding Laboratories, Wilmington, Mass.) were exposed in vitro to 30 nM L-PAM, as previously described [6, 11, 43]. Unless otherwise stated, 60 min after initiation of the L-PAM treatment, the cells were washed three times and cultured in DMEM supplemented with 10% FBS, 5×10^{-5} M 2-ME (Sigma-Aldrich), 1% nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, and 15 mM HEPES buffer (Invitrogen Life Technologies, Carlsbad, Calif.) for the indicated periods of time. In experiments assessing the effect of in vivo administration of L-PAM on B7–1 expression, normal BALB/c mice were given an i.p. injection of 2.0 mg L-PAM per kg body weight (low-dose), and 2 or 3 h later the mice were sacrificed and their spleens were examined for B7–1 expression.

Flow cytometric analysis

In experiments assessing the effect of L-PAM on B7–1 expression on the surface of $B220^+$ cells, untreated spleen cells as well as spleen cells exposed in vitro to 30 nM L-PAM for 1 h were cultured for 23 h. Subsequently, the cells were stained with PE-conjugated anti-B7–1 (16–10A) and with FITC-conjugated anti-B220 monoclonal antibodies (mAb; BD Biosciences-PharMingen, San Diego, Calif.). As a control, we used PE-conjugated isotype matched normal IgG (NIgG). Flow cytometric analysis of 10,000 viable cells was carried out on a Coulter EPICS Elite ESP (Coulter Electronics, Hialeah, Fla.). The experiment was performed four times, and the results of a representative experiment are provided in the form of a histogram.

RT-PCR

Total RNA was extracted from untreated spleen cells, as well as from spleen cells exposed in vitro or in vivo to L-PAM. The RNA was subjected to reverse transcription followed by polymerase chain reaction (PCR), as previously described [11], with sense and antisense primers specific for B7–1. β-actin (Stratagene, La Jolla, Calif.) served as a standard to normalize for the quantity of mRNA subjected to PCR in the various samples within the same experiment. PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized by UV light. The sizes of the PCR products were determined using a standard 100-bp DNA ladder (Invitrogen Life Technologies) and were found to be of the expected size (i.e. ~300 bp). Each experiment was performed at least three times, and the results of a representative experiment are provided.

Assessment of inhibitory activity for L-PAM-induced B7–1 mRNA accumulation

In experiments assessing the importance of RNA synthesis or reactive oxygen species for L-PAM-induced accumulation of B7–1 mRNA, normal spleen cells were treated with actinomycin D (1 μg/ml) [43], or *N*-acetyl-L-cysteine (NAC) (25 mM) [48], respectively, for 1 h prior to their exposure to 30 nM L-PAM as well as during the L-PAM treatment. In experiments evaluating the importance of NF-κB activation for the L-PAM-induced B7–1 gene expression, we used a cell-permeable peptide of the following sequence DRQIKIWFQNRRMKWKKTALDWSWLQTE, which was shown by May et al. [29] to block the binding of the NF-κB essential modifier (NEMO) to the IκB-kinase (IKK) complex (wild-type peptide) thereby selectively inhibiting NF-κB activation. Specifically, normal spleen cells were exposed to 50 μM of the wild-type peptide for 1 h prior to their exposure to L-PAM as well as during the L-PAM treatment. As a control, normal spleen cells were exposed for 1 h prior to their exposure to L-PAM as well as during the L-PAM treatment to 50 μM of a mutant peptide (DRQIKIWFQNRRMKWKKTALDASALQTE) in which W was substituted with A in the two underlined positions and consequently the peptide does not inhibit NEMO binding to the IKK complex [29].

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method of Osborn et al. [36], and 1 μg of nuclear proteins was incubated for 20 min at 25°C with 50 fmol of a ^{32}P-labeled double-stranded oligonucleotide probe. For the evaluation of the activation of AP-1 we used a probe that includes the consensus AP-1 binding site in the promoter region of the B7–1 gene (5'-TCTAGTGTTAGTCACCCCACCC-3') [40], while for the evaluation of the activation of NF-κB we used a probe that includes the consensus NF-κB binding site in the enhancer region of the B7–1 gene (5'-GGGAAAGGGGTTTTCCCAGCAGTCA-3') [52]. Binding reactions contained 10 mM Tris-HCl (pH7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 4% glycerol, 0.1% NP-40, and 0.1–0.250 μg poly dI:dC. The free and protein-bound oligonucleotide probes were separated by electrophoresis on a 5% polyacrylamide gel. Subsequently, the gels were dried and the bands were visualized by autoradiography. The specificity of binding was examined by competition with a 25-fold molar excess of unlabeled specific oligonucleotide, and by competition with a 25-fold excess of an unlabeled mutant oligonucleotide in which the G in the 3rd position and the T in the 8th position from the 5' end of the NF-κB binding site were each substituted with a C. Finally, each experiment was performed at least three times and the results of a representative experiment are presented.

Statistical analysis

To determine the significance of the difference between the percentage of B7–1$^+$ cells among B220$^+$ cells from L-PAM treated spleen cells and the percentage of B7–1$^+$ cells among B220$^+$ cells from untreated spleen cells, Student's *t*-test was employed.

Results

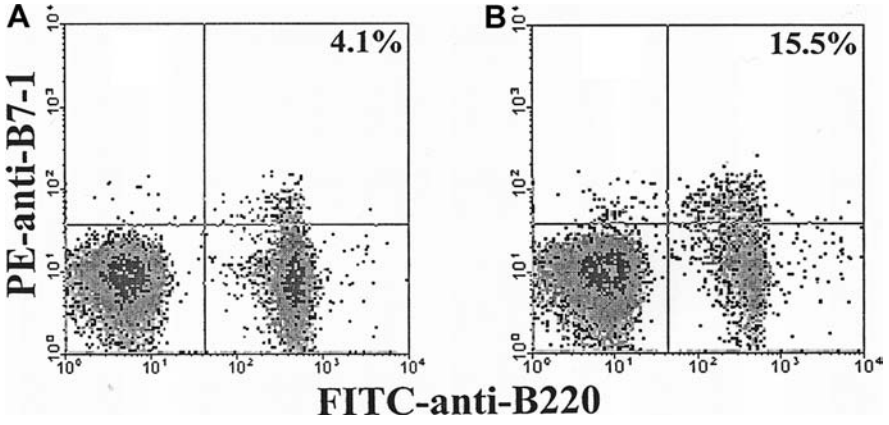
Exposure of normal spleen cells to L-PAM leads to up-regulated expression of B7–1 on the surface of B cells

We have recently shown that in vitro exposure of MOPC-315 or P815 tumor cells to a low concentration of L-PAM leads within 24 h to up-regulated expression of B7–1 (but not B7–2) on their surface [11, 43]. The current experiments were undertaken to determine if exposure of normal spleen cells to L-PAM would also lead to up-regulated expression of B7–1 on the surface of splenic APC. More specifically, we studied the ability of L-PAM to up-regulate B7–1 surface expression on B220$^+$ cells (B cells), as an indication of the ability of L-PAM to up-regulate B7–1 surface expression on host APC. For this purpose, normal spleen cells were exposed in vitro for 1 h to 30 nM L-PAM and then cultured for 23 additional hours before B7–1 surface expression on B220$^+$ was analyzed. A total of 4 separate experiments were carried out and the results of a representative experiment are provided in the form of a histogram. As seen in Fig. 1, exposure of normal spleen cells to L-PAM led to the expression of B7–1 molecules on a substantially higher percentage of B220$^+$ cells. Statistical analysis of the data from all 4 experiments revealed that the mean percentage of B220$^+$ cells that were positive for B7–1 expression increased as a consequence of the L-PAM treatment from 4.2 ± 1.2 to 16.7 ± 3.5 ($P = 0.026$). Thus, exposure to L-PAM, under conditions that were recently shown to up-regulate the expression of B7–1 on the surface of MOPC-315 and P815 tumor cells [11, 43], also leads to up-regulation of B7–1 expression on the surface of normal splenic B cells.

Fig. 1A, B Exposure to L-PAM leads to elevated expression of B7–1 on the surface of normal splenic B220$^+$ cells (B cells). Normal spleen cells were exposed in vitro for 1 h to 30 nM L-PAM or remained untreated and subsequently cultured for 23 h. At the end of the culture period, the untreated (**A**) as well as the L-PAM treated (**B**) spleen cells were stained with PE-conjugated anti-B7–1 and with FITC-conjugated anti-B220 mAb. The number at the top of each histogram represents the percentage of double positive cells among the B220$^+$ cells minus background staining (i.e. staining with NIgG instead of anti-B7–1 mAb)

Exposure of normal spleen cells to L-PAM leads to accumulation of B7–1 mRNA as a result of de novo RNA synthesis

We have recently shown that the L-PAM-induced up-regulation of B7–1 surface expression on P815 tumor cells requires de novo RNA synthesis and is associated with the accumulation of B7–1 mRNA [43]. These findings are not surprising with regard to P815 tumor cells since under normal conditions these cells do not synthesize B7–1 [17, 27, 43] and are negative for B7–1 mRNA expression [11, 43]. However, in contrast to P815 tumor cells, a small percentage of B220$^+$ cells among untreated spleen cells cultured for 23 h are



positive for B7–1 surface expression (Fig. 1), and consequently it cannot be concluded a priori that the L-PAM-induced up-regulation of B7–1 surface expression on normal splenic cells involves L-PAM-induced de novo synthesis of B7–1 mRNA. Therefore, experiments were undertaken to determine if exposure of normal spleen cells, like exposure of P815 tumor cells, to L-PAM would also lead to de novo B7–1 mRNA synthesis. As seen in Fig. 2, in vitro exposure of spleen cells from normal mice to 30 nM L-PAM resulted in accumulation of B7–1 mRNA at 2 and 3 h after initiation of the L-PAM treatment. Pretreatment of the spleen cells with actinomycin D, a known inhibitor of RNA synthesis, before their exposure to L-PAM as well as during the L-PAM exposure, completely prevented the L-PAM-induced accumulation of B7–1 mRNA (Fig. 3). Thus, like with P815 tumor cells [43], L-PAM treatment leads to rapid accumulation of B7–1 mRNA in spleen cells from normal mice and this accumulation requires de novo RNA synthesis, indicating that the regulation is at the transcriptional level.

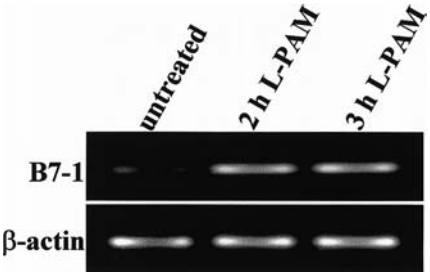


Fig. 2 Rapid accumulation of B7–1 mRNA following exposure of normal spleen cells to L-PAM. Normal spleen cells were exposed in vitro to 30 nM L-PAM. At the indicated time points after initiation of the L-PAM treatment, RNA was extracted from the L-PAM treated spleen cells as well as from untreated spleen cells and subjected to RT-PCR with primers specific for B7–1 or β-actin. The size of the PCR product for the B7–1 was ~300 bp

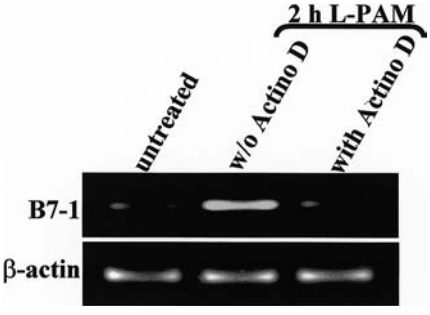


Fig. 3 The RNA synthesis inhibitor actinomycin D inhibits the L-PAM-induced accumulation of B7–1 mRNA in normal spleen cells. Normal spleen cells were exposed in vitro to L-PAM with or without actinomycin D (1 μg/ml). RNA extracted from these cells 2 h after initiation of the L-PAM treatment as well as RNA extracted from untreated spleen cells was subjected to RT-PCR with primers specific for B7–1 or β-actin

Reactive oxygen species are important for L-PAM-induced accumulation of B7–1 mRNA in normal spleen cells

Experiments were initiated to elucidate the molecular mechanisms through which L-PAM leads to up-regulation of B7–1 gene expression in normal spleen cells, since such information may provide some insights into how to manipulate B7–1 expression on normal host cells to the benefit of the host. We focused our attention first on the importance of reactive oxygen species for the L-PAM-induced up-regulation of B7–1 gene expression in normal spleen cells in light of the report by Gorman et al. [21] that L-PAM can increase the levels of intracellular reactive oxygen species in HL60 cells as well as other reports, including ours, that H_2O_2 can induce B7–1 expression on tumor cells [11, 34, 47]. The interest in determining the importance of reactive oxygen species for the L-PAM-induced up-regulation of B7–1 gene expression in normal spleen cells was also fueled by our recent observations that reactive oxygen species are important for L-PAM induced up-regulation of B7–1 gene expression in P815 tumor cells. Thus, we carried out studies to determine the effect of the antioxidant NAC on L-PAM-induced accumulation of B7–1 mRNA in normal spleen cells. As seen in Fig. 4, pretreatment of normal spleen cells with the NAC before their exposure to L-PAM as well as during the L-PAM exposure completely prevented the L-PAM-induced accumulation of B7–1 mRNA. These results indicate that reactive oxygen species are involved in the transcriptional regulation of L-PAM-induced B7–1 gene expression not only in P815 tumor cells [11] but also in normal spleen cells.

NF-κB, but not AP-1, that binds specifically to its cognate element in the enhancer/promoter region of the B7–1 gene is activated in L-PAM treated normal spleen cells

Experiments were carried out to determine if exposure of normal spleen cells to L-PAM leads to activation of the

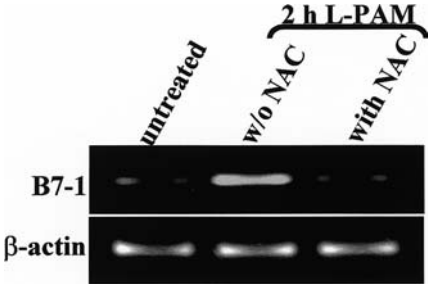


Fig. 4 The antioxidant NAC inhibits the L-PAM-induced accumulation of B7–1 mRNA in normal spleen cells. Normal spleen cells were exposed in vitro to L-PAM with or without NAC (25 mM). RNA extracted from these cells 2 h after initiation of the L-PAM treatment as well as RNA extracted from untreated spleen cells was subjected to RT-PCR with primers specific for B7–1 or β-actin

redox-sensitive transcription factor AP-1 and/or NF-κB [3, 30, 31, 37, 38] that binds specifically to a probe containing the AP-1 or NF-κB binding site in the promoter/enhancer region of the B7–1 gene. These experiments were carried out in light of our observations that reactive oxygen species are important for the transcriptional regulation of B7–1 gene expression in normal spleen cells (Fig. 4), and the enhancer/promoter region of the B7–1 gene has an NF-κB and an AP-1 binding element [40, 51, 52]. In addition, our recent observations that in P815 tumor cells L-PAM leads to activation of NF-κB, but not AP-1, that binds to its cognate element in the enhancer/promoter region of the B7–1 gene, also fueled our interest in determining if in normal spleen cells L-PAM also leads to preferential activation of NF-κB. It should be stressed, however, that the fact that L-PAM activates NF-κB and not AP-1 in P815 tumor cells does not necessarily mean that it would also activate NF-κB and not AP-1 in normal spleen cells. After all, Arts et al. [2] have recently shown that PMA treatment, which led to activation of tissue-type plasminogen activator (t-PA) gene expression in both endothelial and HeLa cells, activates JunB, JunD and c-Fos that binds specifically to a PMA responsive element only in endothelial cells but not in HeLa cells. To determine if L-PAM leads to activation of NF-κB and/or AP-1 that binds specifically to its cognate element in the enhancer/promoter region of the B7–1 gene, nuclear extracts were prepared from untreated normal spleen cells as well as L-PAM treated spleen cells 15 or 30 min after initiation of the L-PAM treatment. The nuclear extracts were then evaluated for binding to a ^{32}P-labeled double stranded oligonucleotide probe that contains the consensus AP-1 binding site in the promoter region of the B7–1 gene (5′-TCTAGTGTTAGTCACCCCACCC-3′) [40], or the consensus NF-κB binding site in the enhancer region of the B7–1 gene (5′-GGGAAAGGGGTTTTCCCAGCAGTCA-3′) [52]. As seen in Fig. 5, which provides the EMSA results with the probe containing the AP-1 binding site, while a protein-DNA complex was evident with nuclear extract from HeLa cells, a protein-DNA complex was not evident with nuclear extract obtained from untreated spleen cells or spleen cells exposed to L-PAM for 15 or 30 min. The specificity of the protein-DNA complex with nuclear extract from HeLa cells is indicated by the fact that the complex was competed out with a 25-fold molar excess of the specific competitor but not with a 25-fold molar excess of an unrelated competitor. Thus, exposure of normal spleen cells to L-PAM, like exposure of P815 tumor cells to L-PAM [11], does not activate AP-1 that binds to the AP-1 binding site in the promoter of the B7–1 gene.

The same nuclear extracts were also evaluated for binding to a probe containing the NF-κB binding site. As seen in Fig. 6A, two shifted protein-DNA complexes were evident with nuclear extract from untreated spleen cells. However, both of these shifted protein-DNA complexes were much more intense with nuclear extract obtained from normal spleen cells exposed to L-PAM for15 min, and to a lesser extent with nuclear extract obtained from spleen cells exposed to L-PAM for 30 min. The specificity of the two protein-DNA complexes is indicated by the fact that the upper and lower complexes were competed out with a 25-fold molar excess of the specific competitor, but not with a 25-fold molar excess of the mutant competitor (Fig. 6B). Thus, exposure of normal spleen cells to L-PAM, like exposure of P815 tumor cells to L-PAM [11] leads to a rapid and transient activation of NF-κB that binds specifically to the NF-κB binding site in the enhancer of the B7–1 gene.

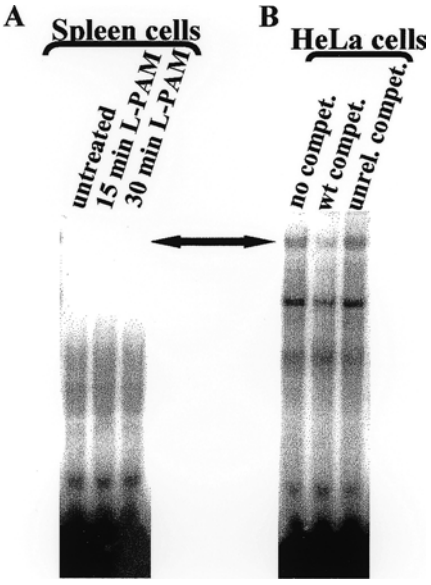


Fig. 5A, B Nuclear extracts from L-PAM treated normal spleen cells do not exhibit increased binding to the AP-1 binding site in the promoter of the B7–1 gene. Nuclear extracts were obtained from untreated normal spleen cells or L-PAM treated spleen cells 15 or 30 min after initiation of the L-PAM treatment (**A**). EMSA were performed using 1 μg of nuclear protein and 50 fM of a ^{32}P-labeled DNA probe containing the AP-1 binding site in the promoter of the B7–1 gene. As a positive control, we performed EMSA with nuclear proteins from HeLa cells (**B**). Specificity of the shifted protein-DNA complex was assessed by incubating nuclear proteins (1 μg) from HeLa cells with a 25-fold molar excess of unlabeled specific DNA probe or a 25-fold molar excess of unlabeled unrelated DNA probe

NF-κB activation is essential for L-PAM-induced B7–1 gene expression in normal spleen cells

Experiments were undertaken to elucidate the importance of L-PAM-induced NF-κB activation for L-PAM-induced B7–1 gene expression in normal spleen cells. For this purpose we utilized a cell-permeable peptide that selectively inhibits NF-κB activation [11, 29] (by blocking the binding of NEMO to the IKK complex [29]), and determined if this peptide would inhibit the L-PAM-induced up-regulation of B7–1 gene expression. Concurrently, we assessed the effect of a mutant peptide, which does not inhibit NF-κB activation [11, 29] (because it does not block the binding of NEMO to

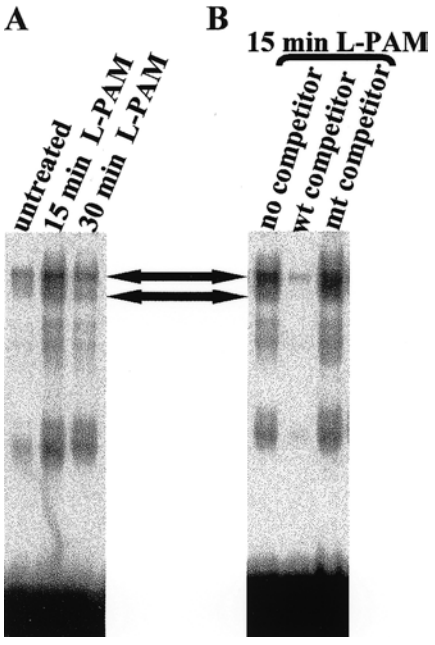


Fig. 6A, B Nuclear extracts from L-PAM treated normal spleen cells exhibit increased binding to the NF-κB binding site in the enhancer of the B7–1 gene. Nuclear extracts were obtained from untreated normal spleen cells or L-PAM treated normal spleen cells 15 or 30 min after initiation of the L-PAM treatment (**A**). EMSA were performed using 1 μg of nuclear protein and 50 fM of a ^{32}P-labeled DNA probe containing the NF-κB binding site in the enhancer of the B7–1 gene. Specificity of the shifted protein-DNA complexes was assessed by incubating nuclear proteins (1 μg) obtained from normal spleen cells that were exposed 15 min earlier to L-PAM with a 25-fold molar excess of unlabeled specific DNA probe or a 25-fold molar excess of unlabeled mutant DNA probe (**B**)

the IKK complex [29]), on L-PAM-induced B7–1 gene expression. Specifically, we incubated normal spleen cells with the wild-type peptide or mutant peptide for 1 h prior to their exposure to L-PAM as well as during the 1 h L-PAM treatment. After the completion of the L-PAM treatment, we evaluated the effect of the wild-type peptide and the mutant peptide on B7–1 mRNA accumulation. As seen in Fig. 7, exposure of normal spleen cells to the wild-type peptide prevented the L-PAM-induced B7–1 mRNA accumulation. In contrast, exposure of normal spleen cells to the mutant peptide did not inhibit the L-PAM-induced B7–1 mRNA accumulation. Thus, L-PAM-induced NF-κB activation is essential for L-PAM-induced B7–1 gene expression in normal spleen cells.

In vivo administration of a low dose of L-PAM to normal mice leads to rapid accumulation of B7–1 mRNA in their spleens

Experiments were carried out to determine if in vivo administration of a low-dose of L-PAM, which was previously shown to potentiate the acquisition of CD8$^+$ T-cell-mediated tumor-eradicating immunity in

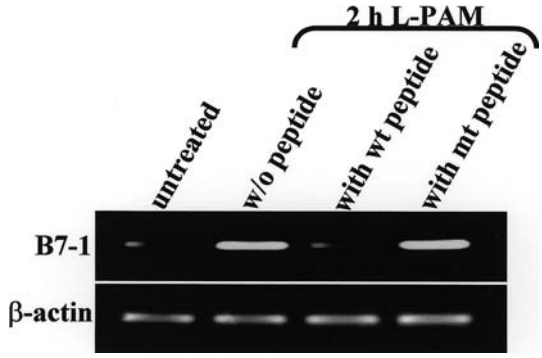


Fig. 7 Selective prevention of NF-κB activation inhibits the L-PAM-induced accumulation of B7–1 mRNA in normal spleen cells. RNA was obtained from untreated normal spleen cells, or normal spleen cells exposed in vitro to L-PAM in the presence or absence of a cell-permeable peptide that blocks the binding of NEMO to the IκB kinase complex (wild-type peptide). RNA was also obtained from normal spleen cells exposed in vitro to L-PAM in the presence of a mutant peptide that does not block the binding of NEMO to the IκB kinase complex. The RNA from the various groups was subjected to RT-PCR with primers specific for B7–1 or β-actin

MOPC-315 tumor bearers [33, 45], can lead to rapid up-regulation of B7–1 mRNA expression in host cells. Accordingly, normal BALB/c mice were given an i.p. injection of 2.0 mg/kg L-PAM and the level of B7–1 mRNA expressed in their spleens was determined 2 and 3 h later. As seen in Fig. 8, elevated B7–1 mRNA expression was evident in the spleens of normal mice at 2 and at 3 h after low-dose L-PAM administration. Thus, in vivo administration of a low dose of L-PAM, which was previously shown to exert an immunopotentiating effect on CD8$^+$ T-cell-mediated tumor-eradicating immunity in MOPC-315 tumor bearers [33, 45], leads to up-regulation of B7–1 expression in host cells.

Discussion

The ability of a wide variety of anticancer drugs to facilitate the acquisition of antitumor immunity in tumor

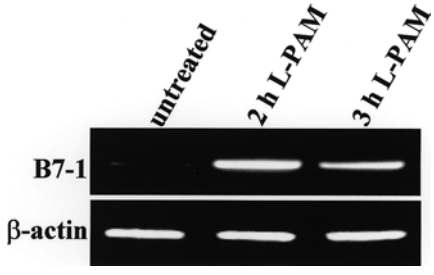


Fig. 8 Rapid accumulation of B7–1 mRNA in the spleen of normal mice injected with low-dose L-PAM. Normal BALB/c mice received an i.p. injection of 2.0 mg/kg L-PAM and 2 and 3 h later their spleen was excised. RNA extracted from the spleens of the L-PAM treated mice as well as RNA extracted from the spleens of untreated mice was subjected to RT-PCR with primers specific for B7–1 or β-actin

bearers has been recognized for some time (for a review please see [4, 32, 33]). Still, the mechanisms through which anticancer drug initiate their immunopotentiating effect for the acquisition of antitumor immunity remained elusive. Recently we found that L-PAM leads to the expression of functional B7–1 molecules on the surface of tumor cells as a result of L-PAM-induced rapid activation of B7–1 gene expression [11]. However, B7–1-expressing tumor cells depend on B7-expressing host APC for the generation of $CD8^+$ T-cell-mediated antitumor activity [23, 28], and therefore it would be beneficial for the generation of $CD8^+$ T-cell-mediated antitumor immunity if L-PAM would lead not only to up-regulation of B7–1 expression on tumor cells but also on host APC.

The current studies were undertaken to determine if exposure to L-PAM, under conditions that were previously shown to lead to rapid up-regulation of B7–1 expression on MOPC-315 and P815 tumor cells [11, 43], would also lead to up-regulation of B7–1 expression on host APCs. As a prototype of host APC, we chose to study the effect of L-PAM on splenic B cells because they are present in normal spleens at a much higher frequency than macrophages and dendritic cells, yet once they are induced to express B7 molecules on their surface they become highly efficient in antigen presentation [9, 14, 41]. Moreover, B7-expressing B cells were implicated in our previous studies in the L-PAM-induced acquisition of CTL activity by $CD8^+$ T-cells from mice bearing a large MOPC-315 tumor [10, 26]. Here we show that within 24 h after exposure of normal spleen cells to a low concentration of L-PAM there is a substantial increase in the percentage of $B220^+$ cells (B cells) that are positive for B7–1 surface expression. In this regard it is interesting to note that the increase in the percentage of B cells that are positive for B7–1 surface expression observed at 24 h after exposure of BALB/c spleen cells to L-PAM was similar to that seen in our previous studies at 24 h after exposure of BALB/c spleen cells to optimal concentration of the activating anti-CD40 mAb 1C10 [10].

We recently carried out studies to elucidate the molecular mechanism through which L-PAM leads to expression of the B7–1 molecule on the surface of P815 tumor cells [11, 43]. The fact that L-PAM, which led within 24 h to the expression of B7–1 on the surface of P815 tumor cells, also led within 24 h to up-regulated expression of B7–1 on the surface of normal B cells, does not necessarily mean that L-PAM is doing so in P815 tumor cells and in normal spleen cells via the same mechanism. For example, it cannot be concluded a priori that the L-PAM-induced B7–1 expression, which is regulated at the transcriptional level in P815 tumor cells [11, 43], is also regulated at the transcriptional level in normal spleen cells. After all, cultured P815 tumor cells are negative for B7–1 expression at both the protein and mRNA level [11] while normal spleen cells cultured for 24 h have some basal level of B7–1 protein expression, and therefore it is theoretically possible that the up-regulation of B7–1 surface expression seen following treatment of normal spleen cells with L-PAM is solely the result of L-PAM-mediated facilitation of B7–1 export to the cell surface. Consistent with such a possibility, Smyth et al. [42] have recently shown that an "intracellular focal concentration" of B7–1 is present in some situations in B7–1 producing cells. Here we show that in normal spleen cells, like in P815 tumor cells [11], L-PAM leads to rapid accumulation of B7–1 mRNA which requires de novo RNA synthesis. Although these results indicate that L-PAM regulates B7–1 expression at the transcriptional level, not only in P815 tumor cells, but also in normal B cells, one cannot a priori conclude that this is achieved via the same mechanism in P815 tumor cells and in normal spleen cells, since the regulation of B7–1 gene expression may be cell-type specific. This is the case, for example, with the expression of the TNF-α gene in response to stimulation with a calcium ionophore, wherein binding of NFAT to both the κ3-NFAT and the –76-NFAT in the tumor necrosis factor-alpha (TNF-α) promoter sites is required for transcriptional induction of the TNF-α gene in T-cells, but binding of NFAT to the κ3-NFAT site is dispensable in B cells [18, 19].

We would like to point out that although accumulation of B7–1 mRNA expression was evident within 2 h after in vitro exposure of normal spleen cells to L-PAM, an increase in the percentage of B cells that express surface B7–1 was evident at 24 h, but not at 12 h (data not shown), after initiation of the L-PAM treatment. The reason for this relatively long delay is not known at present. However, it is consistent with reports by other investigators wherein mRNA for B7–1 was detectable in B cells within 4 to 6 h after surface Ig ligation [16], yet B7–1 protein was evident on the cell surface only after 16 h and peaked at 72 h after the stimulation [16, 39]. This pattern of early mRNA synthesis and late protein expression is reminiscent of that seen with the expression of IL-2 receptor following activation of resting B cells [15].

Our observations that reactive oxygen species are involved in the transcriptional regulation of L-PAM-induced B7–1 gene expression in normal spleen cells, coupled with the fact that the promoter of the B7–1 gene contains an AP-1 binding site [40] that was suggested to be involved in the up-regulation of B7–1 surface expression on a B cell line subjected to surface Ig ligation [22], prompted us to consider the possibility that AP-1 is important for L-PAM-induced up-regulation of B7–1 gene expression in normal spleen cells. Here we show that exposure of normal spleen cells to L-PAM does not lead to activation of AP-1 which binds to its cognate element in the enhancer of the B7–1 gene. In this regard it should be pointed out that although AP-1 was shown to behave as an oxidative stress responsive factor in most studies [3, 31], this was not the case in all studies [3, 30, 38]. These observations led to the conclusion that the effect of oxidative stress on AP-1activation may vary depending on the cell type studied and the modalities

used, which in turn may result in a different pattern of transcription factor activation [3, 30, 38]. Since L-PAM was recently shown to lead to activation of B7–1 gene expression in P815 tumor cells via a mechanism that involves oxidative stress but not AP-1 [11], our current observations that L-PAM also leads to up-regulation of B7–1 gene expression in normal spleen cells via a mechanism that involves oxidative stress but not AP-1, suggest that the failure of L-PAM to activate AP-1 is not due to the cell type used but rather to the modality used. Another redox-sensitive transcription factor that was considered potentially important for L-PAM-induced activation of B7–1 gene expression in normal spleen cells is NF-κB, which was recently shown to be important for L-PAM-induced expression of the B7–1 gene in P815 tumor cells [11]. Here we show that NF-κB that binds to its cognate element in the enhancer of the B7–1 gene is also activated following exposure of normal spleen cells to L-PAM, and NF-κB activation is important for L-PAM-induced up-regulation of B7–1 gene expression. Thus, P815 tumor cells and normal spleen cells apparently use the same pathway in up-regulating B7–1 gene expression following exposure to L-PAM.

We would like to point out at this stage that the B7–1, which is induced on the surface of P815 tumor cells as a consequence of exposure to L-PAM, was shown in our previous studies to be functional [11]. Specifically, we have shown that the proliferation induced following stimulation of $CD8^+$ T-cells from 2C transgeneic mice bred onto a $RAG^{-/-}$ background ($2C/RAG^{-/-}$) with p2Ca (an octapeptide from α-ketoglutarate dehydrogenase) pulsed L-PAM pretreated P815 tumor cells can be inhibited with anti-B7–1, but not with anti-B7–2, mAb or isotype matched immunoglobulin. Although we have not shown directly the functionality of the B7–1 which is induced by L-PAM on the surface of host B cells, since the same dose of L-PAM up-regulates B7–1 expression on the surface of both P815 tumor cells and host B cells via the same mechanism, it would be expected that the B7–1 induced by L-PAM on B cells would also be functional. Consequently, the fact that exposure to L-PAM leads to up-regulation of B7–1 surface expression not only on tumor cells but also on host APCs may have important implications for an immune-potentiating mechanism of L-PAM in clinical settings.

It should be pointed out at this stage that the importance of host APC for the immunopotentiating activity of anticancer drugs was suggested by Mihich and Ehrke almost 15 years ago [12, 13, 32]. Specifically, they concluded from their studies with adriamycin (ADM) that ADM mediates its potentiating effect, at least in part, by increasing the ''accessory cell activity'' of macrophages. Interestingly, the measure of enhanced ''accessory cell activity'' that this group of investigators studied was the ability of ADM-treated macrophages to induce IL-2 production by T-cells that received signal 1 [13]. Although no information is provided in their studies as to why ADM-treated macrophages induced the production of high levels of IL-2 by T-cells that received signal 1, based on our studies it is conceivable that ADM, like L-PAM, led to up-regulation of B7–1 expression on host APCs, and B7–1 in turn interacted with CD28 on T-cells to facilitate IL-2 production [6, 24, 25].

Finally, the information gathered from the current studies with regard to the ability of L-PAM to up-regulate B7–1 gene expression in host cells, not only following in vitro exposure to the drug but also following the in vivo administration of L-PAM, coupled with the molecular mechanism through which L-PAM leads to up-regulation of B7–1 gene expression suggests that other anticancer modalities that increase the level of intracellular reactive oxygen species may also be effective in up-regulating B7–1 expression. This in turn may have implications beyond the acquisition of antitumor immunity since some anticancer modalities (e.g. gamma-irradiation) that increase the level of intracellular reactive oxygen species [3, 37] are used not only in cancer therapy but also as part of treatment regimens in other medical conditions (e.g. transplantation).

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References

1. Allison JP, Hurwitz A, Leach DR (1995) Manipulation of co-stimulatory signals to enhance antitumor T-cell responses. Curr Opin Immunol 7:682
2. Arts J, Herr I, Lansink M, Angel P, Kooistra T (1997) Cell-type specific DNA-protein interactions at the tissue-type plasminogen activator promoter in human endothelial and HeLa cells in vivo and in vitro. Nucleic Acids Res 15:311
3. Baeuerle PA, Henkel T (1994) Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 12:141
4. Bass KK, Mastrangelo MJ (1998) Immunopotentiation with low-dose cyclophosphamide in the active specific immunotherapy of cancer. Cancer Immunol Immunother 47:1
5. Bocian RC, Ben-Efraim S, Dray S, Mokyr MB (1984) Melphalan-mediated potentiation of antitumor immune responsiveness of immunosuppressed spleen cells from mice bearing a large MOPC-315 tumor. Cancer Immunol Immunother 18:41
6. Bour-Jordan H, Bluestone JA (2002) CD28 function: a balance of costimulatory and regulatory signals. J Clin Immunol 22:1
7. Chambers BJ, Wilson JL, Salcedo M, Markovic K, Bejarano MT, Ljunggren HG (1998) Triggering of natural killer cell mediated cytotoxicity by costimulatory molecules. Curr Top Microbiol Immunol 230:53
8. Chen L, Linsley PS, Hellström KE (1993) Costimulation of T cells for tumor immunity. Immunol Today 14:483
9. Constant S, Schweitzer N, West J, Ranney P, Bottomly K (1995) B lymphocytes can be competent antigen-presenting cells for priming $CD4^+$ T cells to protein antigens in vivo. J Immunol 155:3734
10. Donepudi M, Quach DD, Mokyr MB (1999) Signaling through CD40 enhances cytotoxic T lymphocyte generation by $CD8^+$ T cells from mice bearing large tumors. Cancer Immunol Immunother 48:153
11. Donepudi M, Raychaudhuri P, Bluestone JA, Mokyr MB (2001) Mechanism of melphalan-induced B7–1 gene expression in P815 tumor cells. J Immunol 166:6491
12. Ehrke MJ, Ryoyama K, Cohen SA (1984) Cellular basis for adriamycin-induced augmentation of cell-mediated cytotoxicity in culture. Cancer Res 44:2497

13. Ehrke MJ, Maccubbin D, Ryoyama K, Cohen SA, Mihich E (1986) Correlation between adriamycin-induced augmentation of interleukin 2 production and of cell-mediated cytotoxicity in mice. Cancer Res 46:54
14. Falcone M, Lee J, Patstone G, Yeung B, Sarvetnick N (1998) B lymphocytes are crucial antigen-presenting cells in pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice. J Immunol 161:1163
15. Freedman AS, Boyd AW, Berribi A, Horowitz JC, Levy DN, Rosen KJ, Daley J, Slaughenhoupt B, Levine H, Nadler LM (1987) Expression of B cell activation antigens on normal and malignant B cells. Leukemia 1:9
16. Freeman GJ, Freedman AS, Segil JM, Lee G, Whitman JF, Nadler LM (1989) B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. J Immunol 143:2714
17. Gajewski TF, Fallarino F, Uyttenhove C, Boon T (1996) Tumor rejection requires a CTLA-4 ligand provided by the host or expressed on the tumor: superiority of B7–1 over B7–2 for active tumor immunization. J Immunol 156:2909
18. Goldfeld AE, Strominger JL, Doyle C (1991) Human tumor necrosis factor-α gene regulation in phorbol ester stimulated T and B cell lines. J Exp Med 174:73
19. Goldfeld AE, Tsai E, Kincaid R, Belshaw PJ, Schrieber SL, Strominger JL, Rao A (1994) Calcineurin mediates human tumor necrosis factor-α gene induction in stimulated T and B cells. J Exp Med 180:763
20. Geldhof AB, Raes G, Bakkus M, Devos S, Thielemans K, De Baetselier P (1995) Expression of B7–1 by highly metastatic mouse T lymphomas induces optimal natural killer cell-mediated cytotoxicity. Cancer Res 55:2730
21. Gorman A, McGowan A, Cotter TG (1997) Role of peroxide and superoxide anion during tumour cell apoptosis. FEBS Lett 404:27
22. Hsing Y, Bishop GA (1999) Requirement for nuclear factor-κB activation by a distinct subset of CD40-mediated effector functions in B lymphocytes. J Immunol 162:2804
23. Huang AY, Bruce AT, Pardoll DM, Levitsky HI (1996) Does B7–1 expression confer antigen-presenting cell capacity to tumors in vivo? J Exp Med 183:769
24. Janeway CAJ, Bottomly K (1994) Signals and signs for lymphocyte responses. Cell 76:275
25. Jenkins MK, Johnson JG (1993) Molecules involved in T-cell costimulation. Curr Opin Immunol 5:361
26. Kalinichenko TV, Mokyr MB (1998) Limited importance of CD40/CD40L interaction in the B7-dependent generation of anti-MOPC-315 cytotoxic T lymphocyte activity by tumor bearer splenic cells stimulated in vitro in the presence of tumor necrosis factor. Cancer Immunol Immunother 46:293
27. La Motte RN, Rubin MA, Barr E, Leiden JM, Bluestone JA, Mokyr MB (1996) Therapeutic effectiveness of the immunity elicited by P815 tumor cells engineered to express the B7–2 costimulatory molecule. Cancer Immunol Immunother 42:161
28. La Motte RN Sharpe AH, Bluestone JA, Mokyr MB (1999) Host B7–1 and B7–2 costimulatory molecules contribute to the eradication of B7–1-transfected P815 tumor cells via a $CD8^+$ T cell-dependent mechanism. J Immunol 162:4817
29. May M, D'Acquisto F, Madge LA, Glöckner J, Pober S, Ghosh S (2002) Selective inhibition of NF-κB activation by a peptide that blocks the interaction of NEMO with the IκB kinase complex. Science 289:1550
30. Meyer M, Schreck R, Baeuerle PA (1993) H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. Embo J 12:2005
31. Meyer M, Pahl HL, Baeuerle PA (1994) Regulation of the transcription factors NF-kappa B and AP-1 by redox changes. Chem Biol Interact 91:91
32. Mihich E, Ehrke MJ (2000) Anticancer drugs plus cytokines: immunodulation based therapies of mouse tumors. Int J Immunopharmacol 22:1077
33. Mokyr MB, Gorelik L (1997) Melphalan as an immunopotentiating agent in cancer therapy. Biotherapy 11:219
34. Morel A, Fernandez N, de La Coste A, Haddada H, Viguier M, Polla BS, Antoine B, Kahn A (1998) Gamma-ray irradiation induces B7.1 costimulatory molecule neoexpression in various murine tumor cells. Cancer Immunol Immunother 46:277
35. Nishio M, Spielman J, Lee RK, Nelson DL, Podack ER (1996) CD80 (B7.1) and CD54 (intracellular adhesion molecule-1) induce target cell susceptibility to promiscuous cytotoxic T cell lysis. J Immunol 157:4347
36. Osborn L, Kunkel S, Nabe GJ (1989) Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc Natl Acad Sci USA 86:2336
37. Pahl HL, Baeuerle PA (1994) Oxygen and the control of gene expression. Bioessays 16:497
38. Pinkus R, Weiner LM, Daniel V (1996) Role of oxidants and antioxidants in the induction of AP-1, NF-kappa B, and glutathione *S*-transferase gene expression. J Biol Chem 271:13422
39. Ranheim EA, Kipps TJ (1993) Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. J Exp Med 177:925
40. Selvakumar A, White PC, Dupont B (1993) Genomic organization of the mouse B-lymphocyte activation antigen B7. Immunogenetics 38:292
41. Serreze DV, Fleming SA, Chapman HD, Richard SD, Leiter EH, Tish RM (1998) B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Immunol 161:3912
42. Smyth C, Logan G, Weinberger RP, Rowe PB, Alexander IE, Smythe JA (1998) Identification of a dynamic intracellular reservoir of CD86 protein in peripheral blood monocytes that is not associated with the Golgi complex. J Immunol 160:5390
43. Sojka DK, Donepudi M, Bluestone JA, Mokyr MB (2000) Melphalan and other anticancer modalities up-regulate B7–1 gene expression in tumor cells. J Immunol 164:6230
44. Sojka DK, La Motte RN, Mokyr MB (2000) B7–2 expression on tumor cells is important for the acquisition of CTL activity by spleen cells from low-dose melphalan-treated MOPC-315 tumor cells via a mechanism that requires either B7–1 or B7–2 expression on host antigen-presenting cells. Cancer Immunol Immunother 49:10
45. Takesue BY, Pyle JM, Mokyr MB (1990) Importance of tumor-specific cytotoxic $CD8^+$ T-cells in eradication of a large subcutaneous MOPC-315 tumor following low-dose melphalan therapy. Cancer Res 50:7641
46. Townsend SE, Allison JP (1993) Tumor rejection after direct costimulation of $CD8^+$ T cells by B7-transfected melanoma cells. Science 259:368
47. Vereecque R, Buffenoir G, Gonzalez R, Cambier N, Hetuin D, Bauters F, Fenaux P, Quesnel B (2000) Gamma-ray irradiation induces B7.1 expression in myeloid leukaemic cells. Br J Haematol 108:825
48. Verhasselt V, Vanden Berghe W, Vanderheyde N, Willems F, Haegeman G, Goldman M (1999) *N*-acetyl-L-cysteine inhibits primary human T cell responses at the dendritic cell level: association with NF-κB inhibition. J Immunol 162:2569
49. Wu TC, Huang AY, Jaffee EM, Levitsky HI, Pardoll DM (1995) A reassessment of the role of B7–1 expression in tumor rejection. J Exp Med 182:1415
50. Yang G, Mizuno MT, Hellström KK, Chen L (1997) B7-negative versus B7-positive P815 tumor: differential requirements for priming of an antitumor immune response in the lymph nodes. J Immunol 158:851
51. Zhang H, Haasch D, Idler KB, Okasinski GF (1996) Isolation and promoter mapping of the gene encoding murine co-stimulatory factor B7–1. Gene 183:1
52. Zhao J, Freeman GJ, Gray GS, Nadler LM, Glimcher LH (1996) A cell type-specific enhancer in the human B7.1 gene regulated by NF-κB. J Exp Med 183:777