Activation Pathways Implicate Anti-HLA-DP and Anti-LFA-1 Antibodies as Lead Candidates for Intervention in Chronic Berylliosis

Yuan K. Chou, David M. Edwards, Andrew D. Weinberg, Arthur A. Vandenbark, Brian L. Kotzin, Andrew P. Fontenot and Gregory G. Burrows

*J Immunol* 2005; 174:4316-4324; doi: 10.4049/jimmunol.174.7.4316

http://www.jimmunol.org/content/174/7/4316

References

This article cites 57 articles, 23 of which you can access for free at: http://www.jimmunol.org/content/174/7/4316.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Activation Pathways Implicate Anti-HLA-DP and Anti-LFA-1 Antibodies as Lead Candidates for Intervention in Chronic Berylliosis

Yuan K. Chou, David M. Edwards, Andrew D. Weinberg, Arthur A. Vandenbark, Brian L. Kotzin, Andrew P. Fontenot, and Gregory G. Burrows

CD4⁺ T cells play a key role in granulomatous inflammation in the lung of patients with chronic beryllium disease. The goal of this study was to characterize activation pathways of beryllium-responsive bronchoalveolar lavage (BAL) CD4⁺ T cells from chronic beryllium disease patients to identify possible therapeutic interventional strategies. Our results demonstrate that in the presence of APCs, beryllium induced strong proliferation responses of BAL CD4⁺ T cells, production of superoptimal concentrations of secreted proinflammatory cytokines, IFN-γ, TNF-α, and IL-2, and up-regulation of numerous T cell surface markers that would promote T-T Ag presentation. Ab blocking experiments revealed that anti-HLA-DP or anti-LFA-1 Ab strongly reduced proliferation responses and cytokine secretion by BAL CD4⁺ T cells. In contrast, anti-HLA-DR or anti-OX40 ligand Ab mainly affected beryllium-induced proliferation responses with little impact on cytokines other than IL-2, thus implying that nonproliferating BAL CD4⁺ T cells may still contribute to inflammation. Blockade with CTLA4-Ig had a minimal effect on proliferation and cytokine responses, confirming that activation was independent of B7/CD28 costimulation. These results indicate a prominent role for HLA-DP and LFA-1 in BAL CD4⁺ T cell activation and further suggest that specific Abs to these molecules could serve as a possible therapy for chronic beryllium disease. The Journal of Immunology, 2005, 174: 4316–4324.
behavior as a means of obtaining new insights focused on how to prevent this chronic inflammatory and eventually debilitating disease.

With regard to Ag-specific T cell activation, naïve T cells appear to require two signaling events, TCR engagement with Ag/MHC and a second costimulatory signal, which is most frequently mediated by the engagement of CD28 on the T cell with its ligands B7-1 or B7-2, on APCs (23, 24). The BAL beryllium-specific CD4+ T cells are effector memory cells that proliferate and secrete cytokines after TCR engagement without an absolute requirement for CD28-mediated costimulation (25). This result suggested either no dependence on costimulation or the involvement of a different costimulation pathway such as OX40/OX40 ligand (OX40L). OX40 (CD134), a membrane-bound member of the TNFR superfamily, is expressed primarily on activated CD4+ T cells (26). Following engagement of OX40 with OX40L expressed on the APC, OX40 delivers a costimulatory signal that leads to potent, proinflammatory effects (27, 28). However, whether or not human beryllium-specific CD4+ T cells expressed OX40 and, if so, whether the OX40/OX40L costimulatory pathway could play a role in beryllium-induced CD4+ T cell activation has not been explored previously.

In the current study, we evaluated a beryllium-reactive T cell line from the BAL of a CBD patient for the level and the duration of T cell proliferation and cytokine secretion, MHC restriction, and costimulatory molecules upon stimulation with beryllium. These studies substantially enhance our understanding of the immunological features of beryllium-induced activation of BAL CD4+ T cells and provide new data suggesting potential therapeutic avenues for intervention in CBD.

**Materials and Methods**

**Generation of beryllium-specific BAL CD4+ T cells and APCs**

The BAL CD4+ T cell line from a representative HLA-DP2 homozygous CBD patient was developed as previously described (29). Briefly, the diagnosis of CBD was established using previously defined criteria, including a history of beryllium exposure, the presence of granulomatous inflammation upon lung biopsy, and a positive beryllium lymphocyte-proliferation test (30, 31). The BAL cells from an HLA-DP2 homozygous CBD patient were collected and briefly stimulated with 10 μM BeSO4, and 100 μg/ml streptomycin, 0.2 mM glutamine, 1 mM sodium pyruvate, 0.2 mM nonessential amino acids (Invitrogen Life Technologies), 100 μg/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen Life Technologies) followed by the addition of 10 ng/ml rIL-2 (R&D Systems) twice weekly. The induction of EBV-transformed APCs from patient’s PBMC was achieved by using the methods as previously reported (32, 33). HLA typing was performed by standard molecular techniques as previously described (29).

**BAL CD4+ T cell line proliferation assay**

The BAL CD4+ T cell line was selected by stimulating BAL cells with 10 μM BeSO4 in the presence of irradiated (9000 rad) EBV-transformed autologous APCs at a ratio of 1:1 (T:APC) for 3 days, followed by the addition of 10 ng/ml rIL-2 (R&D Systems) twice weekly. The proliferation assay was performed using 4 × 10^5 T cells/well in triplicate in 96-well U-bottom culture plates plus irradiated (9000 rad) EBV-transformed autologous APCs at a ratio of 1:1, alone or in the presence of BeSO4, at a concentration ranging from 0.2 to 80 μM, for 5 days with 0.5 μCi [3H]thymidine (Amersham) for the last 6 h of the culture, before harvesting. The radioactivity was determined by a liquid scintillation counter 1205 Betaplate (Wallac) and proliferation was expressed by either stimulation index (experimental cpm divided control cpm) or net cpm (experimental cpm minus control cpm). Student’s t test was used for determining statistical significance between different cpm (mean ± SD) values. The transfected mouse L cell line DAP3(DP2) encoding HLA-DP2 genes was also used as APCs, irradiated (4500 rad) (33), and cocultured with CD4+ T cells at a ratio of 1:1 for 3 days, alone or in the presence of BeSO4, at the same concentration range as that used for autologous APCs. As irrelevant Ag controls, NitoS (Sigma-Aldrich) and the myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (New England Peptide) were used. Morphological features of BAL CD4+ T cell activation in the culture, with either EBV-transformed autologous APCs or DAP3(DP2)-transfected APCs upon stimulation with 10 μM BeSO4, were studied by microscopy (Leica Microsystems).

**Cell surface marker phenotyping**

BAL CD4+ T cell surface marker phenotyping in an unstimulated state was followed by stimulation with 10 μM BeSO4 at 24, 48, 72, 96, 120, and 144 h in the presence of EBV-transformed autologous APCs and IL-2. All Abs were purchased from BD Pharmingen and BD Biosciences, with the exception of anti-CD28 (CALTAG Laboratories) and anti-04X0L (R&D Systems). All markers were costained with either CD4-FITC or CD4-PE (clone RPA-T4; catalogues nos. 553436 and 553437, respectively). The surface markers were stained with the following mAbs: CD3-PE (clone UCHT1; catalogue no. 553333), CD8-PE (clone RPA-T8; catalogue no. 553367), CD45RO-FITC (clone UCHL1; catalogue no. 554592), CD45RO-PE (clone 2H108.1A-31; catalogue no. 555548), CD25-PE (clone M-A251; catalogue no. 554532), CD69 (clone FN50; catalogue no. 555529), CD134 (clone (Ox40) (clone L016; catalogue no. 340420), CD40L (TNFSF4) (clone 159403; catalogue no. MAB10541), CD28 (clone 15E8; catalogue no. MHC2800-4), CD152-PE (CTLA-4) (clone BN3; catalogue no. 555583), CD80 (clone B7-1) (clone L307.4; catalogue no. 557227), CD86 (B7-2) (clone 2331; catalogue no. 556568), CD50 (ICAM-3) (clone TU41; catalogue no. 555957), CD11a (LFA-1) (clone HI111; catalogue no. 555382), CD24-HLA-DR (clone L243; catalogue no. 347360), HLA-DP (clone B721; catalogue no. 347370), and HLA-DQ (clone SK10; catalogue no. 347450). EBV-transformed APCs and DP2- or DP4-transfected APCs were stained appropriately with CD-19-PE (clone HIB19; catalogue no. 555413), CD11b-PE (Mac-1) (clone ICRF44; catalogue no. 555388), CD209 (DC-SIGN) (clone DCN46; catalogue no. 551186), CD11a (LFA-1), and anti-HLA-DR, -DP, and -DQ mAbs. Purified mAbs were labeled with secondary pan-IgG PE polyclonal Ab (catalogue no. 550589). Each staining sample, with 100,000 cells, was stained appropriately with Abs for 30 min at 4°C. After washing three times with sterile PBS supplemented with 2% FBS (pH 7.2), the cells were analyzed using cellquest software on a FACSscan (BD Biosciences). Cell viability was monitored using Annexin V-PE and 7-aminoactinomycin D from Apoptosis Detection Kit I (catalogue no. 559763; BD Pharmingen) on FACSscan.

**Cytometric bead array (CBA) assay to detect and quantify cytokine production**

The CBA assay was performed using the human Th1/Th2 cytokine I CBA kit (catalogue no. 551809; BD Biosciences), which quantifies the following cytokines: IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α. Cytokine quantification assay sensitivity for each cytokine is listed as follows: IL-2 (2.6 ± 0.2 pg/ml), IL-4 (2.6 ± 0.2 pg/ml), IL-6 (5.0 ± 0.2 pg/ml), IL-10 (2.8 ± 0.2 pg/ml), IFN-γ (7.1 ± 0.3 pg/ml), and TNF-α (2.8 ± 0.2 pg/ml). To obtain an accurate value for absolute IFN-γ production within the limits of the CBA system, a 1/25 dilution of culture supernatants was necessary. Briefly, 50 μl of supernatant, 50 μl of a mixture of capture beads, and 50 μl of Th1/Th2 PE detection reagent were combined and incubated for 3 h at room temperature in the dark. The samples were thoroughly washed and subsequently resuspended in 200 μl of wash buffer and analyzed by flow cytometry (FACSscan). Standard curves were generated for each cytokine using a mixed bead standard to interpolate the concentration of cytokine in the supernatant in picograms per milliliter.

**Blocking T cell response to beryllium by using mAbs and recombinant CTLA-4g**

The purified forms of anti-OX40 (CD134), anti-OX40L (TNFSF4 Ab), anti-CD28, anti-CTLA-4 (CD152), anti-HLA-DR, -DP, and -DQ mAbs were dialyzed against RPMI 1640 with three buffer changes to remove any potential cytotoxic sodium azide. All blocking Abs were subsequently tested in a proliferation assay with 2×10^5 cells/well incubated with 10 μM BeSO4 at 4 × 10^5 cells/well plus irradiated EBV-transformed autologous APCs at a ratio of 1:1 in triplicate in the presence or absence of mAbs at concentrations ranging from 2 to 25 μg/ml for 3 days, followed by [3H]thymidine incorporation for the last 6 h of the culture. Percent inhibition was calculated by subtracting the cpm of mAb treated samples from cpm of 10 μM beryllium-stimulated cells with no mAbs, followed by dividing with the cpm of no mAb × 100%. Recombinant human CTLA-4g (catalogue no. 325-CT;
R&D Systems) was prepared in aliquots of RPMI 1640/1% serum and ensured of noncytotoxicity in a Con A stimulation assay as described above for purified mAbs.

Results

Specificity and proliferation level of BAL CD4+ T cells

The TCR repertoire of blood and BAL CD4+ T cells from patients with CBD are different. From this particular CBD patient, there is an increased percentage of Vβ8.1+ T cells in ex vivo BAL (16.8% compared with 5.1%). After five cycles of BeSO4 stimulation, the increased percentage of Vβ8.1-expressing CD4+ T cells persisted. In addition, a Vβ5.1+ population markedly increased in proportion to the other CD4+ T cells in culture following continued cycles of BeSO4 stimulation. This subset accounted for ~30% of the CD4+ T cells after the fifth cycle of stimulation, and junctional region nucleotide sequencing of Vβ5.1-expressing BAL T cell clones from this line revealed two related clonal T cell populations with an identical CDR3 length (A. P. Fontenot, unpublished observation). These data indicate that as expected, continued selection of BAL cells in the presence of BeSO4 resulted in an oligoclonal expansion of the most highly reactive T cells. BAL CD4+ T cells from this representative HLA-DP2 homozygous CBD patient proliferated strongly in response to BeSO4. As shown in Fig. 1A, BeSO4-specific proliferation was observed over a dose range of almost three orders of magnitude (0.2–80 μM) of beryllium with a maximal stimulation index (SI) >11× at 20 μM BeSO4. The choice of APCs used was of particular importance. Using autologous EBV-transformed cells as APCs (primarily B cells; see Fig. 2C), the CD4+ T cell line responded significantly to 0.2 μM BeSO4 with a SI of 4× that increased dramatically with increasing concentrations of BeSO4. A maximal SI of 11× occurred at 20 μM, then gradually dropped down to a SI of 7–8×, even at BeSO4 concentrations as high as 80 μM (Fig. 1A). Use of a transfected mouse fibroblast cell line DAP3(DP2) encoding human HLA-DP2 (HLA-DPA1*0103/DPB1*0201) genes as APCs resulted in a maximal SI of 4×, 70% lower than that induced by autologous APCs. In contrast, use of a transfected mouse fibroblast cell line DAP3(DP4) encoding the human HLA-DP4 gene (HLA-DPA1*0103/DPB1*0401; identical to HLA-DP2 except at residues 36, 55/56, and 69 within the β-1 domain) as APCs resulted in a maximal SI of only 2×, 85% lower than that induced by autologous APCs, and no greater than that of T cells alone in the presence of BeSO4. The cells were highly specific for beryllium and showed virtually no proliferation to NiSO4 or to an irrelevant Ag peptide, MOG35–55 peptide, which can bind with high affinity to HLA-DP2 (R. Meza-Romero, personal communication).

Potent secretion of IFN-γ and TNF-α by beryllium-stimulated CD4+ T cells

Activation requirements for optimal production of inflammatory cytokines are shown in Table I. Although BAL CD4+ T line cells or APCs alone secreted very low levels of cytokines, the combination of T cells plus APCs produced a synergistic 3- to 10-fold increase in TNF-α and IFN-γ, but no change in IL-2. This beryllium-independent activation conceivably could represent an autologous MLR (AMLR), perhaps driven by recognition of self-MHC class II molecules. A similar degree of cytokine secretion occurred when BAL CD4+ T line cells but not APCs alone were activated by BeSO4. This beryllium-dependent response could result from self-presentation of BeSO4 by T-T interactions or through limiting numbers of BAL-derived APCs that persisted in the T cell line. Of importance, beryllium-dependent activation of BAL CD4+ T cells with added APCs resulted in the superoptimal production of IFN-γ, TNF-α, and IL-2 (Table I).

More specifically, BAL CD4+ T cells were stimulated with 10 μM BeSO4 in the presence of EBV-transformed autologous APCs at a ratio of 1:1 for 3 days and the supernatants were collected and analyzed for Th1/Th2 cytokines using a quantitative CBA assay. BAL CD4+ T cells (40,000/well) cultured in 0.2 ml of medium generated >30 ng/ml IFN-γ and 1 ng/ml TNF-α by 72 h (Fig. 1B). The normalized production of IFN-γ and TNF-α was calculated to be >150 ng IFN-γ and 5 ng TNF-α produced per 1 million BAL beryllium-stimulated CD4+ T cells within 72 h. Along with production of IFN-γ and TNF-α, BAL CD4+ T cells produced IL-2 at >3 ng per million T cells. IL-4, IL-6, and IL-10 were essentially undetectable following stimulation. Thus, beryllium-specific CD4+ T cells showed a predominant Th1 cytokine profile with unexpectedly high levels of secreted IFN-γ and TNF-α. Surprisingly, IFN-γ and TNF-α production by beryllium-stimulated T cells persisted for an extended period (12 wk in culture), when the cells were split with fresh medium containing exogenous IL-2 without washing out the residual beryllium (data not shown). These data suggested that chronic exposure to even low levels of beryllium caused persistent production of IFN-γ and TNF-α.

Phenotypic analysis of BAL CD4+ T cells and EBV-transformed autologous APC

Cell surface molecules were analyzed by FACS with a focus on characterizing the beryllium-induced activation profile of surface...
markers on BAL T cells cultured ex vivo. In addition to having relatively high levels of αβ-TCR (87%), the resting CD3⁺CD4⁺ BAL T cells were virtually 100% positive for CD45RO. Unstimulated BAL T cells also expressed high levels of MHC class II molecules, HLA-DR, -DP, and -DQ, as well as LFA-1 (CD11a) and ICAM-3 (CD50) (Fig. 2A).

Upon exposure to beryllium, BAL T cells showed distinct cell surface molecular changes that provided clear insight into their behavior and, potentially, avenues for therapeutic intervention. OX40 (CD134) and CD25 (IL-2R) were both at low levels in IL-2-supported resting culture, but dramatically increased to levels 90% positive within 24 h (CD25) and 48 h (OX40) in the presence of EBV-transformed APCs at a ratio of 1:1 in comparison to unstimulated cells. Distinct differential expression of each cellular marker at particular time points presented as histograms with both unstimulated and isotype mAb controls is shown on the left. EBV-transformed autologous APC cell surface expression of CD19, CD11b, OX40L, LFA-1 (CD11a), B7-1, B7-2, DC-SIGN (CD209), and HLA-DR, -DP, and -DQ molecules.

Table I. Activation Requirements

<table>
<thead>
<tr>
<th>Condition</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T only</td>
<td>16</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>APC only</td>
<td>102</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>T + APCs</td>
<td>2,305</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>T + Be²⁺</td>
<td>1,728</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>APCs + Be²⁺</td>
<td>113</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>T + APCs + Be²⁺</td>
<td>38,000 b</td>
<td>1,050</td>
<td>710</td>
</tr>
</tbody>
</table>

*Representative of three individual experiments. Each experimental point reflects the mean of two independent measurements. Forty thousand T cells per well with 0.2 ml of medium in triplicate were cultured with APCs at a ratio of 1:1 with or without 10 μM BeSO4 for 72 h.

Fig. 2. Phenotypic analysis of BAL CD4⁺ T cell line cells and EBV-transformed autologous APCs. A. Surface expression of CD3, CD4, TCRαβ chain, CD45RO, LFA-1 (CD11a), ICAM-3 (CD50), and HLA class II, including HLA-DR, -DP, and -DQ molecules by BAL CD4⁺ T cells resting in IL-2-containing medium before restimulation with beryllium. B. Changing surface expression of OX40, IL-2R (CD25), B7-1, B7-2, and CD28 on BAL CD4⁺ T cells upon stimulation with 10 μM BeSO₄ at 24, 48, 72, and 144 h in the presence of EBV-transformed APCs at a ratio of 1:1 in comparison to unstimulated cells. Distinct differential expression of each cellular marker at particular time points presented as histograms with both unstimulated and isotype mAb controls is shown on the left. C. EBV-transformed autologous APC cell surface expression of CD19, CD11b, OX40L, LFA-1 (CD11a), B7-1, B7-2, DC-SIGN (CD209), and HLA-DR, -DP, and -DQ molecules.
cells that increased strongly after 24 h of stimulation (Fig. 2B). CTLA-4 (CD152) was not significantly expressed on BAL T cells (data not shown). Staining of T cells with annexin V and 7-aminoactinomycin D before and after beryllium stimulation documented survival rates typically observed for effector/memory T cells (data not shown) (34, 35).

The EBV-transformed autologous APCs used for these studies were >95% CD19⁺, suggesting that they were predominantly B cells, with high levels of expression of LFA-1 (CD11a), MHC class II molecules, B7-1 and B7-2, and unlike the BAL T cells an alternative costimulation molecule, OX40L (Fig. 2C).

Blocking LFA-1 prevented proliferation and dramatically reduced effector cytokine production

We used a series of mAb blocking experiments, summarized in Table II, to characterize the requirement and relative importance of particular cell surface molecules for proliferation and effector cytokine production of beryllium-specific BAL T cells. In the presence of anti-LFA-1 Ab, both proliferation and, to a lesser degree, secretion of cytokines (IFN-γ, TNF-α, and IL-2) by beryllium-activated BAL T cells were inhibited in a dose-dependent manner (Fig. 3). These data indicate that interactions between the adhesion/trafficking molecule LFA-1 and its ligands contribute to the activation of beryllium-specific T cells.

Blocking HLA-DP prevented proliferation and dramatically reduced effector cytokine production

The absolute requirement of MHC class II molecules for activation of beryllium-specific T cells was clearly documented using Ab blocking experiments. As shown in Fig. 4A, addition of anti-HLA-DP Ab blocked T cell proliferation in a dose-dependent manner, with complete inhibition at 20 μg/ml, strongly reinforcing previous data showing that MHC class II molecules are critical for beryllium-specific proliferation of BAL T cells. Moreover, anti-DP Ab blocked IFN-γ 53% and TNF-α and IL-2 by ~70% (Fig. 4B). Interestingly, anti-HLA-DR Abs also had a blocking effect on proliferation of BAL T line cells (Fig. 4A), but anti-DR Ab had virtually no effect on inhibition of cytokine secretion (Fig. 4B). The anti-HLA-DR Ab used in these experiments reacts with a nonpolymorphic HLA-DR epitope (36–38) and does not cross-react with HLA-DQ or HLA-DP molecules (38). Moreover, we have independently confirmed that the anti-DR Ab does not cross-react with the Ag-binding/TCR recognition domains of either HLA-DR or HLA-DP4 by ELISA (data not shown).

### Table II. Blocking Experiments

<table>
<thead>
<tr>
<th>Blocking mAb Used</th>
<th>Effect (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proliferation</td>
</tr>
<tr>
<td>Anti-LFA-1</td>
<td>−53</td>
</tr>
<tr>
<td>Anti-HLA-DP</td>
<td>−99</td>
</tr>
<tr>
<td>Anti-HLA-DR</td>
<td>−72</td>
</tr>
<tr>
<td>Anti-OX40</td>
<td>nc</td>
</tr>
<tr>
<td>Anti-OX40L</td>
<td>−99</td>
</tr>
<tr>
<td>Anti-CD28</td>
<td>nc</td>
</tr>
<tr>
<td>CTLA4-Ig</td>
<td>−15</td>
</tr>
</tbody>
</table>

*For clarity, only results using the highest dose of blocking mAb are presented. nc, No change from control. Each experimental point reflects the data collected from individual experiments.

![FIGURE 3](http://www.jimmunol.org/)

**Blocking BAL CD4⁺ T cell response to beryllium by a mAb (clone HI111) specific for human LFA-1.** A. Blocking BAL CD4⁺ T cell proliferation to beryllium by anti-LFA-1. BAL CD4⁺ T cells (40,000/well in triplicate) were cultured with EBV-transformed autologous APCs at ratio of 1:1 with and without 10 μM BeSO₄ for 72 h. Anti-human LFA-1 Ab at concentrations of 10 and 2 μg/ml were added into cultures of T cells stimulated with beryllium in the presence of APCs for 72 h. Mouse IgG1 at 10 μg/ml was used as isotype Ab control. T cell proliferation was determined by [³H]thymidine incorporation for the last 6 h of the culture. The percent block of proliferation after addition of anti-LFA-1 Ab was calculated relative to that of the isotype control Ab. Significance was determined by Student’s t test. B. Blocking Th1 cytokine production of beryllium-stimulated BAL CD4⁺ T cells by anti-LFA-1. TNF-α and IL-2 production (picograms per milliliter) were measured using the CBA method on supernatant samples collected from BAL CD4⁺ T cell cultures in triplicate. The percent block of cytokine production by anti-LFA-1 Ab was calculated relative to that of the isotype control Ab and significance was determined by Student’s t test.

![FIGURE 4](http://www.jimmunol.org/)

**Anti-HLA-DP and anti-HLA-DR mAbs significantly blocked the BAL CD4⁺ T cell response to beryllium.** A. BAL CD4⁺ T cells (40,000/well in triplicate) were cultured with EBV-transformed autologous APCs at a ratio of 1:1 with and without 10 μM BeSO₄ for 72 h. Predialyzed nontoxic anti-HLA-DP (clone B7/21) and anti-HLA-DR (clone L243) were added into cultures of T cells stimulated with beryllium at Ab concentrations of 20 and 5 μg/ml for 72 h. Mouse IgG1 at 20 μg/ml served as isotype Ab control. B. Cytokine production (picograms per milliliter) was determined in triplicate using the CBA method. The supernatant samples were collected from T cell cultures as described above. The percent block after addition of Abs was calculated relative to that of the isotype control Ab. Significance was determined by Student’s t test.
beryllium stimulation before declining back to the resting distribution (Fig. 2B). Similar to a previous report (25), blockade of signaling through CD28 using soluble CTLA4-Ig to ligate B7-1 and B7-2 molecules on APCs had a relatively modest inhibition of proliferation (15%), IFN-γ (19%) and IL-2 (23%) secretion, but little effect on TNF-α (Fig. 5B). Moreover, addition of anti-CD28 Ab had no significant effect on activation (Fig. 5A). These data suggest a relatively minor contribution of the CD28/B7 costimulation pathway on beryllium-induced T cell activation.

In contrast to the transient up-regulation of CD28, beryllium-activated T cells had long-lasting high levels of OX40 expression (Fig. 2B), possibly implicating this costimulation pathway. Indeed, blockade of the OX40-OX40L interaction using anti-OX40L Ab resulted in complete (99%) inhibition of proliferation (Fig. 5, A and B). However, there was only a modest inhibitory effect of anti-OX40L Ab on IL-2 secretion (24%) and no detectable effect on IFN-γ or TNF-α levels (Fig. 5C). Similar to anti-CD28 Ab, anti-OX40 Ab also did not inhibit beryllium-induced T cell proliferation (Fig. 5A). These findings suggest a dominant role of the OX40/OX40L costimulation pathway on beryllium-induced proliferation, but further indicate that cytokine release is relatively independent of costimulation by either of these pathways.

**Discussion**

Characterization of pathogenic T cells from patients with CBD has revealed that beryllium-specific CD4⁺ T cells play a major role in initiating and perpetuating granulomatous inflammation (10, 11). The results presented above demonstrate that activation of BAL CD4⁺ T cells is a complex process, involving beryllium-dependent HLA- DP and HLA-DR-restricted stimulation through the TCR, partial dependence on the OX40/OX40L costimulation pathway, and involvement of cell-cell adhesion mediated through ligation of LFA-1. Moreover, activation may also be induced by beryllium-independent interactions between T cells and APCs, reminiscent of an AMLR involving MHC class II recognition. An inherent weakness of our system is the reliance on observations made using mass T cell culture in vitro to faithfully duplicate what occurs in vivo. That being said, in preliminary experiments, ex vivo BAL cells from CBD subjects express high levels of LFA-1 and up to 30% of direct ex vivo BAL CD4⁺ T cells up-regulate OX40 expression by 72 h after beryllium exposure in culture (A. P. Fontenot, unpublished observation). These results indicate that our cultured BAL T cells are representative of beryllium-driven T cells.

Consistent with the severe granulomatous inflammation known to occur in lung tissue in CBD, the in vitro combination of beryllium, APCs, and BAL CD4⁺ T cells induced strong proliferation responses; superoptimal concentrations of the secreted proinflammatory cytokines IFN-γ, TNF-α, and IL-2; and up-regulation of numerous T cell surface markers that would promote T-T Ag presentation. Ab blocking experiments revealed that anti-HLA- DP or anti-LFA-1 Ab strongly reduced proliferation responses and, to a lesser extent, cytokine secretion by BAL CD4⁺ T cells. In contrast, anti-HLA-DR or anti-OX40L Ab mainly affected beryllium-induced proliferation responses with little impact on cytokines other than IL-2, thus implying that nonproliferating BAL CD4⁺ T cells may still contribute to inflammation. Blockade with CTLA4-Ig had a minimal effect on proliferation and cytokine responses, confirming that activation was independent of B7/CD28 costimulation. Because T cell proliferation and secretion of proinflammatory cytokines appear to play a dominant role in the chronic inflammatory process that defines CBD, it would appear that Abs

**Costimulation involved in beryllium-induced CD4⁺ T cell activation: OX40/OX40L pathway plays a dominant role in proliferation**

Memory T cells can proliferate and secrete cytokines after TCR engagement without CD28-mediated costimulation (39), and the CD28 costimulation independence of BAL beryllium-specific T cells has previously been reported (25). We demonstrated above that 98% of BAL beryllium-specific CD4⁺ T line cells were of the CD45RO⁺ memory phenotype, responding to beryllium in the presence of autologous APCs by proliferation and secretion of high levels of IFN-γ and TNF-α. Although CTLA-4 was not present at an appreciable level on resting or beryllium-activated BAL T cells (data not shown), the level of CD28 on the BAL T cell population changed significantly (from 54 to 92%) for 24 h after

**FIGURE 5.** Involvement of costimulation molecules in beryllium-specific activation of BAL CD4⁺ T cell line cells in the presence of EBV-transformed autologous APCs. A, Blocking beryllium-induced BAL CD4⁺ T cell proliferation in the presence of EBV-transformed autologous APCs. Forty thousand BAL CD4⁺ T cells were cultured in the presence of EBV-transformed APCs at a ratio of 1:1 in triplicate with and without 10 μM BeSO₄ for 72 h. Anti-OX40 (25 μg/ml), anti-OX40L, anti-CD28, CTLA4-Ig, and an isotype Ab control (mouse IgG1) were added into cultures of T cells stimulated with beryllium for 72 h. Proliferation was calculated as net cpm above background. The percent block after addition of Abs was calculated relative to that of the isotype control Ab. Significance was determined by Student’s t test. B, Blocking assay on BAL CD4⁺ T cell proliferation to 10 μM BeSO₄ in the presence of EBV-transformed autologous APCs at a ratio of 1:1 was repeated, with and without adding 25 and 10 μg/ml anti-OX40L and CTLA4-Ig, respectively, for 72 h. Proliferation was expressed as net cpm above background. C, Blocking IL-2 production of beryllium-stimulated BAL CD4⁺ T cells by both CTLA4-Ig and anti-OX40L. Cytokine production (picograms per milliliter) was determined in triplicate by the CBA method. The supernatant samples were collected to occur in lung tissue in CBD, the in vitro combination of beryllium, APCs, and BAL CD4⁺ T cells has previously been reported (25). We demonstrated above that 98% of BAL beryllium-specific CD4⁺ T line cells were of the CD45RO⁺ memory phenotype, responding to beryllium in the presence of autologous APCs by proliferation and secretion of high levels of IFN-γ and TNF-α. Although CTLA-4 was not present at an appreciable level on resting or beryllium-activated BAL T cells (data not shown), the level of CD28 on the BAL T cell population changed significantly (from 54 to 92%) for 24 h after

**FIGURE 5.** Involvement of costimulation molecules in beryllium-specific activation of BAL CD4⁺ T cell line cells in the presence of EBV-transformed autologous APCs. A, Blocking beryllium-induced BAL CD4⁺ T cell proliferation in the presence of EBV-transformed autologous APCs. Forty thousand BAL CD4⁺ T cells were cultured in the presence of EBV-transformed APCs at a ratio of 1:1 in triplicate with and without 10 μM BeSO₄ for 72 h. Anti-OX40 (25 μg/ml), anti-OX40L, anti-CD28, CTLA4-Ig, and an isotype Ab control (mouse IgG1) were added into cultures of T cells stimulated with beryllium for 72 h. Proliferation was expressed as net cpm above background. The percent block after addition of Abs was calculated relative to that of the isotype control Ab. Significance was determined by Student’s t test.

A. Blocking assay on BAL CD4⁺ T cell proliferation to 10 μM BeSO₄ in the presence of EBV-transformed autologous APCs at a ratio of 1:1 was repeated, with and without adding 25 and 10 μg/ml anti-OX40L and CTLA4-Ig, respectively, for 72 h. Proliferation was expressed as net cpm above background. B, Blocking IL-2 production of beryllium-stimulated BAL CD4⁺ T cells by both CTLA4-Ig and anti-OX40L. Cytokine production (picograms per milliliter) was determined in triplicate by the CBA method. The supernatant samples were collected to occur in lung tissue in CBD, the in vitro combination of beryllium, APCs, and BAL CD4⁺ T cells has previously been reported (25). We demonstrated above that 98% of BAL beryllium-specific CD4⁺ T line cells were of the CD45RO⁺ memory phenotype, responding to beryllium in the presence of autologous APCs by proliferation and secretion of high levels of IFN-γ and TNF-α. Although CTLA-4 was not present at an appreciable level on resting or beryllium-activated BAL T cells (data not shown), the level of CD28 on the BAL T cell population changed significantly (from 54 to 92%) for 24 h after
specific for HLA-DP and LFA-1 would be well suited for further investigation as a therapeutic strategy for intervention in CBD as well as other hypersensitivity pneumonitides.

T cell proliferation to beryllium salt in vitro was beryllium specific and dose dependent (Fig. 1A), and our analysis clearly excluded nonspecific T cell stimulation by other metal salts, although the molecular details of metal–protein interactions underlying the transport and delivery of metal ions to APCs during their early sensitization phase and the interaction of beryllium with HLA and TCR is still unclear (40). The BeSO₄ dose-dependent T cell proliferation curve consistently showed a decreased maximal stimulation above the peak responsive concentration of 20 μM BeSO₄, but concentrations up to 80 μM still showed almost 70% of the maximal response. These data are consistent with previous observations regarding beryllium toxicity to APCs (41) and suggest that beryllium-specific T cells are truly tolerant of high doses of BeSO₄. Our later studies documented that BAL CD4⁺ T cells retained the ability to secrete large amounts of IFN-γ and continuously live for months under conditions in which exogenous IL-2 was added (Y. K. Chou, personal communication).

Our studies documented that the BAL CD4⁺ T cell line was strongly polarized toward Th1, secreting detectable amounts of IFN-γ even in the absence of beryllium stimulation (Table I). In the absence of APCs, addition of beryllium increased secretion of IFN-γ by >100-fold, directly suggesting T–T interactions play a role in maintaining the chronic milieu characteristic of CBD. In contrast, addition of beryllium to APCs alone resulted in no change in IFN-γ and TNF-α production. However, IFN-γ secretion increased 100-fold merely by culturing the T cells with autologous APCs at a 1:1 ratio, indicating a beryllium-independent activation reminiscent of an AMLR, perhaps involving recognition of self-MHC class II molecules. Unexpectedly, upon beryllium addition to a 1:1 culture ratio of T:APC, the activated T line cells produced an astounding 38,000 pg/ml secreted IFN-γ, as well as >1000 pg/ml TNF-α and 700 pg/ml IL-2. Importantly, in the absence of APCs no IL-2 secretion was observed, clearly documenting the requirement of APCs for the full activation profile of these BAL T cells.

The memory phenotype of BAL beryllium-specific T cells has been reported previously (16–18), and is confirmed by our staining data, with the vast majority of the CD4⁺ T cells staining positive for CD45RO. The features of high-level expression of CD134 (OX40; upon stimulation) and HLA-DR, -DP, and -DQ on the cell surface and the ability to secrete extremely high levels of IFN-γ all confirm that this beryllium-specific BAL CD4⁺ T cell line was highly activated with a T effector/memory phenotype (42–44). The survival of CD4⁺ T effector-memory cells depends on their ability to produce IL-2, whereas cells producing only effector cytokines (e.g., IFN-γ) may be short-lived (45). The level of CD25 (IL-2Rα) expression is considered to be a reflection of recent Ag stimulation and plays a crucial role in the regulation of T cell proliferation (46). In the current study, we found that CD25 was very rapidly up-regulated during the course of stimulation with beryllium, with 5–10% of the resting cells expressing CD25, increasing to >90% within 24 h and remaining at an elevated level for at least 144 h. CD25 expression on the cell surface finally declined 2–3 wk after beryllium stimulation in direct correlation to the concentration of BeSO₄ depleted from the culture medium by serial dilution. T cell proliferation continued as long as IL-2 was added to the culture medium. Of possible diagnostic importance, these data suggest that CD25 can be used as a functional marker for BAL CD4⁺ T cell activation and exposure to BeSO₄.

CD80 (B7-1) and CD86 (B7-2) are members of the Ig supergene family and are coregulators of T cell activation through interactions with CD28 and CTLA-4 on T cells. These molecules are expressed on APCs as well as on activated CD4⁺ cells at late stages after activation (47). We found very high-level expressions of B7-2 even on resting BAL CD4⁺ T cells, and B7-1 was dramatically up-regulated within 24 h upon beryllium stimulation (Fig. 2B). Previous studies suggested that beryllium-specific memory CD4⁺ T cells in blood continued to require CD28 costimulation for proliferation and cytokine responses, whereas BAL effector memory cells were functionally independent of CD28 costimulation (25). One possibility that warrants further investigation is that constitutively high levels of B7-2 allow direct T:T costimulation between resident activated B7-2high/CD28low BAL T cells and effector/memory B7-2high/CD28low CD4⁺ T cells recruited to the lung from the blood, with T–T interactions providing both the primary signal as well as costimulation. Ab blocking studies have clearly helped to identify the most important pathways for activation of BAL CD4⁺ T cells. The ability of anti-HLA- DP to abolish proliferation as well as eliminate 70% of both IFN-γ and TNF-α effector cytokine and IL-2 autocrine secretion point to the central role played by HLA-DR in T cell activation. Future studies will be directed at evaluating how beryllium and HLA- DP interact and further the molecular recognition of beryllium as an HLA- DP-restricted Ag. Blockade of LFA-1 interaction using mAb HI111 (48) consistently inhibited proliferation and IL-2 secretion by >50% and also significantly reduced secretion of IFN-γ and TNF-α. The known ligands of LFA-1 include ICAM-1 (CD54), ICAM-2 (CD102), and ICAM-3 (CD50) (49–52). ICAM-1 and ICAM-2 are expressed on endothelium and have been implicated in leukocyte adhesion and transendothelial migration. ICAM-3 is exclusively present on T and B leukocytes where it appears to be important for the initial scanning of the APC surface by T cells, as well as playing a role in augmenting LFA-1- and ICAM-1-mediated adhesion and signaling via low-affinity interactions (53–55). It is noteworthy that ICAM-1, which has an ~10,000-fold higher affinity for LFA-1 than ICAM-3 (56), is also up-regulated on BAL T cells upon APC plus beryllium exposure (data not shown).

A novel observation from this study is that OX40 expression by beryllium-stimulated CD4⁺ T cells was strongly up-regulated within 24–48 h after stimulation with beryllium and was maintained at high levels throughout the 144-h observation period (Fig. 2B). This activation profile of BAL T cells upon BeSO₄ stimulation is similar to changes observed with peptide-Ag-activated CD4⁺ T cells (57) and suggests that the engagement of OX40 with OX40L expressed on APCs may play a role in activation and increased survival of beryllium-specific memory T cells (58). Interestingly, blockade with anti-OX40 Ab inhibited proliferation responses but not cytokine secretion by BAL CD4⁺ T cells, suggesting an uncoupling of the cell cycle from cytokine production. This finding implies that interruption of the OX40/OX40L costimulation pathway might limit the number of beryllium-reactive T cells without affecting their secretion of inflammatory cytokines. A similar pattern showing inhibition of proliferation but not cytokine responses was observed using anti-HLA-DR Ab, and the possibility exists that BAL CD4⁺ T cells may exhibit a unique activation pathway involving TCR ligation through HLA-DR with OX40/OX40L costimulation.

In summary, our data reinforce previous work concerning the central importance of HLA- DP in beryllium disease, with blockade of HLA- DP being the best approach to eliminate proliferation ability and cytokine production. The ability of anti-LFA-1 to partially inhibit these activities is also of potential therapeutic importance. Since T cell expansion and secretion of proinflammatory cytokines appear to play a dominant role in the chronic inflammatory process
that defines CBD, it would appear that Abs specific for HLA-DR and LFA-1 would be well suited for further investigation as a therapeutic strategy for CBD.

Acknowledgments

We thank Dr. Dennis Bourdette for meaningful discussion regarding CD4+ T cell-mediated chronic inflammation in the CNS and Dorian LatOcha for his assistance with the T cell typing work.

Disclosures

Senior author G. G. Burrows has filed provisional patents with the Oregon Health & Science University Technology and Research Collaboration Office, and coauthors Y. K. Chou, A. A. Vandenbark, and D. Edwards are listed as co-inventors.

References


intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J. Immunol.* 137:1270.


