Autologous High-Killing Cytotoxic T Lymphocytes against Human Lung Cancer Are Induced Using Interleukin (IL)-1β, IL-2, IL-4, and IL-6: Possible Involvement of Dendritic Cells

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ABSTRACT

Although CTLs bear main immune responses in human tumors, stable CTL clones against human lung cancer have rarely been generated. Our previous study demonstrated efficient autologous CTL induction in human gastric cancer and glioblastoma by cytokine combination of interleukin (IL)-1β (167 IU/ml), IL-2 (67 IU/ml), IL-4 (67 IU/ml), and IL-6 (134 IU/ml). In this study, we demonstrated successful induction of autologous stable CTLs in five of six patients with lung adenocarcinoma from mixed-lymphocyte tumor culture using this cytokine combination. All CTLs revealed potent and specific killing activity against autologous target cells (over 75% in CD8+ CTLs and over 50% in CD4+ CTLs at an E:T ratio of 10 for 24 h). Using a series of antibodies, CD8+ CTLs showed to recognize tumor-specific antigens of lung cancer cells through HLA class I. In the separate experiments, failure of CTL induction from monocyte-depleted peripheral blood mononuclear cells and appearance of cells with characteristics of dendritic cells from adherent peripheral blood mononuclear cells in the culture of the same concentration of IL-1β, IL-4, and IL-6 indicated that CTLs can be efficiently generated by this cytokine combination via possible dendritic cell induction. This is the first study of an efficient and reproducible in vitro CTL induction against human lung cancer.

INTRODUCTION

Lung cancer is the most common neoplasm leading to cancer death in a number of developed countries. The majority of NSCLCs are diagnosed unresectable, are relatively resistant to currently available treatment modalities, and, thus, are poor prognosis (1). Immunotherapy has been investigated as a new treatment modality using LAK cells or TILs. These adoptive immunotherapies have been effective on several cancers, but have failed to demonstrate direct antitumor activity in cases of NSCLC (2). Tumor-specific CTLs specifically lyse target cells and show more potent cytotoxic activities than LAK cells or TILs (3–7). Generation of autologous CTLs against human lung cancer, however, has been described in only a limited number of studies despite extensive testing of CTL induction (8, 9). No stable CTLs against lung adenocarcinoma has been generated.

Liu et al. (10) and Tsurushima et al. (11) have previously demonstrated that autologous CTL with high tumor specificity and high cytotoxicity could be induced from MLTC in cytokine combination of IL-1β, IL-2, IL-4, and IL-6. We attempted to induce autologous CTL in patients with lung cancer using this cytokine combination and succeeded in induction of autologous CTLs with specific and high cytotoxicity in five of six cases. We also demonstrated that this cytokine combination could induce CTL effectively through possible generation of DCs in MLTC.

MATERIALS AND METHODS

Patients and Establishment of Lung Cancer Cell Lines.

All research followed the tenets of the Declaration of Helsinki. Of 47 surgically resected NSCLC specimens, six lung adenocarcinoma cell lines (IDACA-1, IDACA-2–1, IDACA-3, IDACA-4, IDACA-5, and IDACA-11) were established as described methods (12). IDACA-2–2 was established as a subline of IDACA-2–1, which shows different morphology and shorter doubling time. These cell lines were maintained in MEM containing 10% fetal bovine serum for at least 6 months. Serological HLA class I typing was performed using PBMCs from the original patients by a microcytotoxicity assay (One Lambda, Canoga Park, CA). For CTL induction from monocyte-depleted PBMCs, IDACA-11 was established and examined.

Generation of CTLs Responsive to Lung Cancer Cells.

PBMCs from five patients whose cell lines had been established as IDACA-1, -2–1, -3, -4, and -5 were isolated by Lymphoprep...
Table 1 Characteristics of established lung cancer cell lines: designated tumor cell lines, patient profiles, and CTL induction

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Age/sex</th>
<th>HLA class I</th>
<th>Histology</th>
<th>Stage/TNM</th>
<th>Expression of HLA on tumor cells</th>
<th>CTL induction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td>(designated name)</td>
</tr>
<tr>
<td>IDACA-1</td>
<td>66/M</td>
<td>A02</td>
<td>B39/55</td>
<td>Cw1/7</td>
<td>+</td>
<td>+ (CTL-1)</td>
</tr>
<tr>
<td>IDACA-2-1</td>
<td>68/M</td>
<td>A02/24</td>
<td>B39/54</td>
<td>Cw1/7</td>
<td>+</td>
<td>+ (CTL-2)</td>
</tr>
<tr>
<td>IDACA-2-2</td>
<td></td>
<td>(subline of IDACA-2-1)</td>
<td></td>
<td></td>
<td>+</td>
<td>+ (CTL-3)</td>
</tr>
<tr>
<td>IDACA-3</td>
<td>63/F</td>
<td>A11/26</td>
<td>B61/62</td>
<td>Cw3/4</td>
<td>+</td>
<td>+ (CTL-4)</td>
</tr>
<tr>
<td>IDACA-4</td>
<td>75/M</td>
<td>A31/33</td>
<td>B44/62</td>
<td>Cw7</td>
<td>+</td>
<td>+ (CTL-5)</td>
</tr>
<tr>
<td>IDACA-5</td>
<td>74/M</td>
<td>A24/33</td>
<td>B44/61</td>
<td>Cw3</td>
<td>+</td>
<td>+ (CTL-5)</td>
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<tr>
<td>IDACA-11</td>
<td>50/M</td>
<td>A02/24</td>
<td>B46/61</td>
<td>Cw1</td>
<td>+</td>
<td>+ (CTL-5)</td>
</tr>
</tbody>
</table>

a) TNM, tumor-node-metastasis.
b) This cell line was used only for CTL induction from monocyte-depleted PBMCs.

Induction of CTL against Lung Cancer

Determination of Surface Markers of Tumor Cells and CTLs. Tumor cells were stained with FITC-conjugated anti-HLA-A B C antibody (W6/32; DAKO Japan, Tokyo, Japan) or FITC-conjugated anti-HLA-DP DQ DR antibody (DAKO Japan). Surface phenotypes of CTLs were determined by flow cytometry after staining with FITC- or PE-conjugated antibodies against CD3, CD4, CD8, CD16, and TCRαβ (Becton Dickinson, Mountain View, CA). Flow cytometry was performed using a FACScan (Becton Dickinson).

Cytotoxic Activities. Cytotoxic activity was determined by 51Cr releasing assay (13) and crystal violet staining assay (14), both after 6 and 24 h. Although 51Cr releasing with a 6-h incubation is widely used as the standard assay, 51Cr releasing with a 24-h incubation is also performed. In our previous study, we showed that the two different methods yield comparable results under a 24-h incubation (10).

Blocking of Cytotoxicities by MAbs. 51Cr-labeled target cells were pretreated with anti-HLA class I (DAKO Japan) or anti-HLA class II MAb (50 μg/ml; DAKO Japan) at 37°C for 1 h. Effector cells (CTLs) were incubated with anti-CD3, -CD4, or -CD8 MAb (50 μg/ml; Ancell, Bayport, MN) at 4°C for 1 h. Lysis of target cells with or without MAbs was determined by a 6-h 51Cr releasing assay.

Induction of DCs from PBMCs. PBMCs were cultured in AIMV medium containing 5% autologous serum, allowing to adhere to plastic dishes. Nonadherent cells were removed after 2 h, and adherent cells were subsequently cultured for 7 days with IL-1β (167 IU/ml), IL-2 (67 IU/ml), IL-4 (67 IU/ml), and IL-6 (134 IU/ml). The medium was changed, and PBMCs were counted every 3 days. Autologous target cells: Δ, IDACA-1; ●, IDACA-2-1; ○, IDACA-2-2. Note that PBMCs mix-cultured with IDACA-2-1 did not expand, despite the fact that another autologous cell line (IDACA-2-2) yielded CTL-2-2.

RESULTS

Patient Profiles, HLA Typing, HLA Expression on Tumor Cells, and CTL Induction. Table 1 summarizes the patient profiles, serological HLA class I types, tumor cell lines, and results of CTL induction. The tumor cell lines used for CTL induction expressed HLA class I only, except IDACA-2-1 and the subline IDACA-2-2, which expressed trace amounts of HLA class II antigen (data not shown). CTLs began to grow
after 1 week of MLTC, and they continued growing by repeated stimulation with live target cells (1 × 10⁶/well) in a 6-well culture plate. CTLs were added to the adherent target cells at an E:T ratio of 10. Photomicrographs were taken through inverted microscopy after 6- and 24-h incubations. Left, mix-culture of CTL-1 (CD8+) and autologous target cells (IDACA-1). A, tumor cells alone; B, 6-h incubation; C, 24-h incubation. Right, mix-culture of CTL-2–2 (CD4+) and autologous target cells (IDACA-2–2). D, tumor cells alone; E, 6-h incubation; F, 24-h incubation.

Table 2  Surface phenotype and cytotoxic activity against autologous target cells

<table>
<thead>
<tr>
<th>CTL</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>1:1</th>
<th>24 h</th>
<th>10:1</th>
<th>24 h</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6 h</td>
<td>24 h</td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td>CTL-1</td>
<td>99</td>
<td>23</td>
<td>78</td>
<td>10.2</td>
<td>36.4</td>
<td>49.0</td>
<td>88.3</td>
</tr>
<tr>
<td>CTL-2–2</td>
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<td>96</td>
<td>8</td>
<td>14.3</td>
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<td>81</td>
<td>(10.5±5.4)</td>
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<td>38.6±4.6</td>
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<tr>
<td>CTL-4</td>
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<td>0</td>
<td>99</td>
<td>10.9±2.2</td>
<td>41.7±4.3</td>
<td>40.7±8.5</td>
<td>76.4±5.0</td>
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<td>CTL-5</td>
<td>97</td>
<td>3</td>
<td>95</td>
<td>30.1±8.3</td>
<td>62.5±7.6</td>
<td>35.8±6.4</td>
<td>76.0±6.5</td>
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*a* Phenotype was determined 2 months after induction. Values represent mean of duplicated experiments.

*b* E:T ratio.

*c* Treatment time.

*d* Target cells were IDAC-2-1 (parent cell line of IDACA-2-2).

Fig. 2  Photomicrographs taken through an inverted microscopy demonstrated strong cytotoxicity of CTLs. After a 12-h preincubation of target cells (1 × 10⁵/well) in a 6-well culture plate, CTLs were added to the adherent target cells at an E:T ratio of 10. Photomicrographs were taken through inverted microscopy after 6- and 24-h incubations. Left, mix-culture of CTL-1 (CD8+) and autologous target cells (IDACA-1). A, tumor cells alone; B, 6-h incubation; C, 24-h incubation. Right, mix-culture of CTL-2–2 (CD4+) and autologous target cells (IDACA-2–2). D, tumor cells alone; E, 6-h incubation; F, 24-h incubation.

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<td>6 h</td>
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<td>24 h</td>
</tr>
<tr>
<td>CTL-1</td>
<td>99</td>
<td>23</td>
<td>78</td>
<td>10.2</td>
<td>36.4</td>
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<tr>
<td>CTL-2–2</td>
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<td>CTL-3</td>
<td>86</td>
<td>5</td>
<td>81</td>
<td>(10.5±5.4)</td>
<td>20.3±9.4</td>
<td>17.3±1.3</td>
<td>38.6±4.6</td>
</tr>
<tr>
<td>CTL-4</td>
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<td>0</td>
<td>99</td>
<td>10.9±2.2</td>
<td>41.7±4.3</td>
<td>40.7±8.5</td>
<td>76.4±5.0</td>
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<tr>
<td>CTL-5</td>
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*a* Phenotype was determined 2 months after induction. Values represent mean of duplicated experiments.

*b* E:T ratio.

*c* Treatment time.

*d* Target cells were IDAC-2-1 (parent cell line of IDACA-2-2).

Cytotoxic Activity of CTLs. Although all CTLs showed specific killing activity for autologous target cells, CTL-1 (CD8+) revealed stronger cytotoxicity than CTL-2–2 (CD4+; Fig. 3, A and B). At an E:T ratio of 10, CTL-1 (CD8+) attached to the autologous target cells and had lysed almost all of them.
Induction of CTL against Lung Cancer also HLA-C matched cells (IDACA-4). CTL-4 showed a high level of lysis against not matched for restriction of HLA class I. In contrast, no significant cytototoxicities against autologous fibroblasts, K562 cells, or HLA-C locus-matched target cells (IDACA-2–1 and -2–2), but negative for CD3, CD8, CD19, and CD83 (specific marker of immature DCs; Fig. 7). The cytokine combination of IL-1β, IL-2, IL-4, and IL-6 as used in the CTL induction, thus, generated immature DCs while lymphocytes proliferated due to the addition of IL-2 (Fig. 6E). These morphological and functional studies indicated that the cytokine combination generated CTLs efficiently via induction of DCs.

**DISCUSSION**

**Immune Responses against Tumor Cells.** The basic concepts of cancer immunology have been derived largely from studies of tumor rejection in tumor-bearing syngeneic mice (16). Although a variety of effector cells (including natural killer cells, TILs, LAK cells, T lymphocytes, macrophages, and neutrophils) are recognized to contribute to the surveillance for developing tumors and the control of tumor progression, CTL response is thought to play a key role in the elimination of tumor cells (17).

In humans, immune responses to melanoma have been well documented. To date, many CTLs to melanoma were induced, and tumor antigens recognized by those CTLs have been identified (3, 4, 18). In contrast to melanoma, reports of induction of autologous CTLs in other tumors, including lung cancer, have been limited (5–9, 19). Our previous studies on induction of DCs generated by GM-CSF and IL-4. They were positive for HLA class I, HLA class II, B7–1, CD1a, CD4+, and CD11c+, but negative for CD3, CD8, CD19, and CD83 (specific marker of mature DCs; Fig. 7). The cytokine combination of IL-1β, IL-2, IL-4, and IL-6 as used in the CTL induction, thus, generated immature DCs while lymphocytes proliferated due to the addition of IL-2 (Fig. 6E). These morphological and functional studies indicated that the cytokine combination generated CTLs efficiently via induction of DCs.

Characterization of CTLs Using Antibodies. The cytotoxic activity of CTL-1 (CD8+) was inhibited by anti-CD3 and -CD8 antibodies, but not by anti-CD4 antibody. Anti-HLA class I, but not HLA class II antibody, inhibited CTL-1 cytotoxic activity against target cells (Fig. 4). Although the killing ability of CTL-2–2 (CD4+) was blocked by anti-CD3 antibody, only partial blocking was observed by anti-CD4 antibody. Neither HLA class I nor HLA class II antibody inhibited CTL-2–2 activity (Fig. 4).

**Involvement of DCs for CTL Induction.** To study whether DCs are involved for CTL induction, monocytes, DC precursor cells, were depleted from PBMCs when PBMCs were used for MLTC. As shown in Fig. 5, CTLs could not be induced from monocyte-depleted PBMCs. However, addition of the cells generated by IL-1β, IL-4, and IL-6 from monocytes into monocyte-depleted PBMCs could induce CTLs as well as addition of DCs generated by GM-CSF and IL-4. The cells from adherent PBMCs cultured with IL-1β, IL-4, and IL-6, but without IL-2, for 7 days showed characteristic morphology of DCs as induced by GM-CSF and IL-4, under phase contrast microscopic observation (Fig. 6A and 6B), whereas no DCs were induced by IL-2 alone or without cytokine stimulation (Fig. 6C, D, and F). May-Giemsa staining of the nonadherent cells also confirmed these observations. In addition, the cells generated by this cytokine combination contained markers typical of immature DCs as induced by GM-CSF and IL-4. They were positive for HLA class I, HLA class II, B7–1, CD1a, CD4+, and CD11c+, but negative for CD3, CD8, CD19, and CD83 (specific marker of mature DCs; Fig. 7). The cytokine combination of IL-1β, IL-2, IL-4, and IL-6 as used in the CTL induction, thus, generated immature DCs while lymphocytes proliferated due to the addition of IL-2 (Fig. 6E). These morphological and functional studies indicated that the cytokine combination generated CTLs efficiently via induction of DCs.

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Priming of CTL responses requires the presentation of the
relevant tumor antigen by professional antigen-presenting cells capable of providing costimulation (20). DCs are only natural professional antigen-presenting cells (21). DCs are now efficiently generated by GM-CSF and IL-4 from PBMCs (15). Although IL-2 has been the primary cytokine used among in vitro systems of CTL induction (18), addition of IL-2 alone cannot induce DCs from PBMCs, as we demonstrated. In our system, however, DCs were generated in the lower concentration of cytokines compared with GM-CSF and IL-4. When monocytes were depleted from PBMCs, induction of CTL was failed. Furthermore, IL-1β can augment the stimulatory capacity of DCs against T cells (22). Generation and functional enhancement of DCs from PBMCs may explain the effective CTL induction by this cytokine combination.

**Table 3** Cytotoxicities of CTLs against allogenic tumor and normal cells

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Cytotoxicity (%)</th>
<th>HLA class</th>
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<tbody>
<tr>
<td></td>
<td>CTL-1</td>
<td>CTL-2-2</td>
</tr>
<tr>
<td>Adeno</td>
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<td></td>
</tr>
<tr>
<td>IDACA-1</td>
<td>49</td>
<td>28</td>
</tr>
<tr>
<td>IDACA-2</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>IDACA-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDACA-4</td>
<td>23</td>
<td>9</td>
</tr>
<tr>
<td>IDACA-5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Leukemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K562</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Daudi</td>
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<td></td>
</tr>
<tr>
<td>Normal Fibroblast</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>(Autologous)</td>
<td></td>
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</tr>
</tbody>
</table>

Table 3 *Percentage of cytotoxicity of 6-h 51Cr releasing assay at E:T ratio of 10. NT, not tested.

**Fig. 4** Inhibition of cytotoxicities by MAbs related with antigen recognition. CTLs were pretreated with MAbs (anti-CD3, CD4, and CD8) at 4°C for 1 h. Target cells were pretreated with MAbs (anti-HLA class I, HLA class II) at 37°C for 1 h. Cytotoxic activities were determined by a 6-h 51Cr releasing assay at an E:T ratio of 10. Cytotoxicity on cells not treated with antibody was determined as 100%. Concentrations of MAbs were 50 μg/ml. □, CD4+ CTL; □, CD8+ CTL.

**Fig. 5** Growth curves of monocyte-depleted-PBMCs with or without DCs stimulated with irradiated autologous tumor cells. Monocyte-depleted PBMCs (5 × 10⁶) with or without DCs (5 × 10⁵) generated by GM-CSF–IL-4 or IL-1β, -4, and -6 were mix-cultured with irradiated autologous tumor cells (IDACA-11). □, complete PBMCs; □, monocyte-depleted PBMCs; □, monocyte-depleted PBMCs with DCs generated by GM-CSF and IL-4; □, monocyte-depleted PBMCs with DCs generated by IL-1β, -4, and -6.

The phenotypes of CTLs were a mixture of CD4+ and CD8+. Although Kurnick et al. (25) generated TILs from lung adenocarcinoma tissues, neither tumor specificity nor the role of MHC molecules was demonstrated. Nevertheless, these studies provided substantial evidence of a cellular immune response to human lung cancers. When Seki et al. (26) induced HLA-A locus-restricted CTLs from TILs in NSCLC, the tumor-specific cytotoxicity only lasted for 1 month.

In contrast, stable CTL clones previously have been established in only two cases of lung squamous cell carcinoma (8, 9). These two CTL clones showed highly specific cytotoxicity against autologous target cells and were identified as MHC class I-restricted CD8+ CTLs. In the present study, CTLs were all proved to be specific to autologous target cells. Furthermore, our CTLs were able to maintain tumor specificity at least 6 months and reached as many as 1 × 10⁹ cells in number after 1 month, a concentration approximately sufficient for practical use in immunotherapy.
Fig. 6 Generation of DCs from PBMCs by cytokine combination. Adherent PBMCs were cultured for 7 days with various cytokine combinations. Photomicrographs were taken after 7 days. A, cells cultured with IL-1β (167 IU/ml), IL-4 (67 IU/ml), and IL-6 (134 IU/ml); B, cells cultured with GM-CSF (1000 IU/ml) and IL-4 (500 IU/ml); C, cells cultured with IL-2 (67 IU/ml); D, cells cultured without cytokine; E, cells cultured with IL-1β, IL-2, IL-4, and IL-6. Note that cells in A, B, and E were nonadherent or easily detached from the dish, but cells in D were tightly attached to the dish.

Fig. 7 Cytofluorographic analysis of DCs grown in the presence of IL-1β, -4, and -6. The cells were stained with a panel of antibodies, as described in “Materials and Methods.” Contaminating lymphocytes were excluded by light scatter properties.
The cytotoxicities of CD8+ CTLs were inhibited by anti-HLA class I antibody, suggesting that CD8+ CTLs are HLA class I-restricted and, thus, may recognize tumor antigen presented in HLA class I molecules (27). Although the HLA loci restriction of CD8+ CTLs were not fully studied, all of the CD8+ CTLs showed HLA-A- and/or -C-restricted cytotoxicity.

The CD4+ CTL was induced in IDACA-2–2 only and showed tumor-specific, but weaker, cytotoxicity. Responses of CD4+ CTLs against tumor cells have been reported recently (28–30). Interestingly, CD4+ CTLs were induced repeatedly in independent experiments from IDACA-2–2, but not from IDACA-2–1, although both cell lines were proved to be derived from one patient by genomic analysis (31).

In conclusion, we reproducibly generated stable CTLs in five of six patients with lung adenocarcinoma by the cytokine combination. The mechanism of efficient CTL induction may be attributed to induction of DCs from PBMCs.

ACKNOWLEDGMENTS

We thank the Ohtsuka Pharmaceutical Co., Ltd. for providing recombinant IL-1β, the Shinonori Pharmaceutical Co., Ltd. for providing IL-2, the Ono Pharmaceutical Co., Ltd. for providing IL-4, and the Kirin Brewery Co., Ltd. for providing IL-6.

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