Activin-A induces regulatory T cells that suppress T helper cell immune responses and protect from allergic airway disease

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Activin-A is a pleiotropic cytokine that participates in developmental, inflammatory, and tissue repair processes. Still, its effects on T helper (Th) cell–mediated immunity, critical for allergic and autoimmune diseases, are elusive. We provide evidence that endogenously produced activin-A suppresses antigen-specific Th2 responses and protects against airway hyperresponsiveness and allergic airway disease in mice. Importantly, we reveal that activin-A exerts suppressive function through induction of antigen-specific regulatory T cells that suppress Th2 responses in vitro and upon transfer in vivo. In fact, activin-A also suppresses Th1-driven responses, pointing to a broader immunoregulatory function. Blockade of interleukin 10 and transforming growth factor β1 reverses activin-A–induced suppression. Remarkably, transfer of activin-A–induced antigen-specific regulatory T cells confers protection against allergic airway disease. This beneficial effect is associated with dramatically decreased maturation of draining lymph node dendritic cells. Therapeutic administration of recombinant activin-A during pulmonary allergen challenge suppresses Th2 responses and protects from allergic disease. Finally, we demonstrate that immune cells infiltrating the lungs from individuals with active allergic asthma, and thus nonregulated inflammatory response, exhibit significantly decreased expression of activin-A's responsive elements. Our results uncover activin-A as a novel suppressive factor for Th immunity and a critical controller of allergic airway disease.

The Journal of Experimental Medicine

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Abbreviations used: Act-RIIA, activin receptor type IIA; AHR, airway hyperresponsiveness; ALK, activin-like kinase; alum, aluminum hydroxide; BAL, bronchoalveolar lavage; DLN, draining LN; EAE, experimental autoimmune encephalomyelitis; FEV_1, forced expiratory volume in 1 s; Foxp3, forkhead box p3; H&E, hematoxylin and eosin; PAS, periodic acid–Schiff; PenH, enhanced pause; r-activin-A, recombinant activin-A.

Immune responses by differentiated effector Th1, Th2, and Th17 cell subsets provide protection against pathogens but can also lead to chronic inflammation, autoimmunity, or allergy if not tightly controlled (Reiner, 2007; Steinman, 2007). Critical controllers of these responses are immunosuppressive cytokines, such as IL-10 and TGF-β1, and regulatory T lymphocytes. Subsets of regulatory T cells suppress responses by other effector Th cells mainly via cell-to-cell interactions (Nakamura et al., 2001) or the release of immunosuppressive cytokines (Asseman et al., 1999; Chen et al., 2003; Hawrylowicz and O'Garra, 2005; Ostroukhova et al., 2006; Li et al., 2007). However, blockade of these cytokines does not completely inhibit immune regulation (von Boehmer, 2005; Tang and Bluestone, 2008; Vignali et al., 2008), suggesting that other, as yet unidentified, cytokines are also involved.

The cytokine activin-A, a member of the TGF-β superfamily, participates in essential biological processes, such as development, hematopoiesis, wound repair, and fibrosis (Vale et al.,

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The Rockefeller University Press $30.00
J. Exp. Med. Vol. 206 No. 8 1769-1785
www.jem.org/cgi/doi/10.1084/jem.20082603

1988; Werner and Alzheimer, 2006). *Activin-A*$^{-/-}$ mice are embryonic lethal (Matzuk et al., 1995b), whereas *activin receptor type IIA* (*act-RIIA*)$^{-/-}$ mice reach adulthood but have major deficiencies in their reproductive systems (Matzuk et al., 1995a). Although most studies have focused on the role of activin-A in developmental and fibrotic processes, certain reports demonstrate elevated levels of this cytokine in immune-mediated diseases such as rheumatoid arthritis (Ota et al., 2003) and inflammatory bowel disease (Hubner et al., 1997; Dohi et al., 2005). Activin-A is also increased in the sera of individuals with allergic asthma (Karagiannidis et al., 2006), and in the lung and bronchoalveolar lavage (BAL) of mice during acute (Rosendahl et al., 2001; Hardy et al., 2006) and chronic allergic airway inflammation and remodeling (Le et al., 2007). In addition, activin-A is induced in human (Karagiannidis et al., 2006) and mouse effector Th2 lymphocytes (Ogawa et al., 2006), which are key players in allergic responses. However, whether activin-A has enhancing or suppressive actions during Th immune responses and subsequent disease remains unclear.

Certain studies indicate that recombinant activin-A (r-activin-A) reduces in vitro nonspecific proliferation of human Th (Karagiannidis et al., 2006) and mouse B cells (Yu et al., 1998; Werner and Alzheimer, 2006), and inhibits certain functions of human natural killer cells (Robson et al., 2009). In other reports, r-activin-A attenuates in vitro endotoxin-induced maturation and phagocytosis of mouse macrophages (Wang et al., 2008; Zhou et al., 2009) and CD40 ligand–dependent cytokine and chemokine release by human monocytes and DCs (Robson et al., 2008). Nevertheless, a few reports have suggested that activin-A has proinflammatory effects, i.e., during in vivo endotoxin administration and allergen challenge in mice (Hardy et al., 2006; Jones et al., 2007). Collectively, these studies indicate a dual nature of this cytokine, a characteristic feature of certain immune mediators (Veldhoen et al., 2006; Zenewicz et al., 2008). Of note, our previous studies revealed a dual role for another cytokine, osteopontin, in allergic airway inflammation (Xanthou et al., 2007).

In the present study, we have investigated the in vivo role of activin-A in Th-mediated immune responses and, more specifically, during Th2-associated allergic airway inflammation. We demonstrate that antibody-mediated depletion of activin-A during pulmonary allergen challenge resulted in significant exacerbation of Th2-mediated allergic airway disease, indicating that endogenous activin-A is suppressive. In fact, our findings reveal that activin-A induces the generation of antigen-specific regulatory T cells that suppress both primary and effector Th responses in vitro and upon adoptive transfer in vivo. Functional in vitro analysis shows that activin-A–mediated suppressive effects on Th responses are dependent on both IL-10 and TGF-β1. Importantly, activin-A–induced antigen-specific regulatory T cells transfer protection against allergic airway disease correlated with decreased DC maturation. Collectively, our data reveal that activin-A can suppress Th responses and represents a critical therapeutic target for allergic asthma.

RESULTS

Depletion of activin-A during allergen challenge in the airways enhances Th2 allergic responses and exacerbates asthmatic disease

We initially investigated the in vivo role of activin-A in Th-mediated responses and, more specifically, in Th2-associated allergic airway inflammation, as activin-A is expressed during Th2-driven allergic responses (Rosendahl et al., 2001; Hardy et al., 2006; Karagiannidis et al., 2006). For this, we administered a neutralizing antibody to activin-A (or Ig control) right before pulmonary allergen (OVA) challenge of OVA/aluminum hydroxide (alum)–sensitized mice (experimental protocol described in Fig. 1 A).

Depletion of activin-A during pulmonary allergen challenge resulted in significant exacerbation of allergic airway disease. This was shown by significantly increased total numbers of BAL-infiltrating cells and eosinophils, a hallmark of allergic airway disease (Humbles et al., 2004; Lee et al., 2004), as compared with Ig treatment (Fig. 1 B). More importantly, airway hyperresponsiveness (AHR) to increasing doses of inhaled methacholine, a clinical measurement of the asthmatic phenotype, was also significantly worsened in mice treated with anti–activin-A (Fig. 1 C). PBS/alum-sensitized and OVA-challenged mice (alum controls) exhibited lower AHR responses in comparison to the other groups (Fig. 1 C). Moreover, pulmonary inflammation and mucus secretion were significantly increased in mice treated with anti–activin-A (Fig. 1 D). The increase in eosinophilic infiltration, observed upon activin-A depletion at challenge, was accompanied by significantly increased expression of the eosinophil-specific chemokine CCL11 in lung homogenates (Fig. 1 G).

We also examined the effects of in vivo neutralization of activin-A on OVA-specific Th2-mediated effector responses by measuring Th cell proliferation and cytokine release in the supernatants of draining LN (DLN) cell cultures stimulated ex vivo with OVA. There was significantly increased OVA-specific Th cell proliferation and significantly increased production of the Th2 cytokines IL-4, IL-13, and IL-10 in stimulation cultures of DLN cells from mice treated with anti–activin-A, as compared with Ig controls (Fig. 1 E). IFN-γ levels in culture supernatants were also significantly increased, possibly resulting from the overall enhanced inflammation (Fig. 1 E). In support, we observed significantly increased lung and BAL levels of IL-4, IL-13, and IL-10 concomitant with decreased production of IL-12 and IL-17, cytokines considered protective when overexpressed in the lungs of allergic mice (Walter et al., 2001; Schnyder-Candrian et al., 2006) upon activin-A depletion in vivo (not depicted). OVA-specific IgE and IgG1 (Th2 isotypes) serum concentrations were significantly increased (Fig. 1 F), whereas OVA-specific IgG2a (Th1 isotype) levels were decreased (Fig. 1 F).

No differences were observed in the recruitment of effector T1/ST2$^+$ Th2 cells among CD3$^+$CD4$^+$ Th cells in the DLNs and lungs between anti–activin-A– and Ig-treated

mice (unpublished data). In support, levels of the Th2 cell–recruiting chemokines CCL17 and CCL22 were not significantly altered between the two groups (Fig. 1 G). This suggested that the overall increase in Th2-mediated allergic responses upon in vivo activin-A depletion was not caused by increased Th2 effector cell trafficking but rather by an enhanced effector function of Th2 cells. To address this, we examined the effects of DLN cells obtained from anti–activin-A–treated mice (or Ig controls) on responses of co-cultured OVA-specific T cell receptor transgenic Th cells

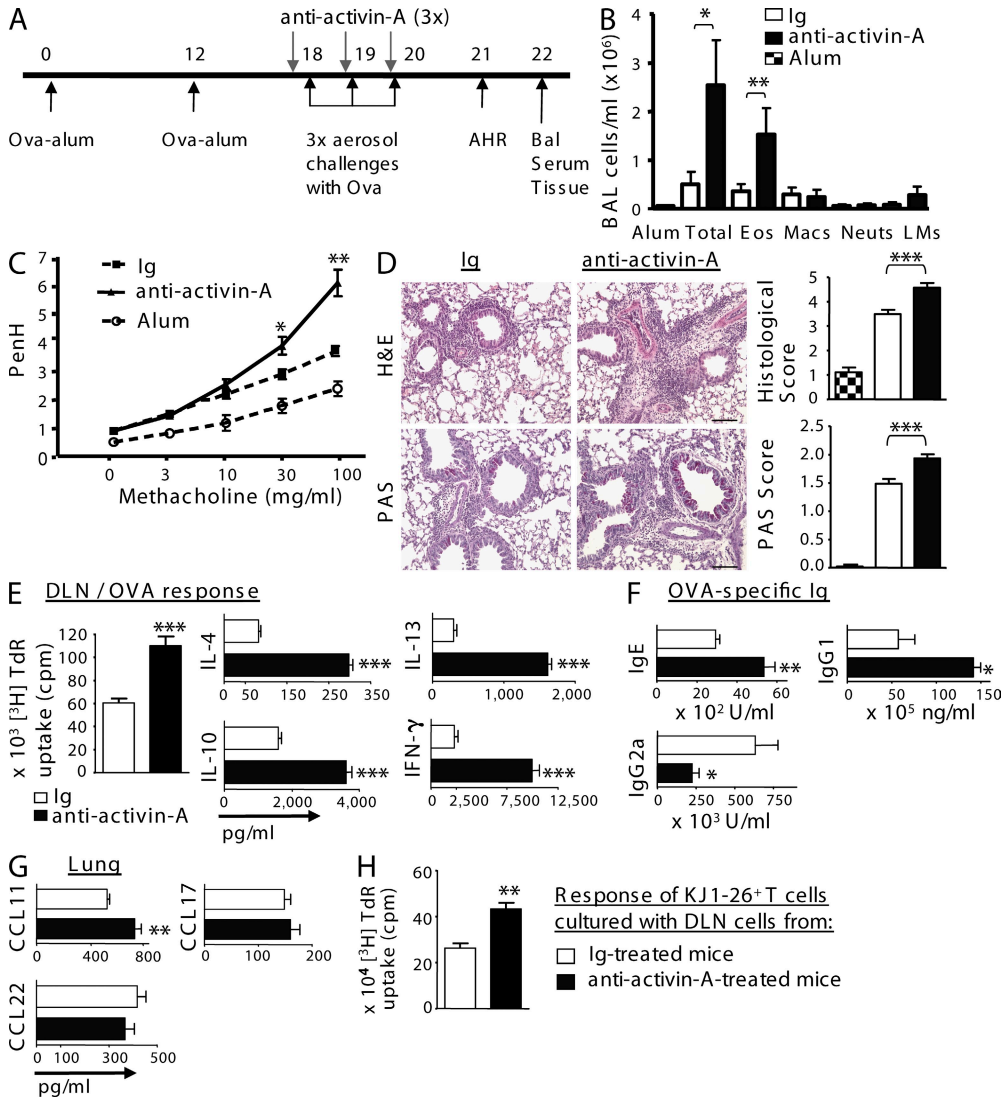

Figure 1. In vivo depletion of activin-A during pulmonary allergen challenge exacerbates allergic airway disease and Th2 responses. (A) Experimental protocol used to block endogenous activin-A during pulmonary challenge. (B) BAL differentials from mice treated with anti–activin-A or Ig control, or from the alum controls are expressed as means ± SEM (n = 5–8 mice per group in four separate experiments). Statistical significance was obtained by an unpaired Student's t test (*, P = 0.0345; **, P = 0.0476). (C) AHR is depicted. Results shown for PenH are expressed as means ± SEM (n = 5–8 mice per group in four separate experiments). Data were analyzed by two-way analysis of variance (ANOVA) for repeated measures, followed by an unpaired Student's t test (*, P = 0.0164; **, P = 0.00047). (D) Representative photomicrographs demonstrating lung inflammation (H&E-stained sections) and mucus secretion (PAS-stained sections). Histological scores of H&E-stained (***, P = 0.0009) and PAS-stained (***, P < 0.0001) sections. Error bars depict means of groups (n = 5–8 mice per group in four independent experiments). Bars, 100 µm. (E) DLN cells were restimulated ex vivo with OVA. Proliferation was measured by [^{3}H]thymidine incorporation. Results are shown as means ± SEM of triplicate wells (n = 5–8 mice per group in four independent experiments; ***, P < 0.0001). IL-4 (***, P < 0.0001), IL-13 (***, P < 0.0001), IL-10 (***, P < 0.0001), and IFN-γ (***, P < 0.0001) in supernatants are shown. Results are means ± SEM of duplicate wells (n = 5–8 mice per group in four independent experiments). (F) OVA-specific IgE (**, P = 0.0060), IgG1 (*, P = 0.0165), and IgG2a (*, P = 0.0218) in the sera of mice. Results are means ± SEM (n = 5–8 mice per group in four independent experiments). (G) CCL11 (**, P = 0.0054), CCL17 (P = 0.9399), and CCL22 (P = 0.5000) in lung homogenates are shown. Results are means ± SEM (n = 5–8 mice per group in four separate experiments). (H) DLN cells from anti–activin-A– or Ig–treated mice were co-cultured with LN KJ1-26$^+$CD4$^+$ T cells (2:1) in the presence of $OVA_{323-339}$ peptide, and proliferation was measured (**, P = 0.001). The results are means ± SEM (n = 5–8 mice per group in two independent experiments). Eos, eosinophils; LMs, lymphomononuclears; Macs, macrophages; Neuts, neutrophils.

from DO11.10 mice (recognized by expression of the clonotypic receptor by the antibody KJ1-26 and hereafter referred to as KJ1-26$^+$) to the $OVA_{323-339}$ peptide. Indeed, DLN cells from anti–activin-A–treated mice lead to significantly increased proliferation of KJ1-26$^+$CD4$^+$ T responders to the $OVA_{323-339}$ peptide (Fig. 1 H). These data further support decreased immune regulation upon blockade of endogenously produced activin-A in vivo. Overall, antibody-mediated depletion of activin-A during pulmonary allergen challenge enhanced allergen-specific Th2 responses and exacerbated allergic airway disease.

Activin-A suppresses antigen-specific primary and effector Th2 responses

Effector Th2 cell–mediated responses are essential for the development of allergic airway inflammation and asthmatic disease. Thus, we hypothesized that activin-A protects against allergic airway inflammation through suppression of antigen-specific Th2 effector responses. To examine this, we isolated OVA-primed Th2 effector cells from DLNs of allergic mice (sensitized with OVA/alum and challenged with OVA) and restimulated them ex vivo with OVA in the presence of r-activin-A or PBS (control). Indeed, r-activin-A treatment suppressed responses of OVA-primed Th2 effector cells, as shown by significantly decreased OVA-specific proliferation (Fig. 2 A) and significantly decreased concentrations of supernatant Th2 cytokines (Fig. 2 A). Similar to what was observed for IL-4 and IL-13, r-activin-A significantly decreased IL-10 production because, in this setting of Th2 effector responses, IL-10 operates mainly as a Th2 cytokine. In support, using the reverse approach, we demonstrated that blockade of either endogenously expressed activin-A or its critical signaling receptor, activin-like kinase (ALK) 4, resulted in significantly increased levels of IL-4, IL-13, and IL-10, and enhanced Th2 cell proliferation, as compared with Ig treatment (not depicted).

We subsequently investigated whether r-activin-A treatment of Th2 effector cells would also render them suppressed during antigenic stimulation in vivo. To address this, r-activin-A– or PBS-treated Th2 effector cells were adoptively transferred into BALB/c-*Rag1*$^{-/-}$ recipient mice, which were later immunized with OVA in alum (experimental protocol described in Fig. 2 B). Adoptively transferred r-activin-A–treated Th2 effector cells retained a suppressed phenotype after in vivo antigenic stimulation, as shown by significantly decreased antigen-specific proliferation (Fig. 2 B).

Subsequently, we investigated activin-A's effects on primary antigen-driven Th responses. We isolated LN KJ1-26$^+$CD4$^+$ T cells and stimulated them in vitro with antigen in the presence of r-activin-A. Treatment with r-activin-A resulted in significantly decreased OVA-induced proliferation of KJ1-26$^+$CD4$^+$ T cells (Fig. 2 C). In addition, IL-4 levels were decreased during r-activin-A treatment as compared with control (Fig. 2 C). The suppressive effect of r-activin-A was accompanied by significantly increased production of IL-10 (Fig. 2 C). Similarly, r-activin-A also significantly suppressed the robust proliferative responses of KJ1-26$^+$CD4$^+$ T cells to the $OVA_{323-339}$ peptide and induced increased production of IL-10 (not depicted). We and other investigators have found that activin-A is produced during primary Th responses (50–500 pg/ml in Th stimulation cultures; Ogawa et al., 2006). As anticipated, addition of a neutralizing antibody to block endogenously produced activin-A resulted in significantly increased proliferation of KJ1-26$^+$CD4$^+$ T cells during OVA-driven stimulation, concomitant with decreased IL-10 levels, as compared with Ig treatment (a two- and fourfold increase, respectively). Similar results were obtained when ALK4 was blocked (a two- and threefold increase, respectively), indicating that the suppressive effects of activin-A were mainly mediated by ALK4. Hence, activin-A suppresses both primary and effector antigen-specific Th2 responses in vitro and renders Th2 effector cells hyporesponsive to immunization in vivo.

Activin-A induces the generation of regulatory CD4$^+$ T cells that are suppressive in vitro and in vivo

Subsequently, we investigated whether activin-A–mediated Th suppression is associated with induction of T cells with regulatory/suppressive function. To address this, we performed an in vitro suppression assay wherein we co-cultured activin-A–treated CD4$^+$ T cells with untreated T cells (KJ1-26$^+$CD4$^+$ T cells) and examined the responses of the latter cells to the $OVA_{323-339}$ peptide. r-activin-A–treated OVA-primed Th2 cells significantly suppressed the proliferation of KJ1-26$^+$CD4$^+$ T cells to the $OVA_{323-339}$ peptide (Fig. 3 A). KJ1-26$^+$CD4$^+$ T cells cultured alone exhibited much higher, compared with the other groups, antigen-specific proliferative responses ($\sim$120,000 cpm). Moreover, r-activin-A–treated T cells significantly inhibited IL-4 and IL-13 release from responder KJ1-26$^+$CD4$^+$ T cells (Fig. 3 B). Treatment with TGF-β1, a well-known immunosuppressive cytokine (Chen et. al., 2003; Li et. al., 2007), also conferred inhibitory effects on OVA-primed Th2 cells. This was shown by significantly decreased antigen-specific proliferation and cytokine release from responder KJ1-26$^+$CD4$^+$ T cells after co-culture with TGF-β1–treated cells (Fig. 3, A and B). Overall, r-activin-A–treated antigen (OVA)-specific Th2 cells had essential features of regulatory T cells; i.e., they were suppressed during antigenic stimulation and suppressive toward antigen-driven responses of other Th cells in vitro.

Next, we examined whether r-activin-A–treated CD4$^+$ T cells were also suppressive toward responses by other Th cells in vivo. For this, we performed an in vivo suppression assay wherein we adoptively cotransferred r-activin-A (or PBS)–treated OVA-primed CD4$^+$ T cells along with untreated KJ1-26$^+$CD4$^+$ T cells into recipient BALB/c-*Rag1*$^{-/-}$ mice and examined antigen-specific responses of KJ1-26$^+$CD4$^+$ T cells after immunization with OVA in alum (experimental protocol described in Fig. 3 C). Flow cytometry revealed that r-activin-A– but not PBS-treated OVA-primed CD4$^+$ T cells decreased OVA-specific expansion of KJ1-26$^+$CD4$^+$ T cells in DLNs of recipient mice (Fig. 3 D). More importantly, r-activin-A–treated CD4$^+$ T cells suppressed the ex vivo proliferation of DLN KJ1-26$^+$CD4$^+$ T cells to the

$OVA_{323-339}$ peptide, as shown by significantly decreased proliferation of DLN cells from mice that received r-activin-A–treated $CD4^+$ T cells as compared with DLN cells from recipients of PBS-treated controls (Fig. 3 E). TGF-β1–treated $CD4^+$ T cells also inhibited OVA-specific expansion and proliferation of $KJ1\text{-}26^+CD4^+$ T cells in DLNs of recipient mice (Fig. 3, D and E). We also observed significantly decreased IL-4 and IL-13 levels in the supernatants of $OVA_{323-339}$–stimulated responder $KJ1\text{-}26^+CD4^+$ T cells in DLNs obtained from mice that received either r-activin-A– or rTGF-β1–treated $CD4^+$ T cells (Fig. 3 F). It was not surprising that IL-10 remained unaltered, as this cytokine also functions as a Th2 cytokine in this setting. Hence, using both in vitro and in vivo suppression assays, we demonstrate that activin-A induces the generation of antigen-specific regulatory T cells that are suppressive toward responses of Th2 effector cells in vitro and upon adoptive transfer in vivo.

Activin-A–induced suppression of Th responses is mediated by both IL-10 and TGF-β1

Our studies so far have shown that activin-A suppresses antigen-driven Th responses. We next investigated whether this cytokine can also affect anti-CD3–driven T cell activation. Purified $CD4^+$ T cells were stimulated with anti-CD3 in the presence of r-activin-A or PBS. Similar to our observations for Th2 effector responses, r-activin-A treatment also suppressed anti-CD3–driven Th stimulation, as shown by significantly reduced proliferation (Fig. 4 A) and IL-2 release (Fig. 4 A). Interestingly, activin-A–mediated suppression was accompanied by increased production of IL-10, a cytokine considered immunoregulatory in the context of, except for Th2, T cell responses (Fig. 4 A; Hawrylowicz and O'Garra, 2005). The immunosuppressive effects of activin-A were not caused by toxicity, as flow cytometry studies showed no increases in annexin V^+ cells (not depicted). In fact, addition of rIL-2 reversed r-activin-A–mediated suppression of Th cell proliferation (Fig. 4 B). This also indicated that activin-A renders Th cells anergic. Notably, r-activin-A treatment also suppressed antigen (OVA)-specific in vitro proliferation of Th1 effector cells (obtained from DLNs of CFA/OVA-immunized mice; Fig. 4 C). Collectively, our results reveal that activin-A not only suppresses Th2 cell–associated responses but also inhibits Th1 cell activation.

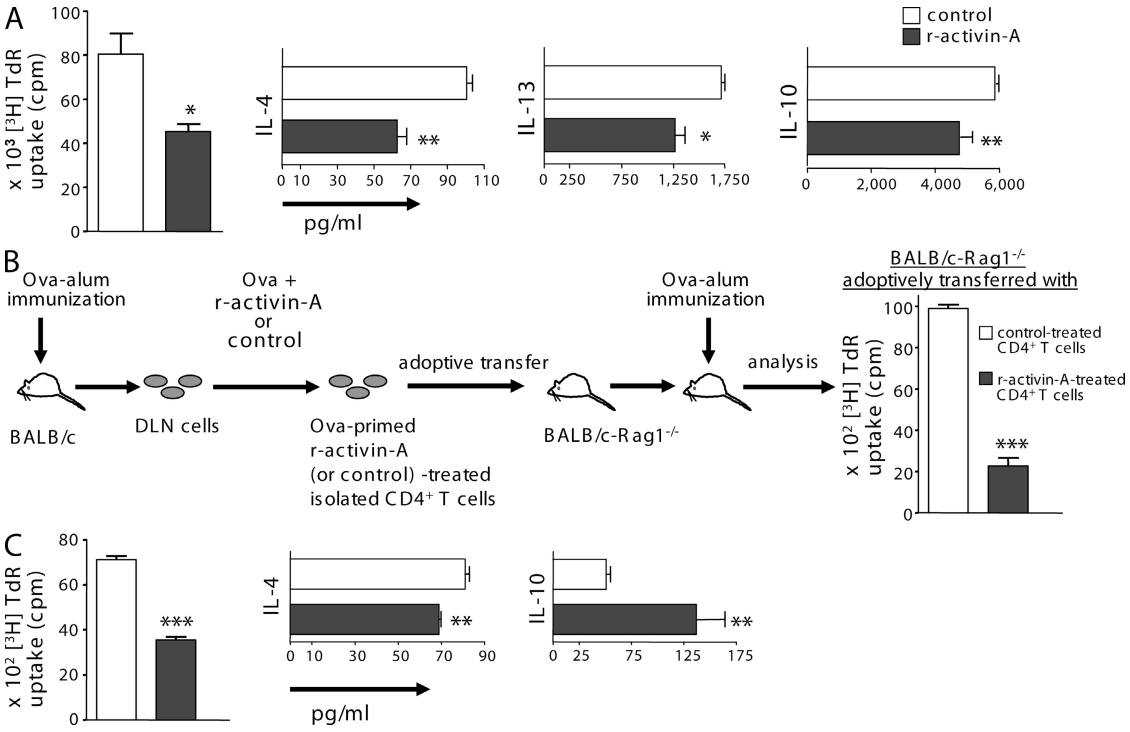

Figure 2. Activin-A suppresses antigen-specific primary and effector Th2 responses. (A) DLN cells were harvested from OVA/alum-immunized and OVA-challenged BALB/c mice, and proliferation was measured after ex vivo OVA restimulation in the presence of PBS (designated as control) or r-activin-A (*, P = 0.0121). Results are shown as means ± SEM from four separate experiments. Statistical significance was obtained by an unpaired Student's *t* test. IL-4 (**, P = 0.0070), IL-13 (*, P = 0.0103), and IL-10 (**, P = 0.0081) in supernatants are shown. (B) DLN cells from OVA/alum-immunized BALB/c mice were restimulated ex vivo with OVA in the presence of PBS or r-activin-A. $CD4^+$ T cells were adoptively transferred into BALB/c-*Rag1*$^{-/-}$ recipients. Proliferation of DLN cells to OVA from mice that received $CD4^+$ T cells treated with PBS or r-activin-A (***, P < 0.0001). The results are means ± SEM of triplicate wells (n = 4–6 mice per group in two separate experiments). Statistical significance was obtained as described. (C) $KJ1\text{-}26^+CD4^+$ T cells were stimulated with OVA and irradiated APCs as indicated. Proliferation was measured (***, P < 0.0001). IL-4 (**, P = 0.0038) and IL-10 (**, P = 0.0073) are shown. Results are means ± SEM of triplicate wells from four separate experiments.

As our studies have demonstrated an increased production of IL-10 under certain immune settings (Figs. 2 C and 4 A), we next investigated the potential role of this cytokine in the immunosuppressive effects of activin-A. Blocking of IL-10, using an antibody against IL-10R, partially reversed r-activin-A–mediated suppression of proliferative Th effector responses (Fig. 4 D). Similarly, anti–IL-10R treatment partially reversed the suppressive effect of r-activin-A on antigen-driven

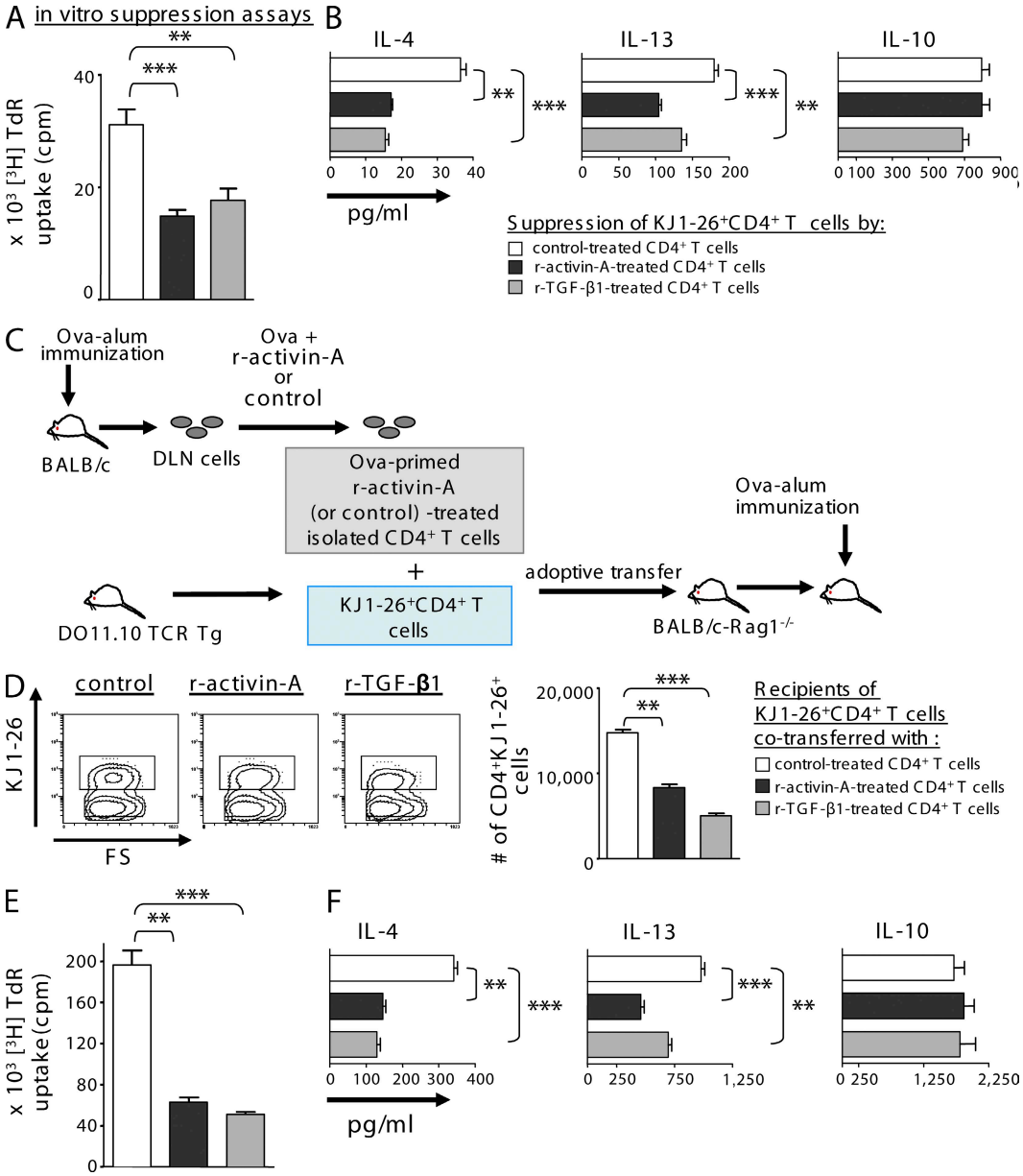

Figure 3. Activin-A induces the generation of antigen-specific regulatory CD4⁺ T cells. (A) DLN cells from OVA/alum-immunized BALB/c mice were restimulated ex vivo with OVA in the presence of PBS, r-activin-A, or rTGF-β1. CD4⁺ T cells were co-cultured with KJ1-26⁺CD4⁺ T cells (2:1) in the presence of irradiated APCs and $OVA_{323-339}$ peptide. Proliferation is shown (***, P < 0.0001; **, P = 0.0010). Results are shown as means ± SEM of triplicate wells in two independent experiments. Statistical significance was calculated by an unpaired Student's *t* test. (B) IL-4 (**, P < 0.0001; ***, P < 0.0001), IL-13 (***, P < 0.0001; **, P = 0.0007), and IL-10 (P = 0.9903; P = 0.0815) in supernatants are shown. Results are means ± SEM of duplicate wells in two separate experiments. Statistical significance was calculated as described. (C) r-activin-A–, rTGF-β1–, or PBS-treated CD4⁺ T cells (obtained as in A) were adoptively transferred along with KJ1-26⁺CD4⁺ T cells (1:1) into BALB/c-*Rag1*$^{-/-}$ recipients. Recipients were immunized with OVA/alum, and DLN cells were harvested. (D) Flow cytometry panels of gated CD4⁺ T cells from recipients stained for KJ1-26. Absolute numbers of CD4⁺KJ1-26⁺ T cells in DLNs of recipients (**, P < 0.0001; ***, P < 0.0001). Values are means ± SEM (*n* = 4–6 mice per group in two separate experiments). (E) Proliferation of DLN cells from recipient mice to $OVA_{323-339}$ peptide is shown (**, P < 0.0001; ***, P < 0.0001). The results are means ± SEM (*n* = 4–6 mice per group from two separate experiments). (F) IL-4 (**, P < 0.0001; ***, P < 0.0001), IL-13 (***, P < 0.0001; **, P = 0.0001), and IL-10 (P = 0.5253; P = 0.7871) in supernatants. Results are means ± SEM (*n* = 4–6 mice per group from two separate experiments).

primary responses of KJ1-26$^+$CD4$^+$ T cells so that r-activin-A– and anti–IL-10R–treated cells exhibited similar proliferation to PBS and anti–IL-10R–treated cells (132,300 ± 6,515 cpm for PBS- and anti–IL-10R–treated cells vs. 120,300 ± 7,752 cpm for r-activin-A– and anti–IL-10R–treated cells). However, blockade of IL-10 did not significantly affect activin-A–induced suppression of IL-2 release (Fig. 4 D). Importantly, blocking of IL-10 also partly reversed the suppressive effects of r-activin-A–induced CD4$^+$ regulatory T cells (OVA-primed Th cells) on the proliferation of KJ1-26$^+$CD4$^+$ responder T cells (unpublished data).

As blocking of IL-10 did not completely reverse the inhibitory effects of activin-A on Th responses, we hypothesized that other immunosuppressive cytokines (such as TGF-β1) may also be involved. Addition of a blocking antibody against TGF-β1 partially reversed activin-A–mediated suppression of Th cell proliferation, whereas it inhibited the suppression of IL-2 release (Fig. 4 D). Notably, addition of both anti–IL-10R and anti–TGF-β1 antibodies completely reversed activin-A–induced suppression of Th responses, indicating a synergistic effect.

Examination of the phenotype of the activin-A–induced regulatory T cells revealed that these cells lacked expression of Forkhead box p3 (Foxp3), the transcription factor that drives differentiation of certain regulatory T cell subsets (Fontenot et al., 2003; Hori et al., 2003; Vignali et al., 2008). In fact, flow cytometry analysis of anti-CD3–stimulated CD4$^+$ T cells showed that there were no differences in the frequency of Foxp3$^+$CD25$^+$CD4$^+$ T cells after r-activin-A treatment as compared with control (280,300 ± 15,150 cells for r-activin-A vs. 335,200 ± 18,950 cells for PBS). In contrast, rTGF-β1 treatment induced a greater number of Foxp3$^+$CD4$^+$ T cells (546,700 ± 20,280 cells; Fig. 4 E) as compared with PBS treatment, as also previously described (Chen et al., 2003; Fontenot et al., 2003; Davidson et al., 2007). In addition, r-activin-A treatment of sorted (CD4$^+$CD25$^-$CD62L$^+$) naive T cells from DO11.10 mice during stimulation with $OVA_{323-339}$ peptide–loaded bone marrow–derived DCs did not induce increased numbers of CD4$^+$Foxp3$^+$ T cells (12,420 ± 2,133 cells for r-activin-A vs. 14,600 ± 1,456 cells for PBS). Similarly, treatment with r-activin-A during restimulation with OVA ex vivo of DLN cells obtained from OVA-primed BALB/c mice did not induce significantly increase Foxp3 expression (34,570 ± 3,123 cells for r-activin-A vs. 30,090 ± 2,827 cells for PBS treatment). Also, activin-A treatment of DLN cells from immunized DO11.10 mice during restimulation with $OVA_{323-339}$ peptide ex vivo did not induce a significant increase in Foxp3 expression, as compared with PBS (30,700 ± 2,170 cells for r-activin-A vs. 28,250 ± 2,350 cells for PBS). Nevertheless, r-activin-A treatment of CD4$^+$ T cells (stimulated with either anti-CD3 or antigen) resulted in an ∼2.5-fold induction of IL-10$^+$CD4$^+$ T cells as compared with PBS (Fig. 4 F). These findings are in agreement with the increased IL-10 levels in CD4$^+$ T cell cultures upon r-activin-A treatment (Figs. 2 and 4). However, rTGF-β1-treatment did not significantly alter IL-10$^+$CD4$^+$ T cell numbers (not depicted), suggesting that, at least in our experimental settings, these two cytokines induce distinct regulatory T cell subsets. Collectively, our findings suggest that activin-A can exert a broad immunosuppressive effect on Th effector responses in vitro that is dependent on both IL-10 and TGF-β1 production.

Activin-A–induced regulatory T cells protect against allergic airway disease

We next asked whether activin-A–induced regulatory T cells can confer protection against Th-mediated disease in vivo. To address this, we adoptively transferred r-activin-A (or control)–treated CD4$^+$ T cells (obtained as in Fig. 3) into BALB/c mice before OVA/alum immunization and OVA aerosol challenge (experimental protocol in Fig. 5 A). Remarkably, transfer of r-activin-A–treated OVA-primed CD4$^+$ T cells resulted in a significant attenuation of all cardinal features of allergic airway disease (Fig. 5). This was demonstrated by significantly decreased total numbers of BAL cells and, notably, eosinophils in mice adoptively transferred with r-activin-A–treated OVA-primed CD4$^+$ T cells as compared with mice transferred with control-treated CD4$^+$ T cells (Fig. 5 B). More importantly, there was a dramatic decrease in AHR responses in mice that received r-activin-A–treated OVA-primed CD4$^+$ T cells that reached levels similar to those of alum controls (Fig. 5 C). A significant decrease (approximately threefold in histological score) was also observed in leukocytic infiltration (Fig. 5 D) and in mucus secretion (approximately threefold; Fig. 5 D) in the lungs of mice transferred with r-activin-A–treated CD4$^+$ T cells, as compared with controls.

In accordance, DLN cells obtained from mice adoptively transferred with r-activin-A–treated CD4$^+$ T cells exhibited significantly decreased proliferation to OVA ex vivo (Fig. 5 E) and produced significantly decreased levels of supernatant IL-4, IL-13, and IL-10 (Fig. 5 E). More importantly, adoptive transfer of r-activin-A–treated CD4$^+$ T cells significantly decreased OVA-specific IgE levels, a critical clinical feature of allergic disease (Fig. 5 F).

DCs are essential for the differentiation and activation of Th responses, and several studies have demonstrated that regulatory T cell subsets can suppress immune-mediated disease through inhibition of the antigen presentation machinery and/or maturation of DCs (Kim et al., 2007; O'Garra and Vieira, 2007; Sakaguchi and Powrie, 2007). Interestingly, examination of the MHC class II molecule I-A^d expression on DLN CD11c$^+$ DCs showed a striking decrease (an approximately ninefold difference, as shown by the mean fluorescence intensity) in I-A^d expression on DCs from mice adoptively transferred with r-activin-A–treated CD4$^+$ T cells, as compared with controls (Fig. 5 G). In support, we observed a decrease in CD86 expression (not depicted). Collectively, our data revealed that activin-A–induced antigen-specific regulatory T cells can protect from allergic airway disease after transfer in vivo, and this is associated with decreased maturation of CD11c$^+$ DCs in lung DLNs.

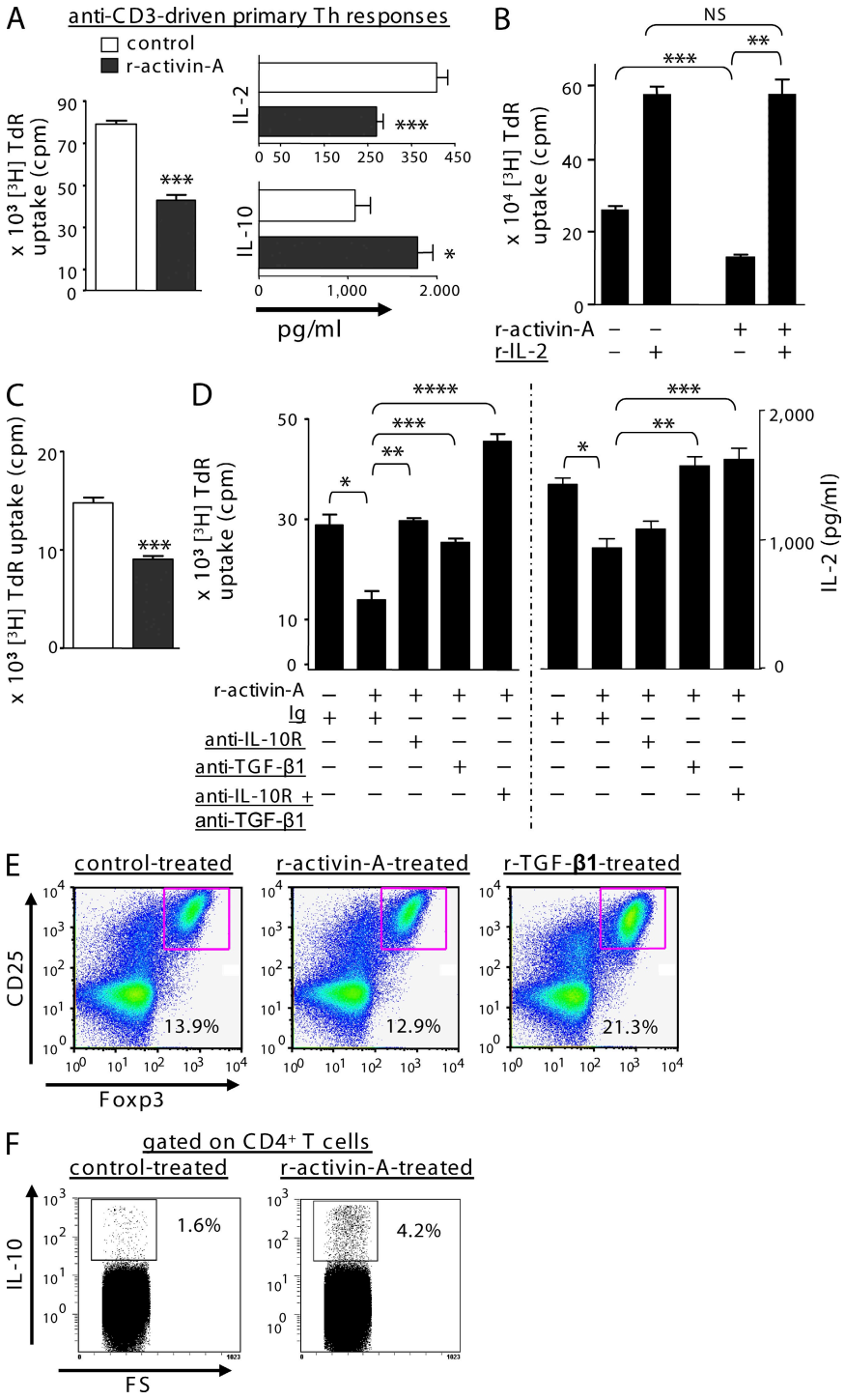

Figure 4. Activin-A–induced suppression is partly dependent on IL-10 and TGF-β1. (A) CD4⁺ T cells were stimulated with anti-CD3 and irradiated APCs as indicated. Proliferation was measured. The results are means ± SEM of triplicate wells from at least three separate experiments. Statistical significance was obtained by an unpaired Student's *t* test (***, P = 0.0009). IL-2 (***, P = 0.0004) and IL-10 (*, P = 0.0463) in supernatants are shown. (B) Proliferation of CD4⁺ T cells stimulated as in A in the presence of r-activin-A and rIL-2 as indicated (***, P < 0.0001; **, P = 0.0010; P = 0.9871). The results are means ± SEM of triplicate wells from three separate experiments. (C) DLN cells, obtained from OVA/CFA-immunized BALB/c mice, were stimulated ex vivo with OVA as indicated. Proliferation was measured (***, P < 0.0001). The results are means ± SEM of triplicate wells from three independent experiments. (D) Proliferation of CD4⁺ T cells stimulated with anti-CD3 and irradiated APCs with PBS or r-activin-A, as indicated (*, P = 0.0054; **, P = 0.0011; ***, P = 0.0012; ****, P = 0.0002). IL-2 (*, P = 0.0005; **, P = 0.0004; ***, P = 0.0003) in supernatants is shown. The results are means ± SEM from two separate experiments. (E) Stimulated (anti-CD3/CD28 with rIL-2) CD4⁺ T cells from PBS-, r-activin-A–, or TGF-β1–treated cultures were stained and analyzed by flow cytometry. Numbers indicate percentages of CD4⁺ gated cells positive for CD25 and Foxp3. Values are expressed as means ± SEM from

Administration of activin-A in vivo suppresses experimental allergic asthma

Based on our data on the suppressive roles of activin-A and activin-A–induced regulatory T cells on Th2 effector responses as well as on the effects of activin-A blockade during allergen challenge, we next examined whether administration of r-activin-A to sensitized mice would protect from disease induction. Indeed, systemic (i.p.) administration of r-activin-A to OVA/alum-immunized animals right before each OVA aerosol challenge resulted in significantly decreased total numbers of cells, and most importantly, eosinophils in the BAL, as compared with treatment with PBS (Fig. 6 A). Moreover, AHR responses were significantly decreased in r-activin-A–treated mice and reached levels similar to those of alum controls (Fig. 6 B). A significant decrease (~1.5-fold in histological score) was also observed in leukocytic infiltration (Fig. 6 C) and in mucus secretion (~2-fold; Fig. 6 C) in the lungs of r-activin-A–treated mice, as compared with PBS controls.

Similarly, DLN cells obtained from mice treated with r-activin-A exhibited significantly decreased proliferation to OVA ex vivo (Fig. 6 D) and produced decreased levels of IL-4, IL-13, and IL-10, as compared with PBS-treated controls (Fig. 6 D). In addition, OVA-specific IgG1 and IgE levels were decreased, whereas IgG2a levels were increased after r-activin-A treatment (Fig. 6 E). Hence, administration of r-activin-A in Th2-primed mice during pulmonary allergen challenge is protective against allergic airway disease induction.

Decreased expression of activin-A signaling components in allergic asthma

Based on the aforementioned findings, one would expect that activin-A production would be down-regulated during aberrant inflammatory processes such as those occurring in allergic asthma. However, we observed increased activin-A expression in lung biopsies from individuals with mild asthma as compared with healthy volunteers (Fig. 7 A, left). Activin-A expression was mainly localized in bronchial epithelial cells and subepithelial inflammatory cells (Fig. 7 A, bottom left). A significant increase in the percentage of activin-A^+ bronchial epithelial cells, as well as in the numbers of activin-A^+ subepithelial cells, was observed in lung biopsies from asthmatics as compared with healthy volunteers (Fig. 7 B). Quite the opposite was evident regarding the expression of activin-A's type I receptor, ALK4, which was significantly decreased in subepithelial infiltrating inflammatory cells (Fig. 7 A, bottom right; and Fig. 7 B). Additionally, the levels of Act-RIIA were significantly decreased, both in bronchial epithelial cells and in subepithelial inflammatory cells, in the lungs of asthmatics (Fig. 7 B). Similarly, we detected decreased expression of Act-RIIB in the lungs of asthmatics (unpublished data). Thus, although activin-A is increased in asthmatic lungs, the expression of its receptors in infiltrating immune cells is markedly reduced, suggesting a down-regulation of its signaling pathway that may lead to reduced suppression of the allergic inflammatory response.

DISCUSSION

Activin-A has been mainly studied in developmental, fibrotic, and monocyte-mediated inflammatory processes (Werner and Alzheimer, 2006). In the present study, our findings reveal that activin-A is a suppressive cytokine for Th-mediated immunity. In fact, we demonstrate for the first time, to our knowledge, that activin-A induces the generation of antigen-specific regulatory T cells that suppress responses by effector Th2 cells and, more importantly, transfer protection against Th2-associated allergic airway disease in vivo. Notably, we show that activin-A–induced regulation also affects Th1-driven responses, pointing to a broader immunosuppressive role for this cytokine.

Initially, our findings demonstrated that antibody-mediated depletion of activin-A during the clinically relevant phase of pulmonary allergen challenge resulted in significant exacerbation of Th2-mediated allergic airway disease (including increased AHR, BAL eosinophilia, Th2 cytokine and chemokine secretion, and OVA-specific systemic Th2 and antibody responses). Importantly, blockade of activin-A during OVA challenge resulted in a rapid (only after three daily doses of anti–activin-A) exacerbation of all cardinal features of the asthmatic disease. This indicated that endogenously produced activin-A is protective against allergic Th2 cell–mediated responses in the lung. In support, we found that DLN cells from anti–activin-A–treated mice cultured with KJ1-26^+CD4^+ T cells induced significantly heightened proliferative responses of the latter cells, pointing to decreased immune regulation. This was also evident by the significantly increased levels of Th2 cytokines. Concomitant up-regulation of IL-10 in these cultures can be explained either by the amplified Th2-mediated response or by the activation of regulatory mechanisms that are struggling to control the enhanced inflammation. We cannot exclude the possibility of an activin-A–mediated effect on lung epithelial and smooth muscle cells as well. Nevertheless, r-activin-A treatment of $CD4^+$ T cells rendered them suppressive, as they inhibited Th2 effector cell responses when adoptively transferred in vivo. This latter experimental approach uncovered the effects of r-activin-A on immune cells only. In another study, activin-A neutralization in vivo by a mouse monoclonal antibody resulted in decreased antigen-specific IgE (Ogawa et al., 2008). The observed differences could be caused by the fact that the investigators of

two separate experiments. (F) $CD4^+$ T cells, stimulated as in A, stained with antibodies to CD3, CD4, and IL-10. Representative dot plots showing $CD4^+$IL-10^+ cells from cultures of PBS- or r-activin-A–treated cells. Numbers above boxed areas indicate the percentage of DLN cells in the outlined gate. Values are expressed as means ± SEM from two separate experiments. FS, forward scatter.

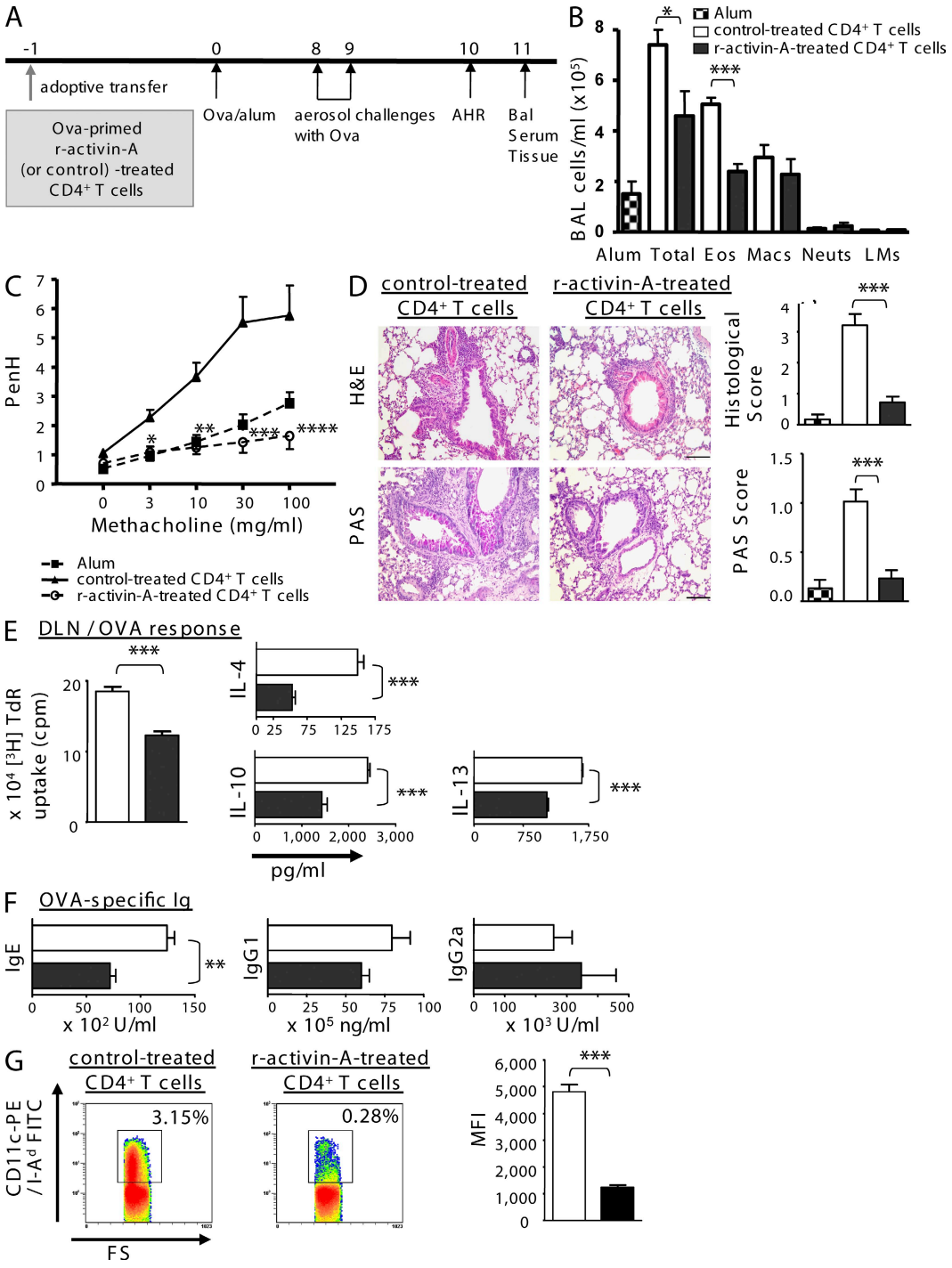

Figure 5. Activin-A–induced regulatory T cells protect from allergic airway disease upon transfer in vivo. (A) CD4+ T cells (obtained as in Fig. 3) were treated with either PBS or r-activin-A and adoptively transferred to BALB/c mice before OVA/alum immunization and OVA challenge. (B) BAL differentials from mice that received r-activin-A– or PBS-treated CD4+ T cells, or from the alum controls. Results are expressed as means ± SEM (n = 5–7 mice per group in two separate experiments). Statistical significance was obtained by an unpaired Student's t test (*, P = 0.0407; ***, P = 0.0007). (C) AHR is depicted. Results shown for PenH are expressed as means (n = 5–7 mice per group in two separate experiments). Data were analyzed by two-way ANOVA for repeated measures, followed by an unpaired Student's t test (*, P = 0.0341; **, P = 0.0003; ***, P = 0.0004; ****, P = 0.0017). (D) Representative photomicrographs and histological scores of H&E-stained (***, P < 0.0001) and PAS-stained (***, P = 0.0004) sections. Error bars depict means of groups. Results are means ± SEM (n = 5–7 mice per group in two independent experiments). Bars, 100 µm. (E) DLN cells were restimulated ex vivo with OVA. Proliferation was measured (***, P = 0.0002). IL-4 (***, P < 0.0001), IL-13 (***, P < 0.0001), and IL-10 (***, P = 0.0008) in supernatants are shown. Results are shown as means ± SEM (n = 5–7 mice per group in two separate experiments). (F) OVA-specific IgE (**, P = 0.0009), IgG1 (P = 0.1259), and IgG2a (P = 0.4556) in the sera of mice. Results are

the latter study used a different experimental protocol that involved several OVA/alum immunizations and a single challenge, whereas they depleted activin-A not only during challenge but at both phases of the allergic response. Alternatively, or in conjunction, these differences may be, at least partly, caused by the use of different activin-A neutralizing antibodies. For example, it is possible that these antibodies blocked distinct activin-A epitopes, leading to different receptor-mediated responses. Nevertheless, in support of the suppressive effects of endogenous activin-A on IgE responses, we showed that systemic administration of r-activin-A into allergic mice right before pulmonary OVA challenge induced a striking (more than threefold) decrease in OVA-specific IgE levels. This was accompanied by significantly suppressed Th2 systemic responses and protection against allergic airway disease. Collectively, our data reveal that administration of r-activin-A can be an effective therapeutic protocol for Th2-driven experimental asthma.

In a different study, local (pulmonary) administration of follistatin, an inhibitor of activin-A, during allergic airway inflammation decreased Th2 cytokines and mucus production, pointing to proinflammatory effects of activin-A locally (Hardy et al., 2006). The different findings between our studies and the aforementioned ones may be, at least partly, caused by the use of distinct activin-A inhibitors. We administered a specific anti–activin-A neutralizing antibody, whereas Hardy et al. (2006) administered follistatin, an approach that does not directly address the effects of activin-A, as follistatin neutralizes all activins and certain bone morphogenetic proteins (Vale et al., 1988; Harrison et al., 2005; Xia and Schneyer, 2009). Moreover, considering that other activins and bone morphogenetic proteins are also induced in the lungs of mice and humans with asthma (Rosendahl et al., 2001; Rosendahl et al., 2002; Kariyawasam et al., 2008), it is plausible that the observed differences are also related to other follistatin functions, such as the inhibition of these proteins.

Activin-A may also exert distinct in vivo effects depending on the route of administration or the site of overexpression. In fact, in contrast to the suppressive effects of systemic activin-A administration on allergic disease, we have showed that local (pulmonary) administration of r-activin-A enhances Th2 effector responses and exacerbates certain disease features (unpublished data). These findings point to proinflammatory effects of excessive amounts of activin-A locally, a feature shared by several cytokines, including immunoregulatory ones. For example, TGF-β1 blockade locally prevents the onset of experimental autoimmune encephalomyelitis (EAE) (Veldhoen et al., 2006), and overexpression in the brain results in more severe EAE, pointing to a proinflammatory role for this cytokine (Wyss-Coray et al., 1997; Luo et al., 2007). In contrast, TGF-β1 neutralization systemically exacerbates EAE (Veldhoen et al., 2006), and systemic (i.p.) TGF-β1 administration protects from disease (Johns et al., 1991; Kuruvilla et al., 1991; Johns and Sriram, 1993). TGF-β1 acts similarly in models of rheumatoid arthritis (Allen et al., 1990; Brandes et al., 1991). Dual effects depending on the route of administration have been also described for IL-10 in models of pneumococcal meningitis and endotoxin-induced uveitis (Rosenbaum and Angell, 1995; Koedel et al., 1996). Collectively, our studies and those by Hardy et al. (2006) emphasize the complex roles of activin-A in the regulation of the immune response.

Importantly, our data reveal that activin-A's immunosuppressive effects were associated with induction of antigen-specific regulatory T cells. Strikingly, these activin-A–induced suppressor T cells inhibited the robust response of KJ1-26^+CD4^+ T cells to the $OVA_{323-339}$ peptide in vitro and suppressed responses of other untreated Th cells upon adoptive cotransfer in vivo. Activin-A–induced regulatory T cells were $CD4^+CD25^-Foxp3^-$. A very recent study demonstrated that TGF-β1–induced conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ T cells in vitro could be further enhanced if activin-A was added in the medium (Huber et al., 2009). However, activin-A was not essential for TGF-β1–induced Foxp3 expression, as neutralization by follistatin or anti–activin-A antibody had no effect on $CD4^+Foxp3^+$ T cell numbers. Moreover, the presence of low levels of TGF-β1 in activin-A–treated cultures was essential for Foxp3 induction, supporting the notion that activin-A alone does not induce considerable Foxp3 expression.

Notably, activin-A induced the generation of a population of $CD4^+IL\text{-}10^+$ T cells, possibly representing Tr1-like T regulatory cells. In support, activin-A treatment increased IL-10 levels in primary $CD4^+$ T cell cultures. Moreover, our in vitro blocking studies revealed that enhanced IL-10 production represents one of the mechanisms of activin-A–induced Th suppression. However, IL-10 levels were unaltered or even decreased during activin-A–mediated suppression of secondary Th2 effector responses. This should not come as a surprise, because, in this setting, IL-10 (along with IL-4 and IL-13) acts predominantly as part of the effector Th2 cytokine profile and its levels follow the general Th2 cell suppression (Wills-Karp, 1999).

In addition, activin-A may induce other suppressive Th cell populations. This possibility explains our finding that blocking of IL-10 did not completely reverse the suppressive function of activin-A–induced regulatory Th cells. Furthermore, TGF-β1 blockade also partially reversed activin-A–mediated Th suppression. Overall, it is conceivable that activin-A induces both IL-10–producing regulatory T cells and other non–IL-10–producing suppressor T cells. The

means ± SEM (n = 5–7 mice per group in two independent experiments). (G) Flow cytometry panels of gated $CD3^-CD11c^+$ DLN cells from recipient mice stained for $I\text{-}A^d$. Numbers above boxed areas indicate the percentage of DLN cells in the outlined gate. The mean fluorescence intensity (MFI) of $CD3^-CD11c^+I\text{-}Ad^+$ cells in DLNs of mice that received $CD4^+$ T cells treated with PBS or r-activin-A is depicted (***, $P < 0.0001$). Values are means ± SEM (n = 5–7 mice per group in two separate experiments). Eos, eosinophils; FS, forward scatter; LMs, lymphomononuclears; Macs, macrophages; Neuts, neutrophils.

chemokine CXCL12 was recently found to inhibit EAE in a similar fashion (Meiron et al., 2008).

Our present studies also revealed that activin-A–induced regulatory T cells can confer protection against AHR and allergic airway disease after adoptive transfer in vivo. In addition, activin-A–induced regulatory T cells suppress Th2 effector responses and, of clinical relevance, decrease allergen-specific IgE. Besides naturally occurring or inducible Foxp3$^+$ T regulatory cells, several studies have shown the existence of antigen-specific Foxp3$^-$ suppressor T cells that are of critical importance, as they

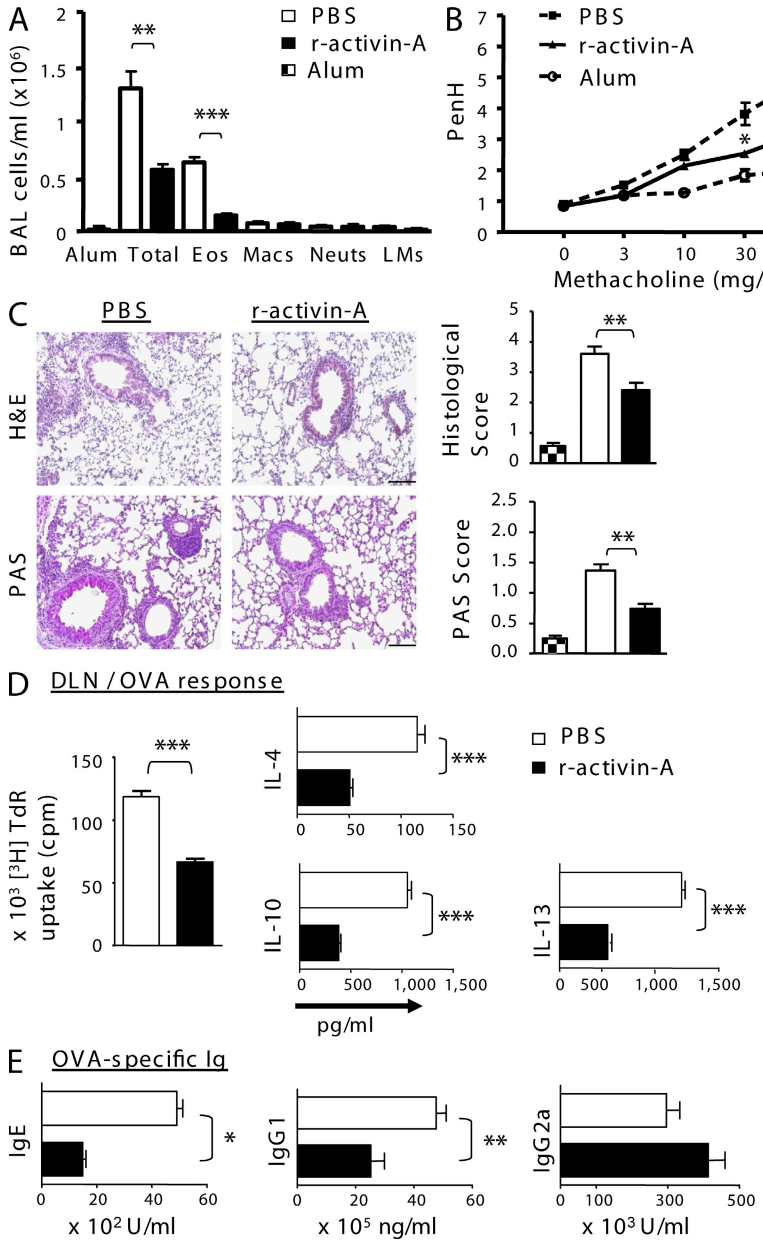

Figure 6. In vivo administration of r-activin-A during pulmonary allergen challenge is protective against allergic airway disease. (A) BAL differentials from mice treated with r-activin-A or PBS, or from the alum controls are expressed as means ± SEM (n = 4–6 mice per group in two separate experiments; **, P = 0.0021; ***, P < 0.0001). Statistical significance was obtained by an unpaired Student's t test. (B) AHR is depicted. Results shown for PenH are expressed as means (n = 4–6 mice per group in two separate experiments). Data were analyzed by two-way ANOVA for repeated measures, followed by an unpaired Student's t test (*, P = 0.0095; **, P = 0.0054). (C) Representative photomicrographs and histological scores of H&E-stained (**, P = 0.0085) and PAS-stained (**, P = 0.0015) sections. Error bars depict means of groups. Results are shown as means ± SEM (n = 4–6 mice per group in two separate experiments). Bars, 100 µm. (D) DLN cells were restimulated ex vivo with OVA. Proliferation was measured (***, P < 0.0001). IL-4 (***, P < 0.0001), IL-13 (***, P < 0.0001), and IL-10 (***, P < 0.0001) in supernatants are shown. Results are means ± SEM (n = 4–6 mice per group from two independent experiments). (E) OVA-specific IgE (*, P = 0.0119), IgG1 (**, P = 0.0081), and IgG2a in the sera of mice. Results are shown as means ± SEM (n = 4–6 mice per group in two independent experiments). Eos, eosinophils; LMs, lymphomononuclears; Macs, macrophages; Neuts, neutrophils.

confer protection against immune-mediated diseases, i.e., in experimental models of multiple sclerosis and inflammatory bowel disease (Roncarolo et al., 2006; Shevach, 2006; Sakaguchi and Powrie, 2007). Hence, the emergence of factors, such as activin-A, that induce suppressor T cells protective against Th-linked diseases is of critical therapeutic importance.

Interestingly, the suppressive effects of activin-A–treated T cells were associated with a striking decrease of DC maturation in lung DLNs. Thus, it is plausible that activin-A also suppresses Th responses through regulation of DC function. In fact, activin-A induces in vitro a dramatic reduction on both MHC class II and CD86 co-stimulatory molecule expression on mouse CD11c$^+$ DCs (unpublished data). In support, recent studies using human DCs have shown that activin-A prevents cytokine-induced HLA-DR up-regulation and promotes a tolerance-inducing DC phenotype (Segerer et al., 2008).

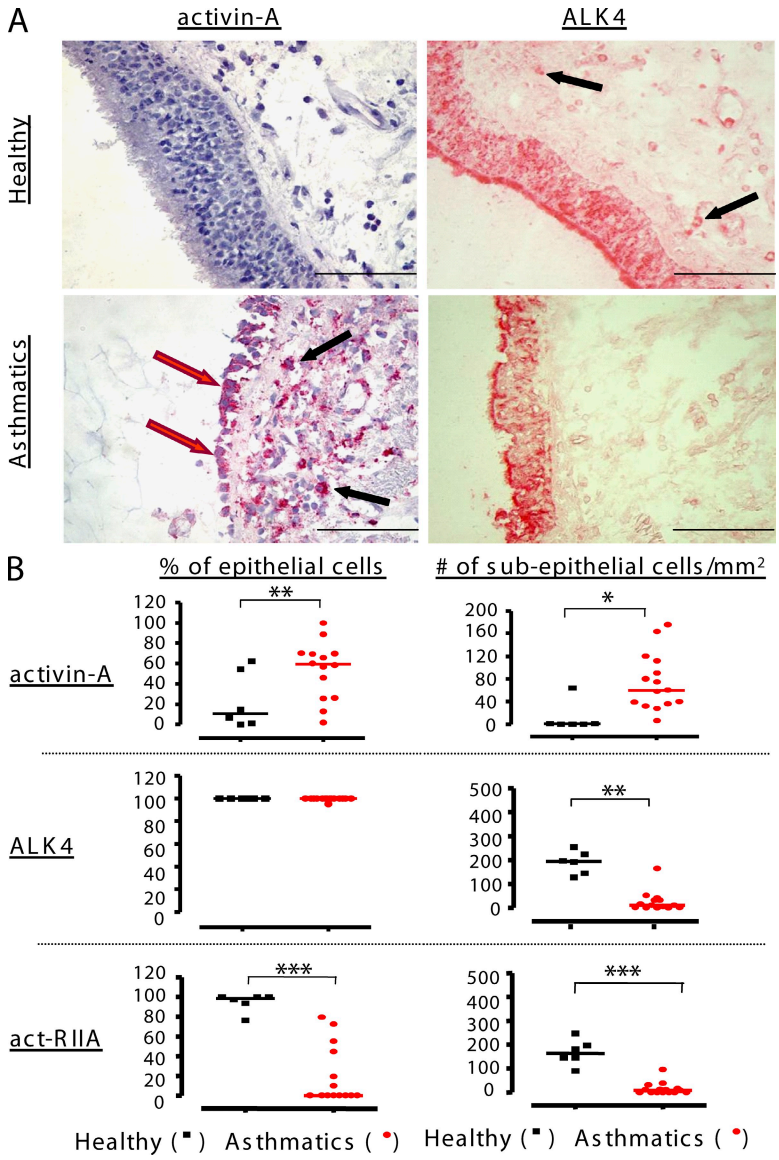

Figure 7. Decreased expression of activin-A's signaling components in allergic asthma. (A) Representative photomicrographs showing expression of activin-A and ALK4 in bronchial biopsies from healthy individuals and asthmatics. The specific staining for activin-A and ALK4 is depicted in red, whereas nuclei are blue (in activin-A photos). Red arrows indicate positively stained epithelial cells and black arrows point to positively stained subepithelial cells. Photomicrographs are representative from experiments performed on lung biopsies obtained from asthmatics (n = 15) and healthy individuals (n = 6). Immunostaining was performed once for each lung section. Bars, 100 µm. (B) Percentages of activin-A$^+$ (**, P = 0.0355), ALK4$^+$, and act-RIIA$^+$ (***, P = 0.0008) bronchial epithelial cells from asthmatics (n = 15) or healthy individuals (n = 6). For each biopsy, data were obtained counting positively stained epithelial cells along the entire basement membrane. Numbers of activin-A$^+$ (*, P = 0.0045), ALK4$^+$ (**, P = 0.0011), and act-RIIA$^+$ (***, P = 0.0007) subepithelial cells were determined by counting the entire section in each biopsy and are expressed as cells per square millimeter. Immunostaining was performed once for each lung section. Cell counts are presented as medians ± interquartile range. Statistical analysis was performed with the Mann-Whitney U test.

We were initially surprised that bronchial epithelial and infiltrating subepithelial cells in lung biopsies from asthmatic individuals exhibited enhanced activin-A expression. Our findings, as well as those from other studies, also revealed increased activin-A expression by Th2 cells and in the lungs of allergic mice (Rosendahl et al., 2001; Hardy et al. 2006; Karagiannidis et al., 2006; Ogawa et al., 2006). Nevertheless, it is not unusual to have increased expression of immunosuppressive mediators during active inflammatory conditions. For example, TGF-β1 is increased in the lungs of asthmatics (Chakir et al., 2003; Flood-Page et al., 2003), whereas up-regulation of αB-crystallin (Ousman et al., 2007) and osteopontin (Xanthou et al., 2007) is observed in multiple sclerosis and asthma, respectively. Importantly, the increase in activin-A was counterbalanced by decreased expression of activin-A's receptors, ALK4 and Act-RIIA and -RIIB, in infiltrating mononuclear cells of asthmatics. This is indicative of reduced activin-A signaling in infiltrating leukocytes, pointing to decreased immunoregulation. In support, blockade of ALK4 significantly enhanced Th2 responses in our experiments. Thus, activin-A up-regulation in the allergen-challenged lung may be part of an inherent protective mechanism, as also suggested by the exacerbation of allergic airway disease upon activin-A neutralization before challenge. Finally, adoptive transfers of regulatory $CD4^+CD25^+$ T cells in sensitized mice not only protected from allergic airway disease (Kearley et al., 2005) but also resulted in significant increase in lung activin-A levels (unpublished data).

Although both activin-A and TGF-β1 belong to the same family of cytokines, it would be unjust to have them considered as redundant during inflammatory processes, as also suggested by several studies (Ogawa et al., 2000; Werner and Alzheimer, 2006; Le et al., 2007; Ogawa et al., 2008; Robson et al., 2009). In fact, our experiments reveal that in vivo blockade of activin-A resulted in significant exacerbation of allergic airway disease despite the presence of endogenous TGF-β1. Moreover, we demonstrate that activin-A and TGF-β1 induce different regulatory T cell subsets, indicating that these two cytokines may also operate through distinct suppressive mechanisms. These findings suggest that, at least in our experimental settings, activin-A has a distinct (to TGF-β1) role.

Our findings introduce activin-A as a new member in the team of immune response regulation factors. Moreover, its implicit protective role in acute allergic airway disease places activin-A as a therapeutic target for allergic asthma. The role of activin-A in several facets of regulatory T cell biology, including generation, maintenance, and function, is of great importance and remains to be further elucidated. Finally, considering its suppressive effects on Th1-driven responses, an involvement of activin-A in the regulation of autoimmunity is highly probable and merits closer examination.

MATERIALS AND METHODS

Mice. BALB/c, BALB/c-*Rag-1*$^{-/-}$, and OVA-specific T cell receptor transgenic DO11.10 mice were purchased from the Jackson Laboratory. Mice were housed at the Biomedical Research Foundation of the Academy of Athens's Animal Facility. Procedures were in accordance with the United States National Institutes of Health Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals (no. A5736-01) and with the European Union Directive 86/609/EEC on the protection of animals used for experimental purposes.

Experimental protocol of acute allergic airway inflammation. Acute allergic airway inflammation was induced in mice using OVA in alum. In brief, BALB/c mice were sensitized with OVA at a concentration of 0.01 mg/mouse in 0.2 ml alum i.p. on days 0 and 12. Control mice received the same volume of PBS in alum (alum controls). Subsequently, all mice received three challenges with aerosolized OVA (5% for 20 min) via the airways between days 18 and 20. For blocking experiments, mice also received an affinity-purified polyclonal neutralizing antibody against mouse activin-A (R&D Systems) i.p. at a dose of 20 μg/mouse, 2–3 h before each OVA challenge. Other groups of OVA-challenged mice received similar doses of isotype control Ig (R&D Systems). In other experiments, mice received i.p. two doses (1.125 μg/mouse/day) of r-activin-A (R&D Systems) or PBS, 4–5 h before each OVA challenge. The optimal dose of r-activin-A was determined after titration experiments and was over endogenous activin-A levels in lungs and DLNs of allergic mice as measured by ELISA. Mice were euthanized 48 h after the final OVA challenge.

AHR. AHR was measured in mice 24 h after the final OVA challenge (day 21) by whole-body plethysmography (Buxco Research Systems) to calculate enhanced pause (PenH). Responses to inhaled methacholine at concentrations of 3–100 mg/ml were measured for 1 min, as described previously (Xanthou et al., 2007).

Analysis of BAL. BAL harvesting was performed as previously described (Xanthou et al., 2007). In brief, inflammatory cells were obtained by cannulation of the trachea and lavage of the airway lumen with PBS. Cytospin slides were prepared by Wright-Giemsa staining. All differential counts were performed blind and in a randomized order at the end of the study.

Lung histology. 4-μm paraffin-embedded sections were stained with hematoxylin and eosin (H&E) to evaluate lung infiltration, as described previously (Xanthou et al., 2007). A semiquantitative scoring system was used to grade the size of lung infiltrates, whereby +5 signified a large (>3 cells–deep) widespread infiltrate around the majority of vessels and bronchioles, and +1 signifies a small number of inflammatory foci. Goblet cells were counted on periodic acid–Schiff (PAS)–stained lung sections using an arbitrary scoring system. In brief, PAS-stained goblet cells in airway epithelium were measured double blind using a numerical scoring system (0, <5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75% goblet cells). The sum of the airway scores from each lung was divided by the number of airways examined (20–30 per mouse) and expressed as mucus score in arbitrary units.

Human subjects. 15 individuals with atopic asthma with a 15% increase in forced expiratory volume in 1 s (FEV_1) to β_2 agonist, or a methacholine PC_{20} of ≤8 mg/ml were recruited. The median age was 25 yr (range = 19–46 yr), with $FEV_{1\%}$ predicted to be 97% (range = 75.4–125.7%) with a methacholine PC_{20} of 2.1 mg/ml (range = 2–3.6 mg/ml; geometric mean ± 95% confidence interval). All subjects demonstrated positive skin prick tests to aeroallergens (ALK). The study design is previously described (Phipps et al., 2004; Kariyawasam et al., 2007). Six healthy volunteers with no history of asthma or atopy with a median age of 30.5 yr (range = 27–42 yr), and with a predicted $FEV_{1\%}$ of 100.4% (range = 80–104.3%) with a methacholine PC_{20} of >16 mg/ml were included. The study was approved by the Royal Brompton and Harefield Hospital Ethics Committee, and subjects gave written informed consent.

Immunohistochemistry. Human lung biopsies were collected in PBS, and transferred into 4% paraformaldehyde (Sigma-Aldrich) and then to 15% sucrose (Sigma-Aldrich), as previously described (Hamid et al., 1991; Robinson

et al., 1993). Biopsies were embedded in Tissuetek OCT (Thermo Fisher Scientific), snap frozen, and stored at −80°C. 5-μm sections were cut and immunostaining was performed, as previously described (Hamid et al., 1991; Robinson et al., 1993). In brief, the alkaline phosphatase–antialkaline phosphatase method was used, and specific antibody binding was visualized using Vectastain ABC-AP kits and the Fast-Red chromogen (Vector Laboratories). All incubations were performed at room temperature. Washes were performed in PBS unless otherwise stated. Normal human serum (10%) was used to reduce nonspecific binding. An affinity-purified polyclonal goat antibody against human activin-A, and an irrelevant species IgG antibody (which served as negative control) were used (R&D Systems). Antibodies against activin-A receptors were made as previously described (Franzén et al., 1993; Rosendahl et al., 2001). Cell counts were performed in a blinded fashion using a microscope (model BH-2; Olympus Corp.), as previously described (Kariyawasam et al., 2007). The numbers of positively stained epithelial cells were counted along the entire basement membrane of each section using a squared eyepiece graticule (Olympus) and expressed as the percentage of positive cells. The numbers of positively stained infiltrating cells that were located below the basement membrane (submucosal cells) were determined by counting the whole section and expressed as cells per square millimeter.

Cell culture, proliferation, and suppression assays. 5×10^4 $CD4^+$ T cells were purified (Dynabeads) from LNs of BALB/c mice and stimulated for 48 h with 2 μg/ml of soluble anti-CD3 and 10^5 irradiated (3,000 rad) T cell–depleted syngeneic APCs in the presence of PBS or 50 ng/ml r-activin-A. Different concentrations of anti-CD3 antibody and r-activin-A were tested. In some experiments, neutralizing antibodies against IL-10R and TGF-β1 (at 10 μg/ml; R&D Systems) or the respective Ig control were added. In others, 20 U/ml rIL-2 (PeproTech) was added. LN cells from DO11.10 mice were stimulated for 48 h with 125 μg/ml OVA in the presence of PBS, 50 ng/ml r-activin-A, 20 μg/ml anti–mouse activin-A, 20 μg/ml anti–mouse ALK4, or 20 μg/ml Ig control (R&D Systems). In other experiments, 5×10^4 $KJ1\text{-}26^+CD4^+$ T cells stimulated for 48 h with 0.25 μg/ml of $OVA_{323\text{-}339}$ peptide and 10^5 irradiated APCs were used. For antigen-driven Th1 and Th2 responses, DLN cells were obtained from OVA/CFA (0.01 mg/mouse of OVA in 0.1 ml CFA) i.p. and OVA/alum (0.01 mg/mouse OVA in 0.2 ml alum), respectively, sensitized BALB/c mice and restimulated ex vivo with OVA. $CD4^+$ T cell proliferation was measured after stimulation for 72 h. Cells were pulsed with 1 μCi/well [^{3}H]thymidine for the final 12 h of culture. [^{3}H]Thymidine incorporation was measured by a scintillation counter (Microbeta-Trilux; PerkinElmer).

For in vitro suppression experiments, BALB/c mice were sensitized with OVA/alum and harvested DLN cells were stimulated ex vivo with 125 μg/ml OVA in the presence of PBS or 50 ng/ml r-activin-A or 2 ng/ml rTGF-β1 for 4 d. Cultures were rested in medium containing 20 U/ml rIL-2 for 6 d. 10^5 $CD4^+$ T cells were isolated and co-cultured with 5×10^4 $KJ1\text{-}26^+CD4^+$ T cells, 10^5 irradiated syngeneic APCs, and 0.25 μg/ml of $OVA_{323\text{-}339}$ peptide. T cell proliferation was measured.

Cytokine analysis. Cytokines were measured in lung homogenates and culture supernatants using commercially available ELISA kits for IL-4, IL-10, IFN-γ, IL-12 (OptEIA; BD), IL-13, activin-A, CCL11, CCL22 and CCL17 (R&D Systems), and IL-2 (Bender Medsystems). In experiments performed in Fig. 6, ELISA kits for IL-4 and IL-10 were obtained from R&D Systems.

Adoptive transfer experiments. DLN cells from OVA/alum sensitized (i.p.) BALB/c mice were harvested and restimulated ex vivo with 125 μg/ml OVA and either PBS or 50 ng/ml r-activin-A for 4 d. 2×10^6 $CD4^+$ T cells/mouse were transferred i.v. into BALB/c-*Rag1*$^{-/-}$ recipients. 36 h later, recipients were sensitized with OVA/alum and euthanized 10 d later. LN cells were restimulated ex vivo with OVA and proliferation was measured.

2×10^6 $CD4^+$ T cells/mouse were obtained as described in the in vitro suppression experiments and adoptively transferred i.v., along with 2×10^6 $KJ1\text{-}26^+CD4^+$ T cells/mouse, into BALB/c-*Rag1*$^{-/-}$ recipients. 36 h later, recipients were sensitized with OVA/alum and euthanized 10 d later. DLN cells were stimulated with the $OVA_{323\text{-}339}$ peptide and T cell responses were measured.

In other experiments, 4×10^6 $CD4^+$ T cells/mouse, obtained as described, were adoptively transferred i.v. into BALB/c mice. 24 h later, mice were sensitized with OVA/alum, and 7 d later they received two challenges with aerosolized OVA (5% for 20 min).

Determination of serum antibody concentration. OVA-specific IgE, IgG1, and IgG2a serum antibodies were measured by ELISA, as previously described (Xanthou et al., 2007). Hyperimmune serum from OVA-alum–sensitized and OVA-challenged BALB/c mice was used for the IgE, IgG1, and IgG2a standards.

Flow-cytometric analysis. Cells were stained with antibodies to CD4, CD3, KJ1-26, CD25, CD11c, I-A^d, CD-86, IL-10 (BD), and T1/ST2 (MD Biosciences). For intracellular IL-10 staining, $CD4^+$ T or $CD4^+KJ1\text{-}26^+$ T cells were stimulated for 24 h with anti-CD3 or $OVA_{323\text{-}339}$ peptide and stained as previously described (Fitzgerald et al., 2007). In brief, cells were restimulated for 4 h with 10 ng/ml PMA (Sigma-Aldrich), 250 ng/ml ionomycin (Sigma-Aldrich), and 1 μl/ml GolgiStop (BD), followed by surface and intracellular staining according to the manufacturer's instructions (BD). For intracellular Foxp3 staining, $CD4^+$ T cells were stimulated with 2 μg/ml anti-CD3, 2 μg/ml anti-CD28, and 20 U/ml rIL-2 for 3 d. Cells were washed and stained with conjugated antibodies to CD4, CD3, and Foxp3 according to the manufacturer's instructions (eBioscience). Sorted ($CD4^+CD25^-CD62L^+$) T cells from DO11.10 mice stimulated with $OVA_{323\text{-}339}$ peptide–loaded bone marrow–derived DCs were also used. In other experiments, DLN cells were harvested from $OVA_{323\text{-}339}$ peptide/alum–immunized DO11.10 mice and restimulated ex vivo for 3 d with $OVA_{323\text{-}339}$ peptide. In others, DLN cells from OVA/alum–sensitized BALB/c mice were stimulated ex vivo with OVA for 4 d and rested in rIL-2 for 6 d before FoxP3 staining. To perform the FACS analysis, we used a cytometer (Cytomics FC 500; Beckman Coulter). Cell death was determined by flow cytometry using annexin V/PI staining (BD).

We are grateful to Dr. P. Verginis for critical reading of the manuscript and helpful discussions. We thank Dr. D. Simoes for help with the statistical analysis and lung function experiments, S. Spyridakis for assistance with flow cytometry, and A. Agapaki for histology preparations. We also thank Dr. S. Pagakis and J. Morianos for assistance with figure preparation.

This work was supported by the Biomedical Research Foundation of the Academy of Athens (to V. Panoutsakopoulou and G. Xanthou); by a grant from the Hellenic Ministry of Development, General Secretariat of Research and Technology (03ED750 to V. Panoutsakopoulou); and by an unrestricted grant for research in respiratory medicine from GlaxoSmithKline (to G. Xanthou). C.M. Lloyd is supported by a Senior Fellowship from the Wellcome Trust (057704). D.S. Robinson is supported by a Wellcome Trust Research Leave Award for Clinical Academics. H.H. Kariyawasam is supported by an Imperial College Trust Fund. A.B. Kay is funded by the Imperial Trust.

The authors have no conflicting financial interests.

Submitted: 17 November 2008
Accepted: 24 June 2009

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