

Aspergillus fumigatus extract differentially regulates antigen-specific CD4⁺ and CD8⁺ T cell responses to promote host immunity

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Abstract: Invasive aspergillosis is a major cause of morbidity and mortality in the severely immunocompromised. The paucity of information about the mechanisms by which *Aspergillus*-derived factors regulate antigen-specific T cell responses in vivo poses a significant hurdle for devising effective immunization strategies to treat or prevent aspergillosis. By monitoring adoptively transferred T cell receptor transgenic, naive CD4⁺ (OT-II) and CD8⁺ (OT-I) T cells specific for distinct peptides of a nominal antigen, chicken ovalbumin (OVA), we demonstrate that sensitization with *Aspergillus fumigatus* (Af) extract plus OVA protein considerably enhances OT-I and OT-II T cell activation, which results in clonal expansion, primarily as a result of increased proliferation. The sensitization provided by Af extract promotes OT-I expansion accompanied by differentiation into interferon- γ -producing cytotoxic cells. It is surprising that no effector differentiation of the induced OT-II response was observed. Moreover, the Af extract-induced OT-I and OT-II T cell expansion was transient, as considerable contraction in the numbers of detectable OT-I and OT-II T cells was evidenced by Day 10. In agreement with these observations, sensitization with Af extract plus OVA marginally promoted host immunity against an OVA-expressing thymoma (E.G7) challenge, and the protection was enhanced by resensitization with Af extract and OVA. Our results demonstrate the ability of Af extract to differentially regulate antigen-specific CD4⁺ and CD8⁺ T cell responses, resulting in limited augmentation of host immunity. This information suggests that strategies to target CD4⁺ T cell effector maturation may promote host immunity to *Aspergillus* and unexpectedly demonstrates the use for Af extract as a CD8⁺ T cell adjuvant. *J. Leukoc. Biol.* 80: 000–000; 2006.

Key Words: rodent · transgenic · cell activation · cell differentiation · cytotoxicity · spleen · lymph nodes · tumor immunity

INTRODUCTION

Aspergillus species are ubiquitous, filamentous fungi, which can cause invasive aspergillosis, a major cause of morbidity

and mortality in immunocompromised hosts. In fact, the number of fatal cases of invasive aspergillosis has increased by several-fold in the 1980s and 1990s in the United States [1]. People most at risk for aspergillosis include patients with prolonged neutropenia, allogeneic hematopoietic stem cell transplant (HSCT) recipients, solid organ transplant recipients, advanced AIDS, and chronic granulomatous disease [2]. Recent studies have shown the predominance of aspergillosis cases in allogeneic HSCT recipients occurring in the postengraftment rather than the neutropenic period [3, 4], and immunosuppressive therapy for graft-versus-host disease is a principal risk factor. Indeed, allogeneic HSCT recipients who received a T cell-depleted or CD34-enriched allograft have an increased risk for invasive aspergillosis in the postneutrophil recovery period [4]. These findings suggest an important role for host adaptive responses in the deterrence of invasive aspergillosis and behooves developing better understanding of the impact of *aspergillus*-derived factors on T cell responses in vivo.

In contrast to invasive aspergillosis, allergic bronchopulmonary aspergillosis (ABPA) occurs in immunocompetent persons and is characterized by recurrent episodes of bronchitis, asthma, pulmonary infiltrates, and lung structural modifications. ABPA is thought to develop from sensitization to *Aspergillus fumigatus* (Af)-derived factors, which promote a Type 2 CD4⁺ T cell response [5]. Af extracts containing the major Af-derived factors have been used to induce airway hyperresponsiveness, eosinophilic inflammation, and a T helper cell type 2 (Th2) response in mice, which mimics ABPA in humans [6, 7]. Administration of CpG sequences attenuated the skewed Th2 responses and was protective in experimental ABPA [8]. Although, several studies have implied that sensitization with Af extract may suppress antigen-specific cellular immunity or deviate T cell responses to the Type 2 phenotype to render host susceptible to *Aspergillus* infection, a few studies have also demonstrated the potential of Af extract to induce Th1 memory responses, which can confer protection against experimental, invasive aspergillosis [9, 10]. It is evident that the priming

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conditions afforded by the innate immune cells determine the effector outcome of an antigen-induced T cell response. The ability of the host's adaptive response to mediate protection against *Aspergillus* infection may require direct cytolytic functions and/or indirect actions via cytokines such as interferon- γ (IFN- γ), which recruits innate immune cells to control aspergillosis. The exact mechanism underpinning host inability to control *Aspergillus* infection remains poorly understood.

A variety of virulence factors, including proteases, reactive oxygen scavengers, phospholipases, and hemolysins, produced by the *Aspergillus* species, mediate their ability to infect and evade host defenses. Several components of the innate immune system, including the mannose-binding lectin pathway [11], chemokines (most notable, ligands of CC chemokine receptor 5 and CXC chemokine receptor 4 [12]), and Toll-like receptors (TLRs) [13, 14], play a role in regulating the quality and extent of an inflammatory response generated by Af. In addition, a number of studies have demonstrated the broad, immunosuppressive properties of gliotoxin, a metabolite produced by Af, which encompasses inactivation of innate and antigen-specific immunity [15–17].

Thus, although some reports show that sensitization with *Aspergillus* hyphal constituents augments protecting Type I immunity, most studies have demonstrated that these factors stimulate allergic (Type II) responses or are immunosuppressive. The seemingly discrepant reports may arise as a result of differences in the animal model used and/or the design of the experiments. Nevertheless, they point to the need for a better understanding of host adaptive responses to *Aspergillus*-derived factors, which can be achieved by longitudinally monitoring an antigen-specific T cell response in vivo.

Several *Aspergillus*-specific antigens have been identified, and T cell responses to them have been studied in patients and animal models of ABPA by ex vivo analysis [7]. Although the information derived has been useful in identifying targets for immunotherapy of aspergillosis and ABPA [18, 19], the low number of *Aspergillus*-specific T cells found in normal hosts possesses a considerable hurdle to better understand the mechanisms by which *Aspergillus*-derived factors impact T cell responses in vivo [17]. To better understand the impact of *Aspergillus*-produced factors on the induction of host CD4+ and CD8+ T cell responses, we have chosen to use the adoptive transfer approach in which ovalbumin (OVA) peptide-specific, naïve T cell receptor (TCR) transgenic CD8+ T cells (OT-I) [20] and CD4+ T cells (OT-II) [21] are transferred into syngeneic recipients and then monitored during a progressive T cell response generated by sensitization with their cognate antigen plus Af extract in vivo. This approach has been used widely to monitor and characterize T cell responses against tumor, viral, and bacteria challenges in vivo and has yielded valuable insights into mechanisms that regulate host adaptive immunity against tumor and infections [22, 23].

Herein, we report that Af extract has adjuvant properties that induce OVA antigen-specific CD4+ and CD8+ T cell activation and proliferation. It is notable that Af extract produced only CD8+ T cell effector differentiation, and the induced T cell responses were transient. In agreement with these observations, sensitization with Af plus OVA showed modest increases in host immunity, as demonstrated by pro-

tection against challenge by OVA-expressing thymoma (E.G7), which was augmented by resensitization. These findings provide new insights into the impact of Af-derived factors on host adaptive T cell responses, and the information presented is likely to benefit development of strategies to harness host T cells to control *Aspergillus* infection.

MATERIALS AND METHODS

Mice

The OT-I [major histocompatibility complex (MHC) class I-restricted] and the OT-II (MHC class II-restricted) TCR transgenic mice [20, 21], on the Rag 2^{-/-} background, were housed under specific pathogen-free conditions at the vivarium at the Roswell Park Cancer Institute (Buffalo, NY) and used in compliance with all relevant laws and institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee of the Roswell Park Cancer Institute. The recipient animals were age- and sex-matched, naïve C57BL/6 mice (Thy1.2, The Jackson Laboratory, Bar Harbor, ME).

Adoptive transfer and immunization

The lymph node (LN) cells from naïve OT-I and OT-II transgenic mice were purified and transferred into Thy1.2+ congenic recipients as described previously [22]. In brief, the LN cells were adherence-depleted, and a 50:50 mix of 2×10^6 (each) OT-I and OT-II T cells was adoptively transferred into congenic, naïve C57BL/6 recipients via tail vein injection, and the purity and activation status of the OT-I and OT-II T cells isolated was confirmed periodically by flow cytometry. To evaluate proliferation, the OT-I and OT-II T cells were labeled with 5 μ M carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), as described previously [24], and the extent of label dilution was monitored in CD8+/Thy1.1+-gated cells after adoptive transfer into Thy1.2+ recipients. For immunization, 10 μ g OVA protein (Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS) and delivered subcutaneously (s.c.) at two sites (base of skull and tail), alone or admixed with 14 μ g Af extract (Greer Laboratories, Lenior, NC) or 1 and 10 μ g *Salmonella typhimurium*-derived lipopolysaccharide (LPS; Sigma-Aldrich). Adoptive transfer of OT-I and OT-II cells was performed on Day -1, and sensitization was performed on Day 0, unless otherwise stated.

Antibodies and flow cytometry

All antibodies used in this study were purchased from BD PharMingen (Mountain View, CA). The mice were killed at indicated times after adoptive transfer, and the spleen, LN cells (from axillary, brachial, cervical, inguinal, lumbar, caudal, and renal nodes) were harvested, counted in a hemocytometer, and then stained with antibodies against CD8, CD4, CD44, CD62L, and Thy 1.1 markers. The stained cells were fixed and analyzed on a FACSCalibur/FACScan flow cytometer using CELLQuest software by BD Biosciences (Mountain View, CA). The gated OT-I (CD8+/Thy1.1+) and OT-II (CD4+/Thy1.1+) cells were evaluated for their activation status and CFSE dilution to determine their rate of cell division.

Intracellular cytokine staining (ICS)

Spleen and LN cells harvested from adoptively transferred and immunized mice were restimulated in vitro with 0.2 μ M OVA_{257–264} (peptide “SIINFEKL” for OT-I cells) or 2 μ M OVA_{323–339} (peptide “ISQAVHAHAHAEINEAGR” for OT-II cells). After 1 h of incubation at 37°C, Brefeldin A, 10 μ g/ml, was added to block protein transport. After 4–5 h at 37°C, the cells were fixed, permeabilized, and stained with antibody to CD8, CD4, Thy1.1, interleukin (IL)-4, and/or IFN- γ . The live OT-I and OT-II T cells were gated and analyzed with CELLQuest software.

In vivo cytolytic T lymphocyte (CTL) activity

Target and reference cells were prepared as described previously [25]. In brief, single-cell suspension of syngeneic B6 mouse spleen cells was pulsed with 0.2 μ M OVA_{257–264} (target) or an irrelevant peptide (reference) for 45 min at 37°C,

washed extensively, and then labeled with high (5 μ M) or low (0.5 μ M) concentration of CFSE, respectively. An equal number (50:50) of target and reference cells was mixed together, and a total of 2×10^7 was tail vein-injected into mice, which were naïve or immunized. After 4 h, the mice were killed, and the spleen cells and LN cells were analyzed for the CFSE-labeled target and reference cells based on their fluorescence intensities by flow cytometry. The percent-specific lysis was calculated by the formula: $1 - (\text{ratio unprimed/ratio primed}) \times 100$; ratio = percent reference/percent target.

Tumor challenge, survival, and statistics

E.G7 cells derived from EL4 murine thymoma cells (syngeneic to C57BL/6), which express the chicken OVA protein, have been used previously in murine tumor models [22]. Naïve C57BL/6 were adoptively transferred with (3×10^6) OT-I and OT-II T cells and sensitized the following day with one of the following regimens as described above: OVA alone; OVA plus Af extract; OVA plus LPS. The E.G7 cells were injected intraperitoneally (i.p.; 5×10^6 cells per mouse) on Day 4 after sensitization. Some mice, which had received OVA plus Af extract, were resensitized on Day 8 after adoptive transfer. Tumor-challenged mice were killed in a blinded manner to the immunization regimen based on prespecified criteria as approved Division of Laboratory Animal Resources protocol guidelines (unable to feed or drink or distress related to ascites). The mice were monitored up to 160 days after challenge with tumor.

Time to euthanasia was plotted using Kaplan-Meier curves and analyzed using the log-rank method (SigmaStat, Point Richmond, CA). A P value < 0.05 between different experimental groups was considered statistically significant. All pair-wise comparisons were made without adjustments for multiple comparisons.

RESULTS

Af extract enhances antigen-specific T cell expansion by augmenting proliferation in vivo

To test the ability of Af extract to regulate antigen-specific T cell responses in vivo, we adoptively transferred (Day -1) naïve (Thy1.1+) TCR transgenic CD4+ (OT-II) and CD8+ (OT-I) T cells into age- and sex-matched C57BL/6 (Thy 1.2+) recipients. On Day 0, the recipients were sensitized (s.c.) with OVA (10 μ g) plus PBS per mouse (antigen-only control), OVA (10 μ g) plus Af extract (14 μ g) per mouse, or OVA (10 μ g) plus LPS (1.0 μ g) per mouse (adjuvant control). The use of 1 μ g per mouse LPS administration was determined after careful titration to be optimal in stimulating OVA-specific T cell expansion in vivo (data not shown). On indicated days after antigen sensitization, mice were killed, and the number of adoptively transferred OT-I and OT-II T cells enumerated from the LNs and spleen by gating on CD4/Thy1.1- or CD8/Thy1.1-positive cells using flow cytometry. Rounds of cell division in adoptively transferred, antigen-specific T cells were evaluated by CFSE dilution.

Sensitization with Af extract plus OVA produced significantly greater clonal expansion of the adoptively transferred OT-I and OT-II T cells in comparison with OVA alone or OVA + LPS, optimally detected on Day 4 in the LNs and spleen (Fig. 1). As shown in Figure 1, A and B, sensitization with LPS plus OVA in comparison with OVA alone (normalized to 1) induced greater antigen-specific CD4+ and CD8+ T cell clonal expansion in the LNs (approximately two- and 1.5-fold, respectively) as well as spleen (approximately 2.1- and twofold, respectively). However, the animals sensitized with Af extract and antigen showed greater clonal expansion of OT-I and OT-II T cells than those sensitized with LPS plus OVA in the LNs

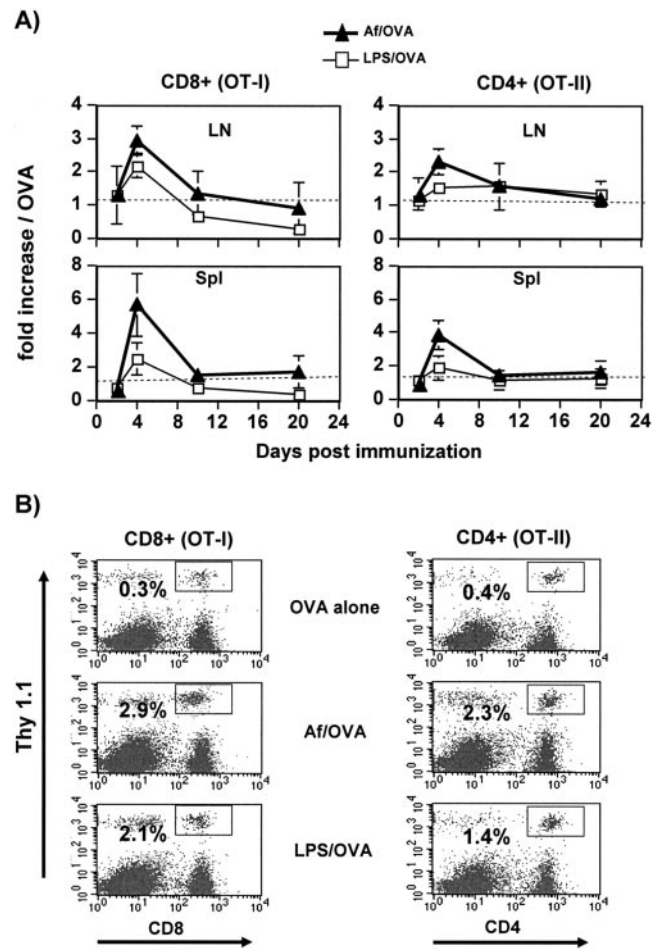


Fig. 1. Sensitization with Af extract enhances antigen-specific OT-I and OT-II T cell expansion. The number of OT-I and OT-II T cells detected in the LN and spleen (Spl) of animals sensitized with OVA alone or LPS/OVA or Af/OVA was obtained from the percentage of gated CD8+/Thy1.1+ and CD4+/Thy1.1+ T cells detected at various days by flow cytometry. (A) The number of OT-I and OT-II cells in “OVA alone”-sensitized animals was defined arbitrarily as one (dashed line), and the fold increase over OVA alone was calculated. The fold increase in OT-I and OT-II numbers was obtained from four independent experiments with two animals at each time-point/experimental group. Error bars show the standard deviation of the mean values. (B) A representative dot-plot analysis of the OT-I and OT-II T cells in the LN of animals at Day 4. The percentage of OT-I and OT-II T cells detected in the total cell population is indicated. A representative dot plot is shown.

(approximately three- and 2.5-fold, respectively) and spleen (approximately six- and fourfold, respectively). This observation from more than four independent experiments provided direct evidence for the adjuvant properties of Af extract in terms of priming a naïve, antigen-specific T cell response in vivo. By Day 10, the Af extract as well as LPS-induced CD4+ and CD8+ T cell expansion underwent considerable contraction. The numbers of OT-I and OT-II T cells detected in adjuvant-treated animals were comparable with the OVA alone groups in the LNs and in the spleen on Day 20 (Fig. 1A: Days 8–20).

To ascertain whether the adjuvant-induced T cell proliferation contributed to the observed clonal expansion, we labeled naïve OT-I and OT-II with CFSE prior to adoptive transfer and then monitored dilution of the CFSE dye in T cells harvested

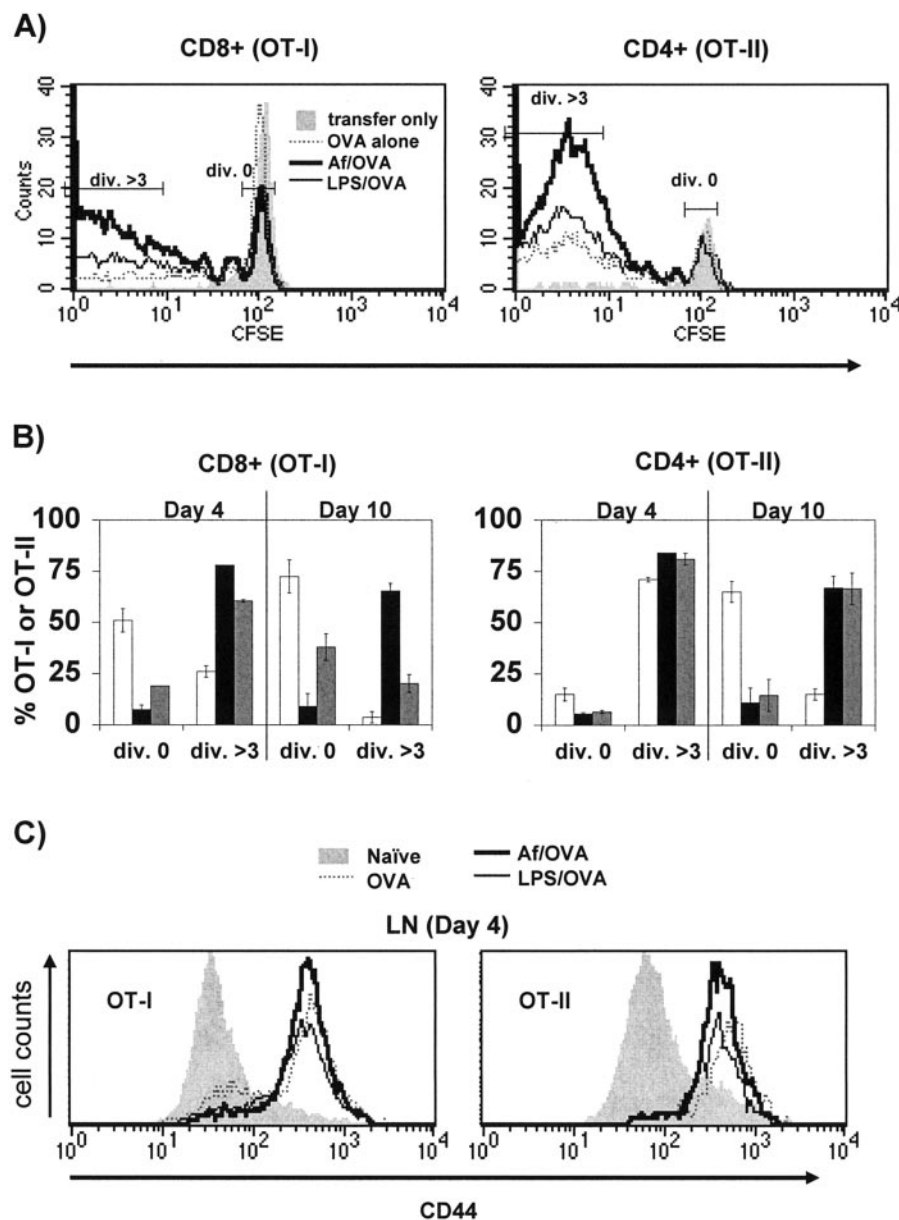
from differentially antigen-sensitized animals. At the peak of the OT-I and OT-II cell expansion in the LNs (Day 4), the Af extract plus OVA-treated animals had the largest percentage of OT-I cells undergoing at least one and more than three rounds of cell division (**Fig. 2A**). In contrast, the Af extract was unable to promote a greater percentage of the OT-II T cell to undergo cell division. Although a larger percentage of OT-II T cells underwent three or more rounds of cell division in mice sensitized with Af extract compared with LPS at Day 4 (**Fig. 2A**), the actual numbers of OT-II T cell detected with less than three rounds were relatively unchanged (**Fig. 2B**). Af extract and LPS activated OT-I and OT-II T cells equivalently, as determined by CD44 expression profiles on Day 4 (**Fig. 2C**). These results indicate that sensitization with the Af extract promotes clonal activation and proliferation of OT-I and OT-II T cells but unlike CD8+ T cells, the CD4+ T cell expansion was not robust. This may be a result of qualitative differences in priming required for CD8+ and CD4+ T cells, thereby

producing differential outcomes in terms of attrition and/or clonal exhaustion.

Af extract promotes CD8 T cell effector differentiation

To further characterize the differential adjuvant properties of Af extract on CD4+ and CD8+ T cell responses, we next studied the impact of Af extract on OVA-induced differentiation of OT-I and OT-II T cells into effector cells. A relatively high frequency of OT-I but not the OT-II T cells, obtained from LPS and Af extract-sensitized mice on Day 4, produced IFN- γ , as determined by intracytoplasmic staining and flow cytometry (**Fig. 3**). The percentage of IFN- γ -positive OT-I T cells detected in Af-sensitized animals persisted in the spleen (Day 20) but not in the LN (**Fig. 3A**). However, as a result of the decreases in the actual numbers of OT-I T cells (**Fig. 1**), the total numbers of IFN- γ -producing OT-I T cells were considerably lower on Day 20 compared with earlier time-points.

Fig. 2. Af extract enhances antigen-induced T cell proliferation but not their activation profile. The OT-I (CD8+ Thy 1.1+) and OT-II (CD4+ Thy 1.1) cells detected in the LNs on Day 4 of animals, sensitized with OVA alone, LPS/OVA, or Af/OVA, were gated and evaluated for CFSE dilution by flow cytometry. (A) An overlay histogram of the CFSE profile of OT-I (left) and OT-II (right) cells. The markers "div. 0" indicate the undivided population and "div. >3", the population that has undergone more than three cell divisions. A representative histogram is shown. (B) The bar graphs representing the mean percentage of OT-I (left) and OT-II (right) T cells detected, which had undergone div. 0 and div. >3 in animals sensitized with OVA/PBS (open bars), LPS/OVA (shaded bars), or Af/OVA (solid bars). The percentage of OT-I and OT-II cells obtained from four independent experiments with two animals at each time-point/experimental group is shown. Error bars show the standard deviation of the mean values. (C) The expression of CD44 on gated OT-I and OT-II T LN cells obtained from differentially sensitized animals on Day 4. A representative histogram from three independent experiments is shown.



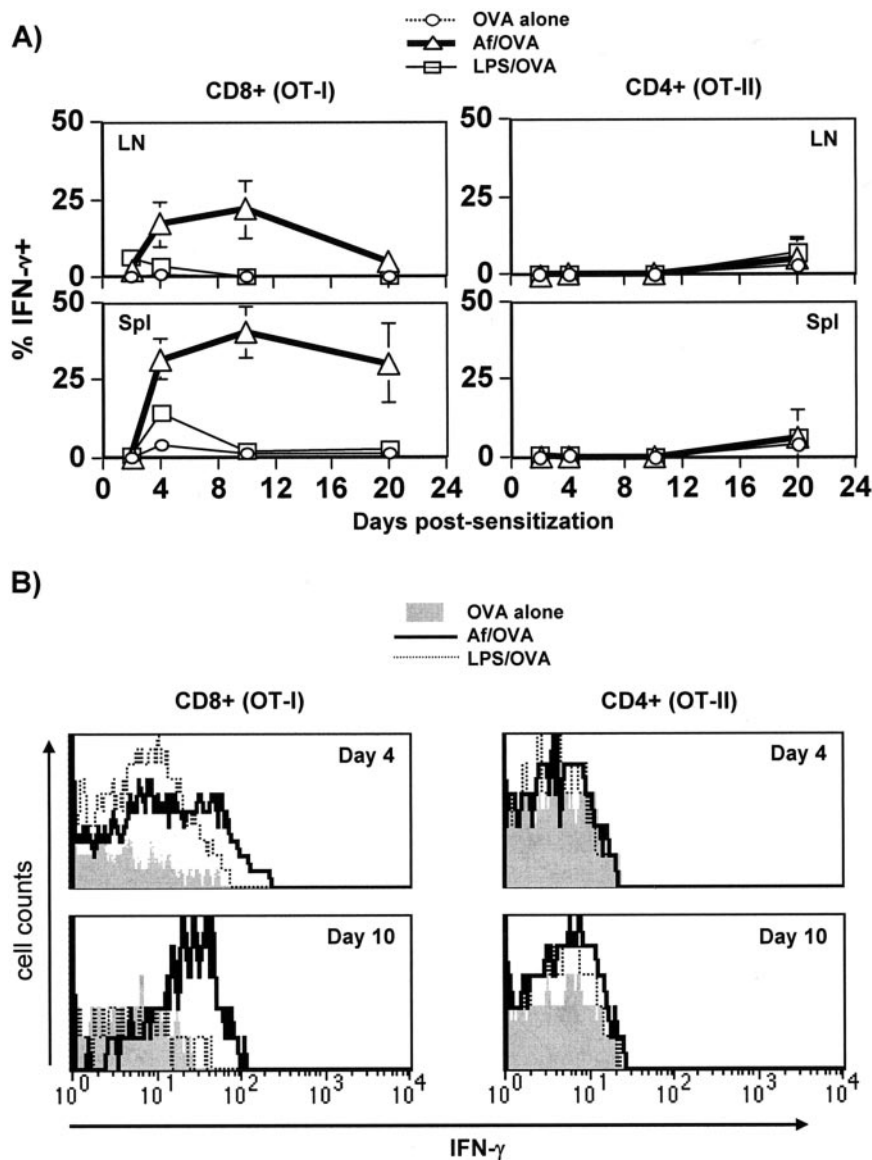


Fig. 3. Af extract induces OT-I but not OT-II T cell differentiation into IFN- γ + cells. The spleen and LN cells from mice sensitized obtained at different time-points were restimulated with OVA-derived peptides in the presence of Brefeldin A for 4 h and stained for CD8/Thy1.1 and then for IFN- γ by ICS. The OT-I and OT-II T cells were gated and analyzed by flow cytometer for IFN- γ production. (A) Kinetics of percent IFN- γ -producing OT-I (left) and OT-II (right) cells from LN (upper) and spleen (lower) after immunization. The average percentage of OT-I and OT-II positive for IFN- γ from four independent experiments with two animals at each time-point/experimental group is plotted, and error bars represent the standard deviation of the mean values. (B) A representative overlay histograms of IFN- γ detected by ICS in gated OT-I (left) and OT-II (right) T cells obtained from spleens on Day 4 (upper) and Day 10 (lower) after immunization.

Adjuvants such as LPS and Af extract could lead to deviation of OT-II cells into Type 2 effector cells. To test the notion that Af extract induces T cell effector deviation, we also determined the levels of IL-4 production in OT-I and OT-II T cells by intracytoplasmic staining, and no IL-4 was detected in either of the T cells (data not shown). To confirm lack of IL-4

production, Day 4 OT-I and OT-II T cells were activated *ex vivo* with their cognate antigen, and 24 h supernatants were harvested, analyzed by multiplex bead array (BD Biosciences). As shown in **Table 1**, OT-I T cells produced detectable amounts of IFN- γ tumor necrosis factor α (TNF- α) but not IL-4. Moreover, supernatants from OT-II-stimulated cultures

TABLE 1. Af Extract Enhances Effector Differentiation of CD8+ but not CD4+ T Cells^a

In vivo sensitization	IFN- γ (pg/ml)		IL-4 (pg/ml)		TNF- α (pg/ml)	
	CD4	CD8	CD4	CD8	CD4	CD8
PBS	85 (20)	90 (15)	92 (19)	101 (27)	95 (19)	97 (12)
OVA	91 (18)	108 (22)	114 (21)	113 (15)	85 (11)	118 (16)
OVA + LPS	115 (25)	450 (39)	126 (28)	146 (31)	124 (23)	390 (35)
OVA + Af extract	100 (14)	545 (45)	98 (12)	128 (24)	112 (16)	310 (20)

^a Recipients of naïve OT-I and OT-II T cells were sensitized with vehicle control (PBS), soluble OVA (10 μ g), OVA plus LPS (1 μ g), or OVA plus Af extract (14 μ g). The CD8+ T cells purified from the draining lymph node on Day 4 were stimulated with beads presenting SIINFEKL *in vitro* for 24 h; the supernatants harvested and analyzed for IFN- γ , IL-4, and TNF- α in a multiplexed bead array assay by flow cytometry. The mean values with SD (in parentheses) from three mice are representative of two independent experiments. The sensitivity range for detection of cytokines in one of the representative experiments: IFN- γ (11–23,000 pg/ml), IL-4 (10–11,000 pg/ml), and TNF- α (8–20,000 pg/ml).

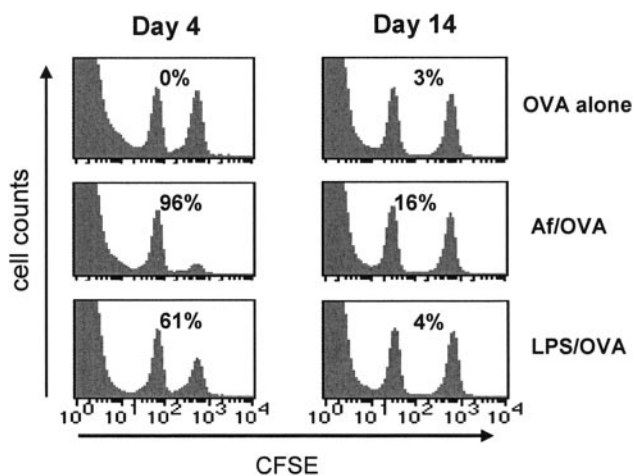


Fig. 4. Af extract generates antigen-specific in vivo CTLs. The recipients of OT-I and OT-II T cells were sensitized and at indicated days, postsensitization-injected with equal numbers of target (antigen-pulsed) and reference cells (pulsed with irrelevant peptide), which were labeled with high and low concentrations of CFSE, respectively. LN and spleen cells were harvested after 4 h and analyzed by flow cytometry for the relative loss of CFSE^{high} (target) versus CFSE^{low} (reference) cells. The percent lysis is indicated. A representative histogram from four independent experiments with similar outcomes is shown.

contained no increases in all cytokines tested. These observations confirm the notion that Af extract sensitizes CD4⁺ T cell activation and proliferation but does not promote effector differentiation into cytokine-producing cells.

To characterize the ability of Af extract to promote full effector differentiation of OT-I T cells, we conducted in vivo CTL assays. At indicated times after sensitization, the sensitized mice were transferred with equal numbers of splenocytes pulsed with OVA peptide (OVAp) SIINFELK (CFSE^{high}) or an irrelevant K^b-restricted peptide SIYREGL (CFSE^{low}). After 4 h, spleen cells harvested were analyzed by flow cytometry for the relative percentage of CFSE^{high} (antigen) versus CFSE^{low} (reference) cells. As shown in **Figure 4**, on Day 4, the relative percentage of CFSE^{high} (antigen-pulsed) target cells was reduced significantly in LPS and Af extract-treated animals. The cytotoxic capacity detected was in concert with the observed increases in IFN- γ production in the Af extract-sensitized animals. The CTL activity generated by LPS was lost on Day 14, although some residual CTL ability was still evident in Af extract-sensitized animals (Fig. 4). The observed in vivo CTL activity was a result of CD8⁺ T cells, as antibody-mediated depletion of CD8⁺ resulted in complete loss of target lysis, and CD8⁺ T cells purified from Af extract-sensitized mice showed lytic ability against OVAp-pulsed target cells directly ex vivo in a 4-h chromium release assay on Day 10 (data not shown). These results demonstrated the ability of Af extract to promote robust type I differentiation of CD8⁺ T cells. However, the number of differentiated CTLs underwent considerable attrition over time (Figs. 3 and 4 and Table 1). In contrast, CD4⁺ T cell differentiation was not observed, allowing us to conclude that Af extract was a T cell adjuvant, which selectively induced CD8⁺ but not CD4⁺ T cell differentiation.

Sensitization with Af extract and antigen protects against tumor challenge

To evaluate whether the transient but effective CD8⁺ T cell response generated with the Af extract could empower host immunity, we challenged Af extract-sensitized mice with 5×10^6 syngeneic thymoma cells (E.G7), which express the OVA protein, and then monitored the percentage of tumor-bearing animals that survived the tumor challenge. Decisions about euthanasia were made blinded to the sensitization regimen based on the prespecified requirements of our approved animal use protocols. A single sensitization with Af extract plus OVA was more effective in promoting a modest but statistically significant survival benefit in tumor-challenged mice than sensitization with OVA alone (**Fig. 5**; log-rank analysis comparing Af extract plus OVA vs. OVA alone, P value=0.003). In contrast to the observed effectiveness of Af extract over LPS sensitization in terms of OT-I and OT-II responses, the survival after tumor challenge with either adjuvant was similar. As sensitization with Af extract plus OVA produced only a transient T cell response, we predicted that sensitization twice with Af extract would augment tumor immunity. In agreement with this prediction, our results demonstrate that a modest but significant extension of survival was achieved twice in Af-sensitized animals compared with a single sensitization (Fig. 5; log-rank analysis, P value=0.04). In fact, three out of 15 animals sensitized with Af extract had morbidity-free survival for the entire experiment (160 days); in contrast, all animals receiving a single sensitization with Af extract and OVA showed 100% tumor-related fatality. These results indicate that the transient clonal expansion and associated CD8⁺ T cell effector differentiation produced by Af extract sensitization can

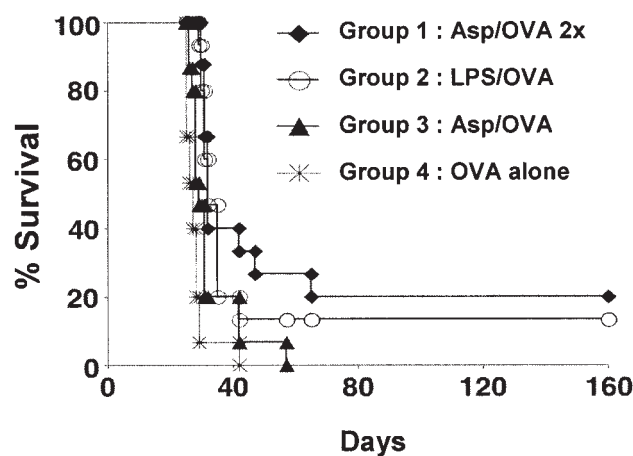


Fig. 5. Immunization with Af extract promotes survival of syngeneic tumor-challenged hosts. Mice ($n=15$ per group) were adoptively transferred with OT-I and OT-II T cells (2×10^6 per mouse) on Day -1 and sensitized with one of the following: OVA alone (Day 0), LPS and OVA (Day 0), Af extract (Asp) and OVA (Day 0), or Af extract and OVA 2 $\times$ (Days 0 and 7). Mice ($n=15$ per group) were challenged by i.p. injection of E.G7 thymoma (5×10^6 /mouse) on Day 4. The result shown is representative of two independent experiments. The Kaplan-Meier survival curves were generated, and all pair-wise comparisons were made by the log-rank method without adjustments for multiple comparisons. The following comparisons produced statistical significance: Group 1 versus 4 ($P=0.00008$); Group 2 versus 4 ($P=0.0006$); Group 3 versus 4 ($P=0.003$); and Group 1 versus 3 ($P=0.04$).

confer protection against tumor challenge, and strategies for its optimal use are warranted.

DISCUSSION

By monitoring the response of adoptively transferred TCR transgenic, naïve, antigen-specific T cells to Af extract and nominal antigen OVAp, our results indicate that Af extract sensitization promotes transient, OVA-specific CD4⁺(OT-II) and CD8⁺ (OT-I) T cell expansion in the LNs and spleen as a result of proliferation. The increased proliferation of OT-I cells was accompanied by differentiation into IFN- γ -producing CTLs, but OT-II T cell activation and expansion were devoid of detectable effector differentiation. IFN- γ is known to enhance CTL activity against tumor cells, at least in part, as a result of increased surface expression of MHC-I and display of target antigens [26]. Moreover, the repeated sensitization with Af extract and OVA was more effective than a single sensitization in conferring protection against a syngeneic, OVA-expressing thymoma challenge, confirming the transient induction of CTL activity by the Af extract.

The consistent observation that sensitization with Af extract when administered s.c. produced substantially better antigen-specific T cell activation than LPS, an established T cell adjuvant in our studies, is not entirely unexpected, as previous studies have demonstrated the ability of *Aspergillus* extract to confer protection against experimental aspergillosis. In fact, Ito and Lyons [10] have shown that immunization with *Aspergillus* extract conferred protection against lethal pulmonary *Aspergillus* challenge in corticosteroid-treated mice, whereas the results generated by Cenci et al. [9] demonstrated that immunization of immunocompetent mice with an *Aspergillus* crude filtrate resulted in memory Th1 CD4⁺ T cell responses, which could confer protection against Af upon adoptive transfer into neutropenic mice. Ramadan et al. [27] demonstrated that dendritic cells pulsed with a pentadecapeptide pool from the *Aspergillus* antigen, Asp f16, were capable of inducing polyclonal human leukocyte antigen-Class I-restricted, *Aspergillus*-specific CTLs, which were cidal to *Aspergillus* in vitro. This study raises the potential of augmentation of CTL responses in vivo as a strategy for conferring protection against invasive aspergillosis.

The host immune responses to Af extract are likely to be influenced by several variables including extract composition, presence or absence of gliotoxin, and the route of sensitization. Systemic priming followed by intranasal sensitization with Af extract induces asthma in mice, which is characterized by increased airway hyper-responsiveness, eosinophilic infiltration of the lung, and Type 2 cytokine responses [6]. The carbohydrates, glucan, chitin, and galactomannan, are the principal components of the cell wall of Af, which are thought to modulate T cell-mediated, allergic responses [28]. The Af extract used in our studies was obtained from a commercial source, and preliminary characterization shows no detectable gliotoxin (data not shown). Moreover, we sensitized the animals to the Af extract by s.c. injection; it can be argued that sensitization by other routes (e.g., intranasal or intravenous) may differentially influence the adaptive response observed.

Nevertheless, our results clearly indicate the ability of Af constituents to alert the host adaptive response and provide a strong rationale for understanding the role of specific constituents of the Af extract and the route of delivery in modulating immunologic responses for effectively using Af extract in immunotherapy strategies.

The ability of Af extract sensitization to induce OVA antigen-specific immunity observed in our studies may testify to the ability of Af extract to induce activation of innate immune cells by ligating TLR-dependent pathways, and TLR-dependent antifungal pathways are highly conserved in nature, as demonstrated by their presence in *Drosophila* [29]. The TLRs recognize motifs on *Candida* species [30], *Cryptococcus neoformans* [31], and various *Aspergillus* species [32] to regulate innate immune cell-mediated inflammation. Recognition of *Aspergillus* motifs is coordinated by TLR2 and TLR4, and recent evidence suggests that each receptor is likely to activate specialized antifungal effector functions [13]. Netea et al. [14] reported that Af evades immune recognition during germination through loss of the ligand for TLR4, thus, attenuation of proinflammatory cytokine responses in macrophages. In addition, the *Aspergillus*-induced cytokine profiles and asthma were altered considerably in mice deficient for the mannose-binding lectin-deficient (MBL^{-/-}) [11]. The use of specific TLR (TLR2 and -4), myeloid differentiation primary-response protein 88, and TNF receptor-associated factor knockout mice can further delineate the role of key host defense pathways that respond to *Aspergillus* products and will identify potential targets for the generation of adaptive host immunity.

Sensitization with Af extract induced OT-I and OT-II proliferation, but the effector maturation reflected by cytokine (IFN- γ , TNF- α) production and CTL activity occurred only in OT-I cells. Although the IFN- γ production is typically correlated with CTL activity against tumor cells, in part as a result of increased surface expression of MHC class I proteins and display of target antigens, the precise role for IFN- γ versus CTL activity in the observed tumor immunity remains to be delineated clearly. It is possible that the lack of effector maturation observed in OT-II T cells is a result of the regulatory effects of the Af extract on the antigen-presenting cells, such as dendritic cells, leading to their inability to promote CD4⁺ T cell differentiation. This observation is not entirely unexpected, as distinct requirements for naïve CD4⁺ and CD8⁺ T cell activation and differentiation have been noted previously [33]. It is important that our observations indicate that the CD8⁺ T cell differentiation occurs independent of CD4⁺ T cell maturation. In addition to the selective CD8⁺ T cell differentiation, Af extract-induced CD4⁺ and CD8⁺ T cell responses were transient, as by Day 10, the OT-I and OT-II T cell numbers underwent considerable attrition, and the detectable CTL activity in vivo had waned. The role for defective CD4⁺ T cell effector maturation in the unsustainable, induced CD8⁺ T cell response is suspected, as CD4⁺ help is required to achieve a durable CD8⁺ T cell response in several infectious diseases [34–36]. However, as CD4⁺ T cells were not required for vaccine-induced resistance against experimental fungal pulmonary infections with two pathogens—*Blastomyces dermatitidis*, an extracellular, and *Histoplasma capsulatum*, a facultative, intracellular pathogen [37]—it can be envisioned

that in the absence of Th cells, cytokines produced by CD8+ T cells in response to recognition of fungal antigens and adjuvant factors could achieve immunity upon vaccination.

Although the adoptive transfer of OT-I and OT-II cells enables a detailed visualization of immune responses in vivo using a nominal OVA antigen, a number of limitations to our experimental design exist. Sensitization with Af extract may affect the homing and survival of transferred cells, directly or indirectly, by modulating the response of recipient cells. For example, the lack of sustained expansion of transferred OT-I and OT-II cells may be a result of homeostatic mechanisms occurring at the encounter with recipient cells, which is arguably unexpected to play a major role, as the OT-I and OT-II T cells are transferred into intact recipients that do not promote homeostatic expansion. The Af extract used in our studies contains a broad spectrum of antigens and has been used to induce allergic responses in mice [11]. However, the extract obviously cannot model the full repertoire of immunologic responses induced by a live organism. Another limitation relates to contamination with LPS. The concentration of LPS was 0.05 ng/ml, as measured by the Limulus amebocyte lysate (LAL) assay, a standard method for LPS detection. However, the LAL assay is problematic, as it cross-reacts with β -glucan, a fungal cell-wall constituent, and may therefore overestimate the level of LPS contamination. Thus, although the *Aspergillus* extract was more effective in priming CD8 T cell responses in vivo compared with LPS (1.0 ug/mouse), we cannot rule out a combined effect of *Aspergillus* constituents and low levels of LPS contamination in priming T cell responses.

Our results clearly demonstrate the ability of Af extract to promote CD8+ T cell activation, proliferation, and differentiation, which afford limited protection against tumor challenge in the absence of CD4+ T cell effector maturation. It appears that the inability of Af extract to induce CD4+ T cell differentiation may contribute to the transient CD8+ T cell expansion, thereby leading to modest efficacy against tumor. Nevertheless, by applying the adoptive transfer approach, we have developed a unique model system, which is amenable to detailed study of the host/fungal pathogen interplay to further dissect the mechanisms regulating disease outcomes. It is surprising that our findings have identified the potential for exploiting Af extract as a CD8+ T cell adjuvant in other disease settings such as cancer.

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