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Proteasome Activity Is Required for T Lymphocyte Aggregation After Mitogen Activation

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Abstract The proteasome is a multicatalytic complex of proteases involved in T lymphocyte proliferation and activation through multiple mechanisms. In this study, we investigated its role in lymphocyte aggregation. We found that blocking proteasome activity by a proteasome-specific inhibitor lactacystin (LAC) prevented clustering of T lymphocytes after stimulation with various mitogens. Expression of adhesion molecules ICAM-1 and LFA-1 at cell surfaces of activated T cells was decreased after treatment with LAC. Mechanisms by which the proteasome intervenes in the expression of these adhesion molecules were different. LAC inhibited ICAM-1 expression at the mRNA level, whereas LFA-1 inhibition was probably at a post-translational level. Downregulation of these molecules after proteasome inhibition likely contributes to the observed repression of T cell aggregation. Our results show that the proteasome plays an important role in cell–cell interaction during T cell activation. J. Cell. Biochem. 81:347–356, 2001. © 2001 Wiley-Liss, Inc.

Key words: proteasome; T lymphocytes; ICAM-1; LFA-1; cell adhesion

The proteasome is a large multicatalytic complex of proteases responsible for 70–90% of nonlysosomal protein breakdowns in both cytosol and nuclei [Rock et al., 1994]. The structure of the 20S proteasome resembles a hollow cylinder composed of four rings of seven subunits each. The two outer rings are composed of α subunits, and the two inner rings of β subunits. Active catalytic sites of proteases are inside the β subunits [Groettrup et al., 1996]. The 26S proteasome is formed by association of 19S regulatory complexes with both ends of the 20S proteasome. The 26S proteasome degrades proteins in a ubiquitination- and ATP-dependent fashion [Jentsch and Schlender, 1995]. Proteins destined to be degraded are selectively ubiquitinated, and the ubiquitinated proteins are then recognized by the proteasome. [Jentsch and Schlender, 1995; Ciechanover and Schwartz, 1998]. Ubiquitination involves a series of well-defined reactions catalyzed by several classes of enzymes called ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) [Jentsch and Schlender, 1995]. Recently, a novel ubiquitination factor (E4) has been described [Koegl et al., 1999]. The 20S proteasome can also bind at its extremities 11S activators (PA28) that accelerates degradation of large peptides into small ones in an ATP-independent way [Realini et al., 1994].

Recently, we realized that the proteasome is involved in much more than degradation of spent proteins in cells or generation of antigenic peptides associated with MHC class I molecules. The proteasome actually controls many cellular processes by selectively destroying regulatory proteins involved in cell cycling such as cyclins

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and a cyclin-dependent kinase inhibitor $p27^{kip}$ [Deshaies et al., 1995; Pagano et al., 1995; Yaglom et al., 1995; Hoyt 1997], in cell proliferation such as p53 [Scheffner et al., 1993], and in regulation of transcription factors such as IκB [Palombella et al., 1994]. It is also essential in processing precursors of regulatory factors into active ones [Palombella et al., 1994; Lin et al., 1998].

Inhibitors of the ubiquitin-proteasome proteolytic pathway have been used to investigate physiological functions of the proteasome. Lactacystin (LAC), a microbial metabolite, was reported by Fenteany et al. [1995] as a proteasome-specific inhibitor. It inhibits three major peptidase activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide-hydrolyzing activities) of the proteasome, and inhibition of the first two is irreversible in in vitro assays. LAC does not affect other proteases such as calpain, cathepsin B, chymotrypsin, trypsin and papain [Fenteany et al., 1995]. Our previous study using LAC shows that the proteasome is essential in lymphocyte activation and proliferation. Moreover, proteasome inhibition by LAC leads to apoptosis of activated but not resting cells [Wang et al., 1998]. During our study, we noticed that aggregation of T cells after mitogen activation was inhibited by the proteasome inhibitor LAC. A detailed examination on this phenomenon is reported herewith.

MATERIALS AND METHODS

Reagents

RPMI 1640, Fetal calf serum (FCS), penicillin/streptomycin, L-glutamine and Trizol Reagent were purchased from Life Technologies (Burlington, Ont., Canada). Lactacystin was obtained from Dr. E.J. Corey [Fenteany et al., 1995]. Phorbol myristate acetate (PMA), phytohemagglutinin (PHA) and streptavidin-R-phycoerythrin were purchased from Sigma (St. Louis, MO), and concanavalin A (Con A) from Boehringer Mannheim (Mannheim, Germany). Lymphoprep was purchased from Nycomed (Oslo, Norway). The FITC-conjugated hamster anti-mouse CD54 (ICAM-1) monoclonal antibody (mAb clone 3E2), FITC-conjugated rat anti-mouse CD25 (IL-2 receptor α chain) mAb (clone 7D4), FITC-conjugated rat anti-mouse CD44 (Pgp-1) mAb (clone 1M7), FITC-conjugated rat anti-mouse L-selectin mAb (clone MEL-14) were obtained from PharMingen (San Diego, CA). FITC-conjugated anti-mouse CD11a (LFA-1α) mAb (clone 121/7), FITC-conjugated anti-mouse CD18 (LFA-1β) mAb (clone C71/16) and biotin-conjugated anti-mouse CD90 (Thy1.2) were ordered from Cedarlane (Hornby, Ont., Canada).

Cell Culture

Total spleen cells from BALB/c mice were obtained after lysis of red blood cells with 0.84% NH_4Cl. Mesenteric lymph node cells were directly used after being flushed out of the node. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors with Lymphoprep according to manufacturer's instructions. Highly purified human T cells were obtained by sheep erythrocyte rosetting as described previously [Luo et al., 1992], followed by one passage on a nylon wool column [Coligan et al., 1991]. All cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine and antibiotics in six-well flat bottom plates at a density of 3×10^6 cells/ml for periods as shown in the absence or presence of different stimulators and LAC.

Flow Cytometry

Two-color staining was performed on mouse spleen cells as described previously [Luo et al., 1993]. Splenocytes were stained with biotin-anti-Thy1.2/streptavidin-phycoerythrin along with an FITC-conjugated mAb against various adhesion molecules as indicated. The samples were analyzed on a Coulter Profile I flow cytometer.

Northern Blot

Total cellular RNA from mouse spleen cells was extracted with Trizol Reagent. Northern blot analysis was performed as described in our previous publication [Shan et al., 1994] and RNA was blotted onto nylon membranes. A 1897-bp mouse ICAM-1 cDNA and a 2856 bp mouse LFA-1β cDNA were excised from pUC19 constructs (ATCC, Rockville, MD). A 468 bp mouse LFA-1α EST clone (clone ID: IMAGE 1149832, Incyte Genomics, St. Louis, MI) was excised from a pBluescript SK construct. These cDNAs were labeled by random priming with ^{32}P-dCTP, and were used as probes. Even loading of RNA in different samples was shown on basis of intensity of 18S and 28S ribosomal RNA.

RESULTS

Effect of Proteasome Inhibition on Lymphocyte Aggregation in Mouse and Human Cells Stimulated With Different Mitogens

Roles of the proteasome in immune cell interaction were first studied using mouse lymph node cells. The cells were activated with a T cell mitogen Con A (2 μg/ml) for 20 h and large clumps of cell aggregation were obvious as expected (Fig. 1A). In the presence of LAC (8 μM), which was added at the beginning of the culture, the clumps failed to form, suggesting that proteasome activity is essential for the cell aggregation. To exclude a possibility that such an effect is species-specific, a similar experiment was carried out using human PBMC. After 20 h stimulation by a T cell mitogen PHA (1 μg/ml), PBMC formed clumps of aggregates (Fig. 1B). However, in the presence of LAC (8 μM), the

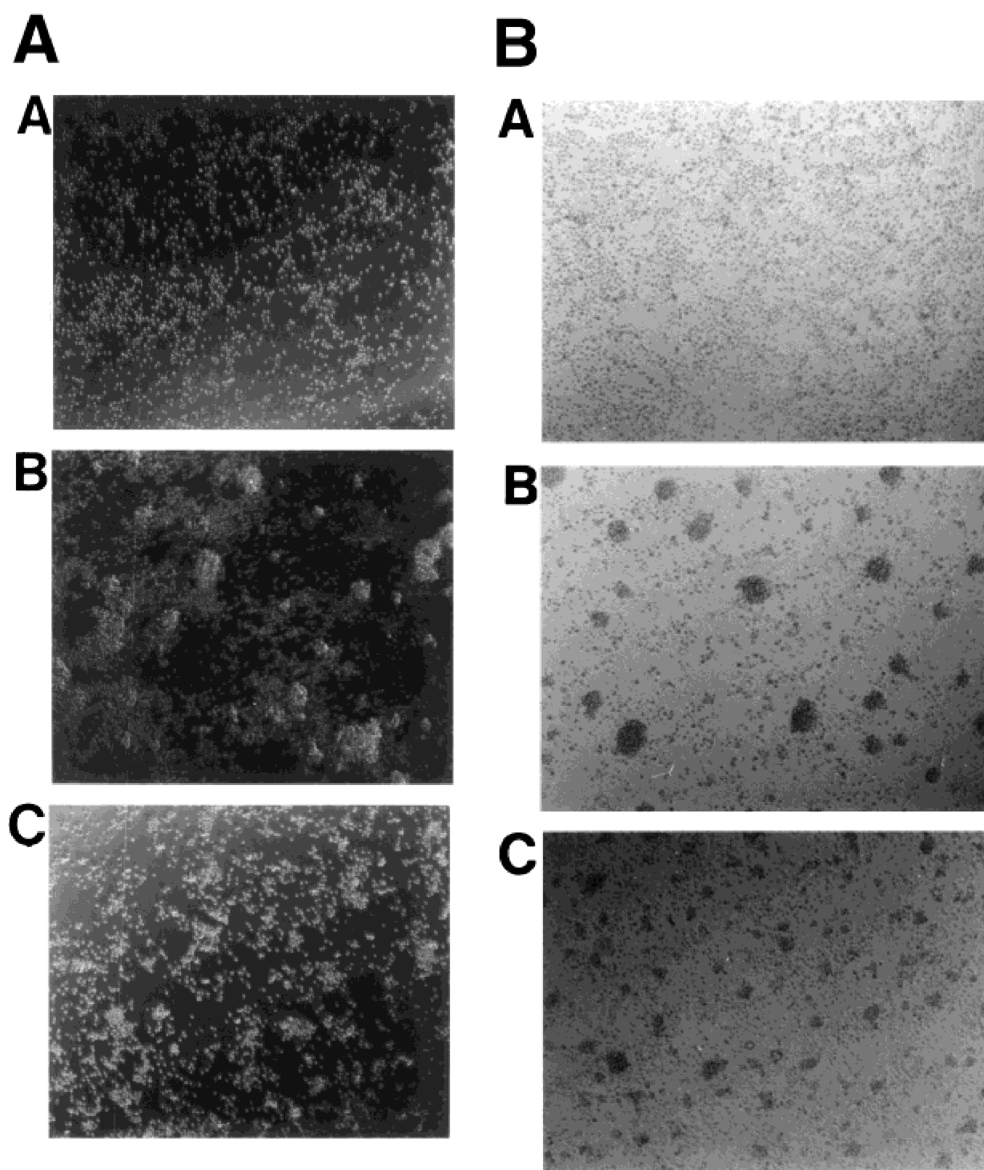
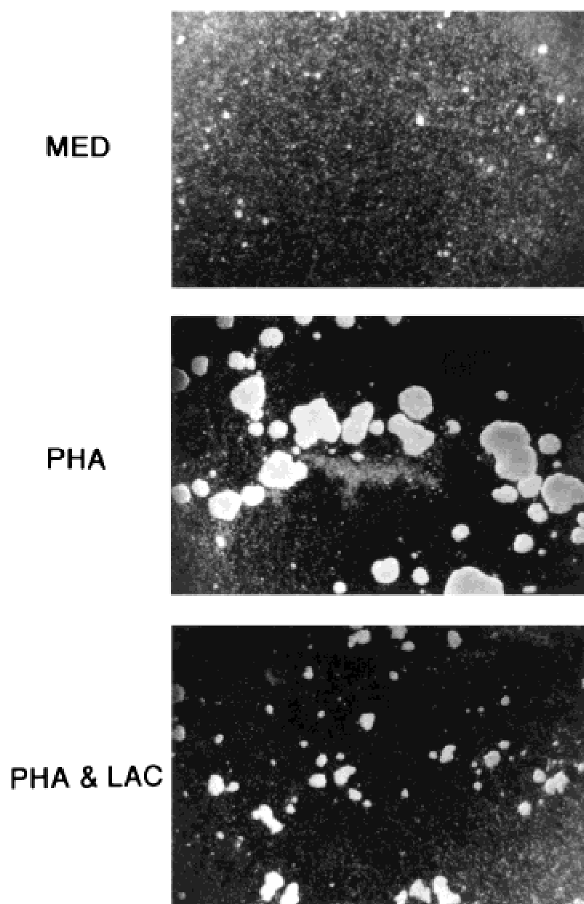


Fig. 1. LAC inhibits clustering of mouse and human lymphocytes. Lymphocytes were stimulated with various mitogens as indicated, and LAC (8 μM) was added at beginning of overnight cultures. **A**: Mouse lymph nodes cells cultured in medium (A), activated with Con A (2 μg/ml) (B), or treated with Con A plus LAC (C). **B**: Human PBMC cultured in medium (A), stimulated with PHA (2 μg/ml) (B), or treated with PHA plus LAC (C). **C**: Highly purified human T lymphocytes from PBMC were culture in medium in the presence of absence of PHA (2 μg/ml) and LAC as indicated. **D**: Highly purified human T cells were cultured with PMA (1 nM) plus ionomycin (1 μg/ml) in the absence or presence of LAC as shown.



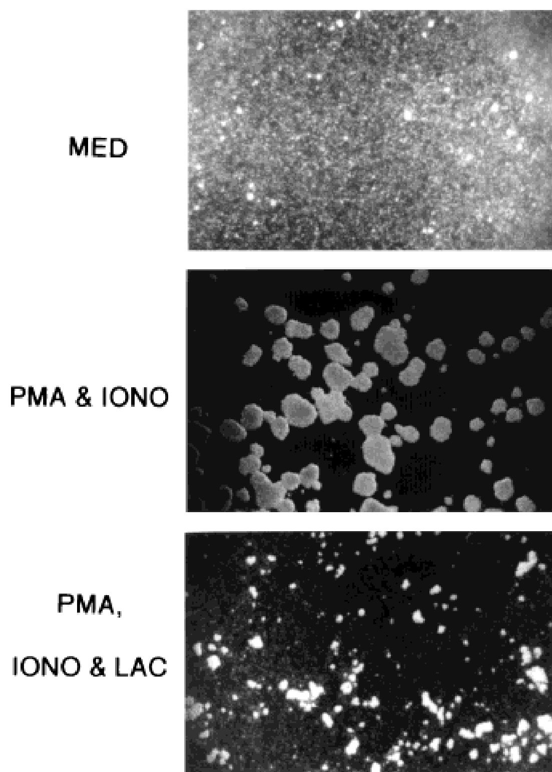


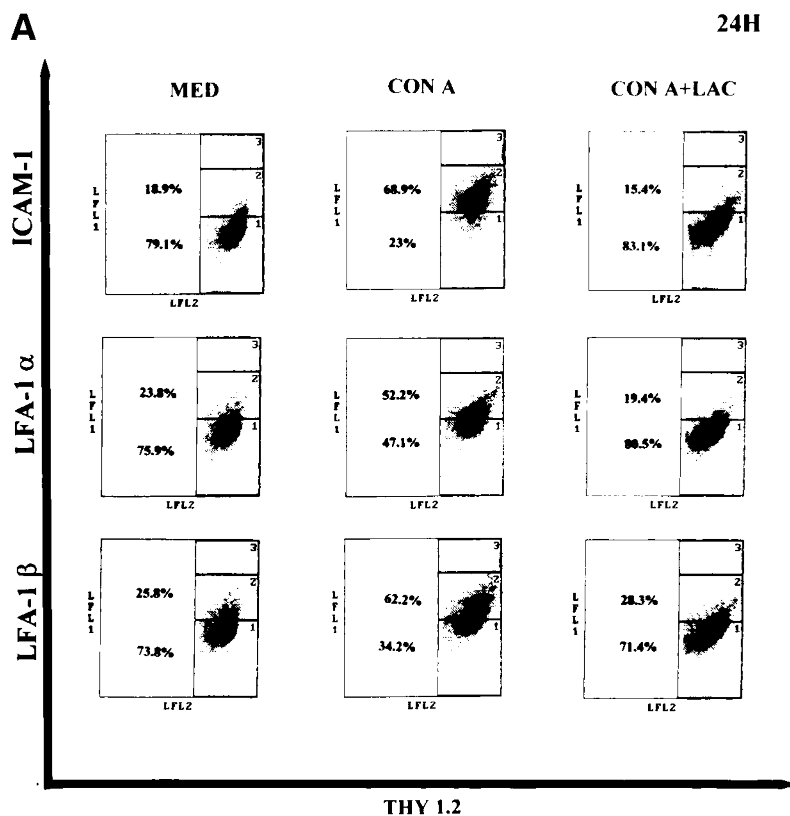
Fig. 1. (*Continued*)

clumps were of much smaller size. The mouse lymph node cells and human PBMC are heterogenous cells, containing B cells, macrophages, dendritic cells, etc., in addition to T cells. To understand whether the proteasome is required in T cell–T cell interaction, we next used highly purified T cells (97% CD3 positive) from human PBMC. PHA (1 μg/ml) induced formation of large T cell clumps within 20 h, and in the LAC-treated culture, only small clumps appeared (Fig. 1C). To exclude a possibility that the effect of LAC was due to interference of direct agglutinating property of the lectins (Con A and PHA) used in stimulation, we used PMA and ionomycin as stimulators for the highly purified human T cells. As shown in Figure 1D, LAC could effectively inhibit T cell aggregation after 20-h stimulation with PMA and ionomycin. These results show that the proteasome activity is required for interaction between subpopulations of T cells during their activation.

Involvement of the Proteasome in Expression of Cell Surface Molecules in Activated T Lymphocytes

After observing the effect of proteasome inhibition on T cell aggregation at the morphological level, we investigated whether certain adhesion molecules were affected. Our focus was on expression of two prominent adhesion

Fig. 2. LAC inhibits ICAM-1 and LFA-1 α and β expression on cell surfaces of activated mouse T cells. Splenocytes were stimulated with Con A (2 μg/ml) for 24 h (**A**) and 48 h (**B**), in the presence and absence of LAC (8 μM) as indicated. ICAM-1 and LFA-1 α and β expression on T cells was evaluated by two-color flow cytometry. The cells in the histograms were a Thy-1.2 positive population according to gating.


A
24H
MED
CON A
CON A+LAC
ICAM-1
LFA-1 α
LFA-1 β
18.9%
79.1%
68.9%
23%
15.4%
83.1%
23.8%
75.9%
52.2%
47.1%
19.4%
25.8%
73.8%
62.2%
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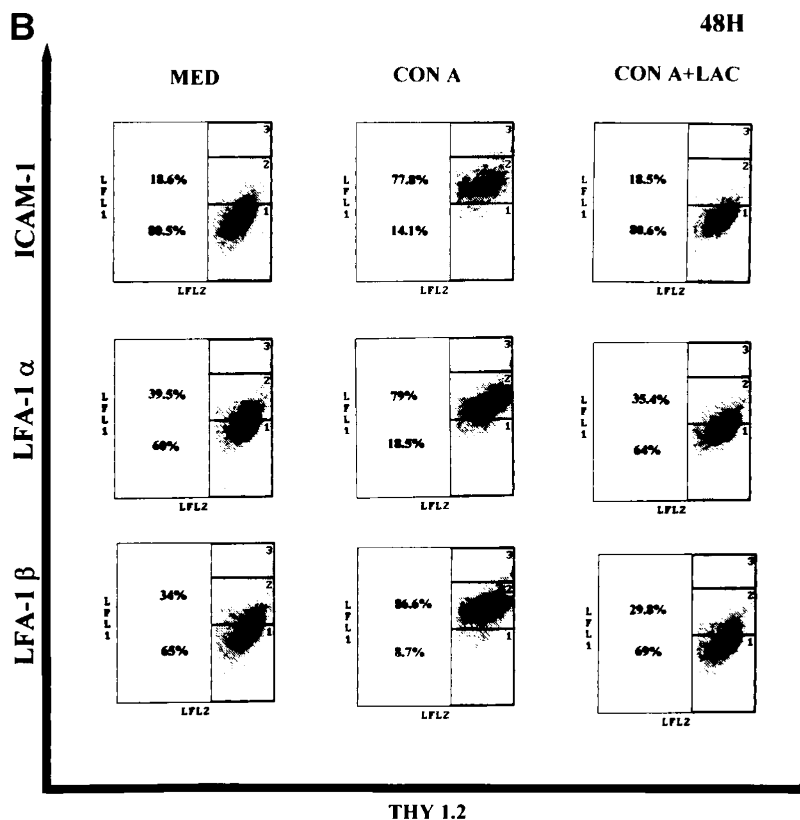
28.3%
71.4%
LFL1
LFL2
THY 1.2


B
48H
MED
CON A
CON A+LAC
ICAM-1
LFA-1 α
LFA-1 β
18.6%
77.8%
14.1%
18.5%
39.5%
60%
79%
18.5%
35.4%
64%
34%
65%
86.6%
8.7%
29.8%
69%
LFL1
LFL2
THY 1.2

moieties, ICAM-1 and LFA-1 (α and β), the interaction of which is clearly involved in interaction between lymphocytes and between T cells subpopulations [Pardi et al., 1992]. Mouse spleen cells were cultured for 24 and 48 h, and LAC (8 μM) was added at beginning of the culture along with a mitogen (Con A, 2 μg/ml). Viability of the spleen cells was evaluated with trypan blue exclusion. Spleen cells cultured in medium for 24 and 48 h had viability of 88 and 82%, respectively. This is a normal phenomenon due to which cells were deprived of cytokines and cell–matrix interactions of the in vivo environment. The cells stimulated with Con A had a better viability of 94 and 83% at 24 and 48 h, respectively. The enhanced viability is largely due to cytokines produced after T cell activation. The viability of the cells in the presence of Con A and LAC (8 μM) was 69 and 62% at 24 and 48 h, respectively. These results correspond well to observations we reported in our previous publication [Wang et al., 1998]. The drop of viability could be due to a variety of reasons, such as lack of cytokines in the culture and a light non-specific toxic effect of LAC. In any case, when these cells were evaluated by flow cytometry, only live cells were gated. As shown in Figure 2, T cells activated for 24 and 48 h, had augmented expression of ICAM-1 and both α and β chains of LFA-1 on cell surfaces. These augmentations were repressed to basal levels in the presence of LAC.

Is the proteasome activity required universally for expression of activation-related cell surface molecules? Expression of additional three molecules in this category, i.e., CD25, CD44, and MEL-14 was studied. Expression of CD25, the alpha subunit the IL-2 receptor, was increased after Con A stimulation at 24 h as expected (Fig. 3A) and the increase was more pronounced at 48 h (Fig. 3B). Such an expression pattern confirmed that the T cells were properly activated. The upregulation of CD25 was repressed in the presence of LAC at both time points (Fig. 3A and B), as with ICAM-1 and LFA-1. Another adhesion molecule CD44 was also upregulated after Con A activation, but its expression at 48 h with or without LAC was similar (Fig. 3B). This indicates that the observed repression of ICAM-1, LFA-1 and CD25 by LAC is not due to general deterioration of cell condition. MEL-14 (L-selectin) is an adhesion molecule implicated in interaction between lymphocytes and endothelial cells, and consequently in lymphocyte trafficking. It is constitutively expressed on T cells and its expression was transiently downregulated after T cell activation at 24 h, and then returned to its original level at 48 h (Fig. 3A and B). In the presence of LAC, the downregulation was more prominent and the expression remained low at 48 h. It seems then the constitutive high expression of MEL-14 is also a proteasome-dependent process. In all these experiments, we used Thy-1.2 to gate the T cells in two-color flow cytometry. Thy-1.2 was constitutively expressed on T cell surface and its expression was not modulated by LAC (data not shown). The results of this section show that the proteasome was preferentially needed for a certain adhesion molecules and activation-related molecules. Such modulation is not due to general toxic effect of LAC, because (1) the cells only had slight decrease of viability in the presence of LAC, (2) only live cells were gated for analysis, and (3) the expression of an activation-related molecule CD44 or a constitutively expressed molecule Thy1.2 was not modulated by LAC.

LAC's Effect on ICAM-1 and LFA-1 Expression at the mRNA Level

The cell surface expression of ICAM-1 and LFA-1 was both repressed by LAC. Did LAC affect their levels of steady-state mRNA? Northern blot analysis was conducted to address this question. Mouse spleen cells were stimulated with Con A (2 μg/ml) for 20 h in the presence or absence of LAC (8 μM), and total cellular RNA of these cells was analyzed by Northern blot. As shown in Figure 4, ICAM-1 mRNA of the cells was upregulated after Con A stimulation, and the upregulation was suppressed by LAC. On the contrary, the activated T cells had no increase in expression of LFA-1 α or β at mRNA levels, and LAC did not affect steady-state mRNA of these two molecules. In this experiment, the same membrane was sequentially hybridized with ICAM-1, LFA-1α and LFA-1β probes, and they served as internal controls for each other. The lack of upregulation of LFA-1α and β expression in the presence of Con A was not due to insufficient activation since the ICAM-1 signal was upregulated; inhibition of ICAM-1 expression in the presence of LAC was not due to less RNA loading since the LFA-1 signals of LAC-treated samples were not reduced. The results of this section show that the proteasome regulates the ICAM-1 expression at

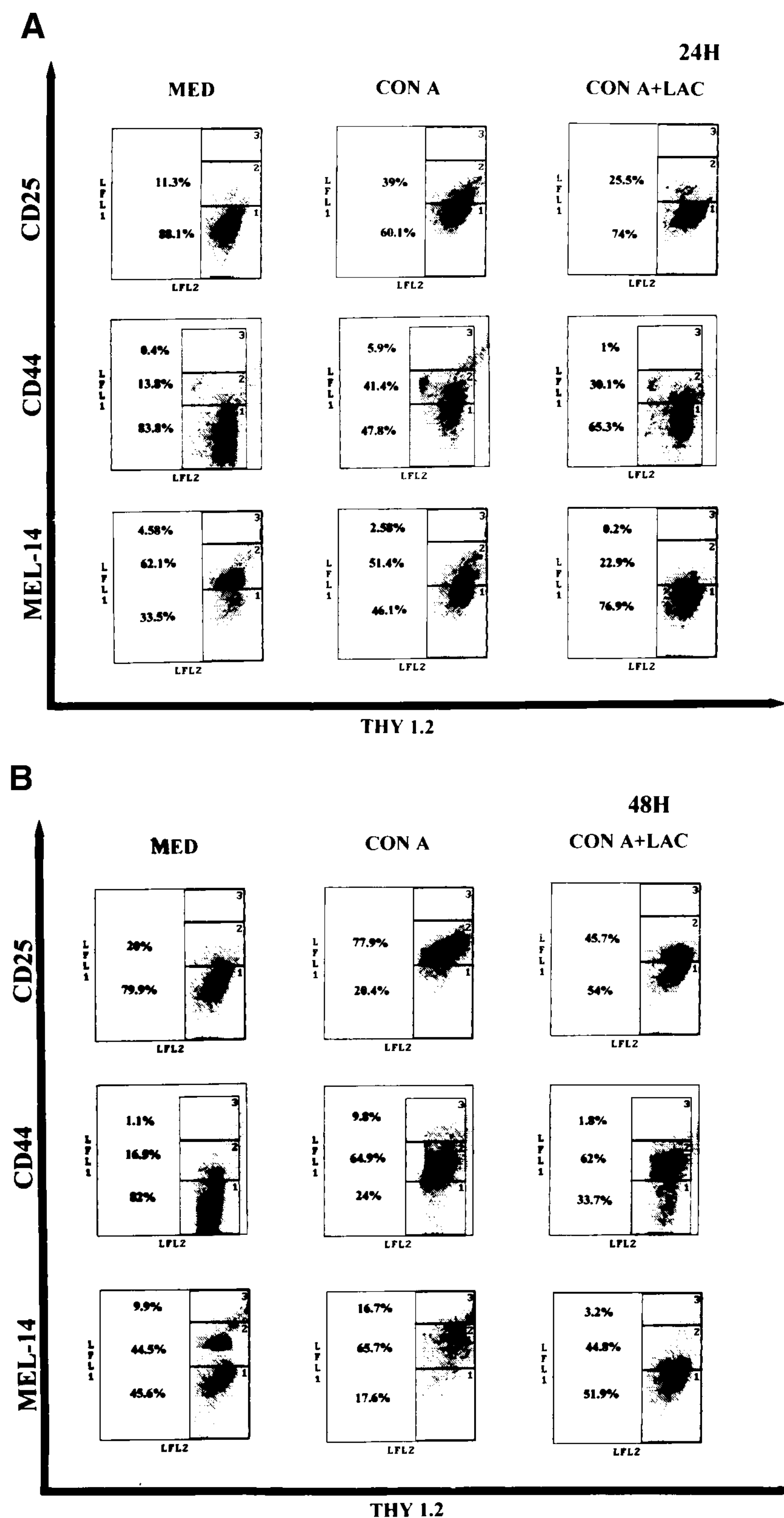


Fig. 3. Effects of LAC on expression of CD25, CD44 and MEL-14. Mouse splenocytes stimulated with Con A in the presence or absence of LAC for 24 h (**A**), and 48 h (**B**). CD25, CD44 and MEL-14 expressions on T cells were evaluated with two-color flow cytometry. The cells in the histograms were a Thy-1.2 positive population according to gating.

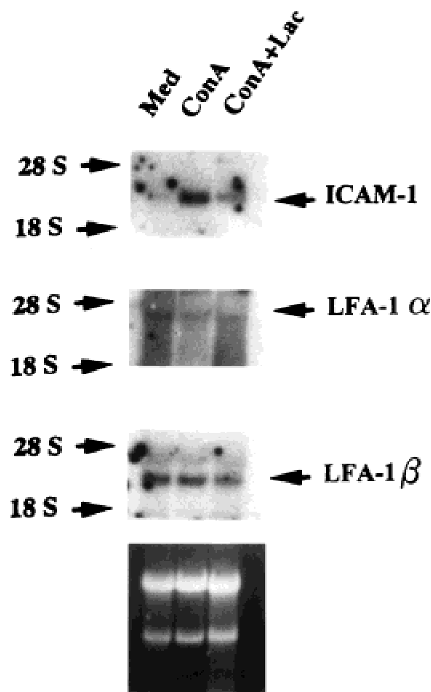


Fig. 4. Northern blot analysis of steady-state mRNA of ICAM-1, LFA-1 α and LFA-1 β after proteasome inhibition. Total spleen cells were cultured for 20 h at a concentration of 3×10^6 cells/ml. Con A (2 μg/ml) and LAC (8 μM) were added as shown. Total cellular RNA was analyzed by Northern blot. The membrane was sequentially hybridized with ICAM-1, LFA-1α and LFA-1β probes. Bands corresponding to mRNAs of ICAM-1 (1.9 kb), LFA-1 α (3.5 kb) and LFA-1 β (2.8 kb) are indicated as such, and even loading of RNA is evidenced according to ethidium bromide staining of 18S and 28S ribosomal RNA (the bottom panel).

the mRNA level, whereas its modulation on cell surface expression of LFA-1 is probably at a post-translational level.

DISCUSSION

Cell-cell interactions mediated by adhesion molecules play an important role in lymphocyte activation and in regulation of immune responses [Springer, 1990]. In this study, we have found that the proteasome activity is necessary for lymphocyte aggregation, since inhibition of the proteasome activity by LAC prevents clustering of lymphocytes and interferes with expression of certain adhesion molecules, i.e., ICAM-1, LFA-1α and LFA-1β, at T cell surfaces.

ICAM-1 and LFA-1 are critical in T cell response to antigens. Antibodies directed against either ICAM-1 [Cosimi et al., 1988], or LFA-1 [Fisher et al., 1986; Talento et al., 1993; Hourmant et al., 1994, 1996; Spillner et al., 1998] can prolong survival of grafts in transplantation of bone marrow and solid organs. A combination of both anti-ICAM-1 and anti-LFA-1 antibodies has even pronounced inhibitory effects on allograft rejection [Isobe et al., 1992]. We have reported that proteasome inhibition can strongly repress T cell activation and proliferation via several mechanisms related to cell cycle control [Wang, et al., 1998]. Obviously, inhibition of interaction between T cells or between T cells and other immune cells via ICAM-1 and LFA-1 is an additional factor contributing to the LAC-mediated inhibition of T cell activation.

Although expressions of ICAM-1, LFA-1α and β on T cell surfaces were all repressed after proteasome inhibition, the underlying mechanisms seem different. The former but not the latter two was affected by LAC at the mRNA level. After examining the genes of these three molecules, we noticed that the promoter region of ICAM-1 contains DNA sequences that are recognized by the nuclear transcription factor κB (NF-κB) [Baeurle et al., 1994; Van de Stolpe, 1994]. NF-κB is a heterodimer composed of a p50 and a p65 subunit, and is in the cytoplasm of most cells in its inactive form that binds to an inhibitory protein IκB [Baldwin, 1996]. The proteasome can interfere with NF-κB activation in two ways. First, the precursor of the p50 subunit of NF-κB is generated by a unique cotranslational processing requiring the activity of the 26S proteasome [Lin et al., 1998]. Secondly, the proteasome is needed to degrade IκBα [Palombella et al., 1994] and IκBβ [Cui et al., 1997]. After degradation of these inhibitors, NF-κB can then translocate from cytosol into nuclei to play its role. By interfering with the translation of the NF-κB p50 subunit, or by inhibiting the degradation of IκB, LAC could prevent activation of the gene encoding for ICAM-1. On the other hand, the lack of NF-κB binding sequence in the promoters regions of LFA-1α and β might explain the lack of modulation of these two molecules at the mRNA level by LAC. Thus, the repression of LFA-1 α and β by LAC is likely at a post-translational level. Several possible mechanisms could be perceived. The proteasome might be required for chaperons that are necessary for transportating LFA-1 to cell surfaces, or simply for stability of LFA-1 before its cell surface expression. These

speculations are apparently worth further exploration.

Adhesion molecules are essential in interaction between T cells and antigen-presenting cells, between T help cells and T effector cells, and between T cells and endothelial cells. In this study, we have shown that the proteasome activity is required for the expression ICAM-1, LFA-1 and MEL-14, which are involved in the processes described above. Obviously, the proteasome could affect cell–cell interaction through mechanisms other than ones investigated here.

In summary, this study demonstrates that proteasome inhibitors can repress cell–cell interactions in the immune system, and thus have potential therapeutic applications in controlling undesirable immune responses.

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