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Blockade of Allergic Airway Inflammation Following Systemic Treatment with a B7-Dendritic Cell (PD-L2) Cross-Linking Human Antibody

Suresh Radhakrishnan, Koji Iijima, Takao Kobayashi, Moses Rodriguez, Hirohiito Kita, and Larry R. Pease

We present a novel immunotherapeutic strategy using a human B7-DC cross-linking Ab that prevents lung inflammation, airway obstruction, and hyperreactivity to allergen in a mouse model of allergic inflammatory airway disease. Dendritic cells (DC) have the ability to skew the immune response toward a Th1 or Th2 polarity. The sHIgM12 Ab functions in vitro by cross-linking the costimulatory family molecule B7-DC (PD-L2) on DC up-regulating IL-12 production, homing to lymph nodes, and T cell-activating potential of these APCs. Using chicken OVA as a model Ag, the administration of sHIgM12 Ab to BALB/c mice blocked lung inflammation, airway pathology, and responsiveness to methacholine. Even after animals were presensitized and a Th2-polarized immune response was established. This therapeutic strategy was ineffective in STAT4-deficient animals, indicating that IL-12 production is critical in this system. Moreover, the polarity of the immune response upon antigen re-exposure with Ag is changed in wild-type mice, with a resulting decrease in Th2 cytokines IL-4 and IL-5 and an increase in the immunoregulatory cytokine IL-12 production is critical in this system. Moreover, the polarity of the immune response upon antigen re-exposure with Ag is changed in wild-type mice, with a resulting decrease in Th2 cytokines IL-4 and IL-5 and an increase in the immunoregulatory cytokine IL-12. These studies demonstrate that the immune responses to hyperreactive responders can be modified using B7-DC cross-linking Abs, preventing allergic airway disease upon re-exposure to allergens.

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2 S.R. and K.I. made equal contributions to this study.
3 Address correspondence and reprint requests to Dr. Larry R. Pease, Department of Immunology, Mayo Clinic College of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail address: pease.larry@mayo.edu
4 Abbreviations used in this paper: DC, dendritic cell; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; Penh, enhanced pause; pHIGM, polyclonal human IgM; sHmgm12, serum-derived human IgM Ab 12.
airway inflammation. Strikingly, this Ab treatment protected mice from allergic symptoms even when administered 14 days after hyper-sensitization. Moreover, the polarity of T cells isolated from the spleens of therapeutically treated mice was changed from strong Th2 to weak Th1. We conclude that B7-DC cross-linking Ab treatment protects mice in both a prophylactic and, more importantly, a therapeutic setting in a murine model of allergic airway inflammation.

Materials and Methods

Mice and reagents

Six- to 8-wk-old BALB/cJ and BALB/c-STAT6<sup>−/−</sup> mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the animal house facility, Mayo Clinic, as per the institutional guidelines for further studies. OVA protein was purchased from Sigma-Aldrich (St. Louis, MO). These studies were approved by the Mayo Clinic Institutional Animal Care and Use Committee. The IgM human Ab, sHgM12, is derived from a patient with Waldenstrom’s macroglobulinemia, as described previously (11). Five hundred milligrams of Ab have been purified. The polyclonal human IgM (pHgM) Ab used as a control in these studies has been described (18).

Immunization and airway challenge

The sensitization and challenge procedure with OVA was modified from the method described by Zhang et al. (19). Briefly, mice were sensitized by an intranasal injection of 100 μg of OVA adsorbed to 1 mg of alum (Pierce, Rockford, IL). Experimental mice were intranasally challenged with 100 μg of OVA in PBS under tribromoethanol anesthesia.

Treatment with B7-DC cross-linking Ab

In the prophylactic regimen, mice were treated with sHgM12 or the control pHgM Ab i.v. at 10 μg per day on days −1, 0, and 1, prior to sensitization with OVA in alum. This schedule was designed to determine whether Ab treatment would prevent the establishment of the Th2-polarizing response, typically elicited by the use of an adjuvant in the animal model presentation. In the therapeutic regimen, the Ab treatments were conducted at the same dose and time points as the prophylactic regimen. The sensitization and challenge procedure with OVA was modified from the method described by Zhang et al. (19). Briefly, mice were sensitized by an intranasal injection of 100 μg of OVA adsorbed to 1 mg of alum (Pierce, Rockford, IL). Experimental mice were intranasally challenged with 100 μg of OVA in PBS under tribromoethanol anesthesia.

Measurement of airway responsiveness to methacholine

Airway responsiveness was assessed in conscious mice by methacholine-induced airflow obstruction in conscious mice by using whole body plethysmography (Buxco Electronics, Troy, NY). Pulmonary airflow obstruction was measured by enhanced pause (Penh) with a transducer connected to plethysmograph modules and analyzed by system software to measure methacholine responsiveness. Mice were exposed to 2 μl of PBS followed by incremental dosages of aerosolized methacholine. F100 sustained Penh was monitored for each dose.

Collection of bronchoalveolar lavage (BAL) fluid

Immediately after measuring airway hyperreactivity (AHR), animals were injected i.p. with a lethal dose (250 mg/kg) of pentobarbital (Abbott Laboratories, Abbott Park, IL). The trachea was cannulated, and the lungs were lavaged twice with 0.5 ml of HBSS. After centrifugation, the supernatant was collected and stored at −20°C. The cells were resuspended and counted using a hemocytometer. BAL cell differentials were determined with Wright-Giemsa stain; ≥200 cells were differentiated using conventional morphologic criteria. IL-5 in the BAL fluid supernatants was measured by ELISA, as directed by the manufacturer (R&D Systems, Minneapolis, MN).

Histology

After BAL fluid collection, the lung was fixed in 10% formalin and embedded in paraffin. Sections were obtained and stained with H&E, and in some cases with anti-CD3 Ab (using a peroxidase-labeled secondary developing reagent). The sections were evaluated by microscopy at ×100 and ×400 magnification.

In vitro cytokine production and proliferation

On day 27, splenocytes from the control Ab or the sHgM12 Ab-treated mice were harvested and processed. Briefly, after making a single cell suspension, RBC were lysed by hypotonic shock using ammonium chloride/potassium bicarbonate/EDTA. Cells were counted and resuspended at 3 million cells/ml in RPMI 1640 (Cambrex, Walkersville, MD). OVA was made to a final concentration of 2 mg/ml and was titrated at half log dilutions. Supernatants were added at 3 × 10<sup>6</sup> cells in 100 μl. Supernatants were harvested after 48 h and stored for cytokine assay. Cells were pulsed with [3H]thymidine during the last 18 h of the 72-h assay. Cells were harvested and counted for incorporation of [3H]thymidine (Packard Instrument, Boston, MA).

Stored supernatants were analyzed for IL-4, IL-5, IL-10, IFN-γ, and TNF-α by ELISA, per the manufacturer’s protocol (R&D Systems).

Statistical analysis

Data were analyzed using a two-way repeated measures ANOVA or Student’s t test for normally distributed data and the Whitney rank sum test for nonparametric data.

Results

Prophylactic treatment with sHgM12 Ab prevents bronchial AHR in murine model of allergic airway inflammation

Treating bone marrow derived murine DC in vitro with sHgM12 B7-DC cross-linking Ab resulted in secretion of IL-12 (12). IL-12 is a key cytokine in determining the polarity of the immune response, supporting the Th1 phenotype by promoting the secretion of IFN-γ (20). Treatment with the Th1-polarizing cytokine TNF-α can convert Th2 toward Th1 phenotype by promoting allergic disease such as asthma (21). To test whether cross-linking of B7-DC on murine DC with sHgM12 Ab resulted in skewing the immune response away from the Th2-homologous Th2 phenotype induced by immunization with the adjuvant alum, we examined the effect of sHgM12 Ab treatment during the initial immunization with OVA in the induced asthma-like condition in BALB/c mice. Mice were administered sHgM12 Ab daily before, the day of, and 1 day after immunization with OVA (Fig. 1A). This regimen of Ab treatment resulted in a significant reduction in airway responsiveness to methacholine challenge compared with the control Ab-treated mice. Mice treated with the sHgM12 B7-DC cross-linking Ab, however, were significantly protected from airway responsiveness to methacholine challenge relative to animals treated with isotype control Abs (e.g.,
The OVA model of allergic asthma is characterized by pulmonary inflammation reflected by a statistically significant increase in the number of total cells in the BAL, an increase in the number of eosinophils in the BAL, and perivascular and peribronchial cellular infiltrates in lung tissue sections (22). The number of total cells in the BAL fluid was significantly reduced in sHlgM12-treated mice (Fig. 2B, *p* = 0.013). Moreover, sHlgM12 treatment also resulted in prevention of eosinophils migrating to the lungs (Fig. 2C, *p* = 0.015). This failure to detect significant eosinophilic infiltrates correlated with the reduced levels of IL-5 found following treatment with sHlgM12 Ab (Fig. 2D, *p* = 0.008). IL-5 is a cytokine that plays a pivotal role in migration of eosinophils (23). Most striking was the finding that sHlgM12 treatment totally abrogated lung inflammation, the thickening of bronchial epithelium, and the accompanying accumulation of mucus plugs that were readily evident in mice treated with isotype control Abs (Fig. 3A and B).

**Figure 2.** Prophylactic effects of sHlgM12 B7-DC cross-linking Ab on OVA-induced AHR and inflammation. A, Responsiveness of mice to methacholine challenge. Mice that received either the isotype control polyclonal IgM Ab or the B7-DC cross-linking Ab sHlgM12 were challenged with increasing dosages of methacholine. AHR was measured by Penh, as described in Materials and Methods. Data are represented as means ± SEM (*n* = 10 per group). B, Cellular infiltration in lungs. Mice were sacrificed after measuring the AHR to methacholine. Cells present in BAL were counted. Data are represented as means ± SEM (*n* = 10/group). C, Eosinophilic infiltration in the lungs. The cells from the BAL fluid were stained with Wright-Giemsa for differential counts. Data are represented as the percentage of eosinophils with respect to the total number of cells counted. D, IL-5 in the BAL. The amount of IL-5 in the BAL was determined by ELISA. Data are represented as means ± SEM (*n* = 10/group).
mice. Similar to our findings with the prophylactic treatment regimen, there was no detectable eosinophilic infiltration in the mice treated therapeutically with sHIgM12 (Fig. 3J, p = 0.001).

To further characterize the T cell response pattern, the supernatants from the homogenized lungs of the various groups of mice were analyzed for the prototypic Th1 cytokine, IFN-γ, and the Th2 cytokine, IL-4. The mice that received sHIgM12 showed significantly reduced amounts of both IL-4 (Fig. 4D, p = 0.008) and IFN-γ (Fig. 4E, p = 0.016) in comparison with the control Ab-treated mice. Although IL-5 levels were not measured in this experiment, the complete absence of eosinophilia suggests that IL-5 levels were most likely low in the sHIgM12-treated animals. This cytokine pattern suggests that sHIgM12 treatment does not lead to a proinflammatory cytokine environment in the lungs by switching the polarity of the T cell response from Th2 to Th1, but rather blocks the development of either kind of T cell response. Immunohistochemistry analysis of lung tissue using CD3-specific Ab as a probe supported this conclusion. Few T cells were present in the lungs of naive animals (Fig. 5A). In contrast, lungs from mice treated with PBS or polyclonal IgM control Ab contained extensive T cell infiltrates (Fig. 5B and C). Remarkably, the lungs from sHIgM12-treated mice showed no signs of infiltration (Fig. 5D), resembling the lungs of untreated mice (Fig. 5A). In addition, there was no lung pathology in animals that received the therapeutic treatment protocol of B7-DC cross-linking Ab (Fig. 5F). In direct contrast, animals that were treated with PBS (Fig. 5D) or isotype control Ab (Fig. 5E) exhibited severe distortion of their bronchial architecture and substantial inflammatory infiltration.

Our therapeutic strategy was predicated on our early observations that B7-DC cross-linking Ab induces IL-12 production by DC in vivo (12). To determine whether the IL-12 signaling pathways are important for the in vivo therapeutic effects of sHIgM12 Ab treatment, we assessed the ability of Ab to modulate inflammatory airway disease in STAT4-deficient animals. STAT4 is a requisite intermediary that mediates IL-12 signaling (24). STAT4-deficient animals are known to develop highly polarized Th2 responses, as their ability to develop immune responses with Th1 character is severely compromised by the mutation (25, 26). The severity of induced airway inflammatory disease was substantially greater in STAT4-deficient animals (Fig. 3I) relative to wild-type mice (Fig. 3G). Therapeutic treatment of the STAT4-deficient animals with B7-DC cross-linking Ab had no effect (Fig. 3J), while in the same experiment wild-type mice were completely protected from airway inflammatory disease (Fig. 3H). This finding indicates that the ability to activate the STAT4 signaling pathway is critical for the treatment effect of sHIgM12 Ab and provides mechanistic evidence that IL-12 may be important for altering the polarity of the response by presensitized animals.

Treatment with sHIgM12 alters cytokine production in the spleens of allergen-presensitized mice

In the absence of inflammation in the lungs of sHIgM12-treated animals, we examined splenocytes from Ab-treated animals in vitro for the nature of their recall response to OVA challenge to determine whether the Th2 polarity characteristic of an allergic response was altered toward a Th1 polarity. Mice were treated with sHIgM12 Ab or isotype control Ab on days 13, 14, and 15 post-sensitization with OVA in alum adjuvant. The splenocytes were
harvested at day 28 and were restimulated in vitro with OVA. The proliferative response of T cells in response to Ag was enhanced 10-fold in mice that had received sHlgM12 treatment in comparison with the control Ab treatment (Fig. 6A). This finding is consistent with our previous observation that treatment of DC with our B7-DC cross-linking Ab enhances their ability to stimulate T cells (11). Furthermore, it demonstrates the potential of this Ab to stimulate cellular responses against isolated proteins, an observation that may have important implications for the development of vaccines. The supernatants from the stimulated cultures were harvested and tested for the presence of cytokines. Although mice that received sHlgM12 produced significantly higher levels of IFN-γ than mice treated with isotype control Ab, neither treatment group produced substantial levels (Fig. 6B, p = 0.008). The same trend was observed for the amount of TNF-α produced, in which splenocytes from mice treated with sHlgM12 secrete small amounts of TNF-α, while no TNF-α was detected in control Ab-treated splenocytes (Fig. 6C, p = 0.029). In contrast to these Th1 cytokines, the prototypic Th2 cytokines IL-4 and IL-5 were substantially lower in cultures from mice treated with sHlgM12 Ab. Splenic cultures from mice treated with sHlgM12 contained very small quantities of IL-4 (Fig. 6D, p = 0.004) or IL-5 (Fig. 6E, p = 0.048). These data indicate that sHlgM12 skews the T cell response toward a Th1 polarity, but that even the Th1 response remains weak, despite a strong proliferative response to secondary Ag challenge. The presence of substantial levels of IL-10 in secondary cultures pretreated with the B7-DC cross-linking Ab sHlgM12 (Fig. 6F, p = 0.008) might explain the absence of inflammation in the lungs of mice challenged intranasally with experimental allergen. Despite the ability of T cells in these animals to secrete IFN-γ and TNF-α, T regulatory cells might dampen this tendency and prevent them from tracking to the lungs. Taken together, these data support the notion that sHlgM12 treatment protects presensitized individuals from allergic airway inflammatory disease by the ability of the Ab to prevent a Th2 type of environment and also due to its ability to promote the secretion of the anti-inflammatory cytokine, IL-10.

Discussion

The incidence and severity of allergic airway inflammation are increasing nationally (28). The general improvement in hygiene during the last century may be responsible as the immune system seems to be also influenced by childhood infections. The hypothesis is that a lack of early infection during childhood leads to a predisposition later in life to develop immediate responses to inhaled environmental Ags (28). We recently described a novel approach for the potentiation of the immune response to Ag using a human mAb that targets to mature human DC (11). This treatment differs from conventional pulsed treatment with CD40L, LPS, or CpG-oligodeoxynucleotides used to activate DCs in that maturation phenotypes induced by up-regulation of CD80, CD86, and MHC molecules are not induced. Animals treated with conventional DC activation experience splenomegaly, while mice treated with sHlgM12 do not. Treatment of DCs with sHlgM12 Ab in vitro induces IL-12 and alters the migration pattern of DCs transplanted into mice (12). Remarkably, systemic treatment of mice with very small quantities (30 μg) of Ab can induce the same migration of DC to draining lymph nodes and potentiate Ag presentation of Ag-pulsed transplanted DC that reach the nodes (12).

The mechanism of DC activation by sHlgM12 Ab is not fully understood. We have shown that Ab binding to DC is dependent on expression of the costimulatory molecule B7-DC, and that B7-DC-binding ligands and B7-DC-specific IgG Abs can partially block the binding of the human mAb. Because monomers of the pentameric IgM Ab fail to activate, and in fact block, the activation by the native Ab, we have concluded that the cross-linking ability of the Ab is a critical feature of this reagent. We are now acquiring data (not described in this work) demonstrating conclusively that treatment of cultured DCs (both human and mouse) with sHlgM12 Ab activates specific signaling pathways and reproducibly up-regulates subsets of genes. These new findings tend to support the hypothesis that the systemic immunologic consequences of treatment with sHlgM12 are related to Ab-induced changes in resident DCs. Another, nonexclusive possibility is that Ab administered in vivo interferes with the interaction of B7-DC with a natural ligand. One such ligand is PD-1, a known negative regulator of T cell function (29). The possibility that additional undefined ligands exist is also plausible. Just how disruption of interactions between B7-DC and its ligands in vivo might influence the course of immunity is not known at present.

One possible explanation of the therapeutic effect of sHlgM12 is that B7-DC cross-linking by sHlgM12 Ab might lead to development of new sets of T cells that are skewed to secrete IFN-γ in response to IL-12 and IFN-γ secreted by the DC. This new lineage of T cells might then suppress the development of effector Th2
cells from the memory Th2 cell population, and, hence, IL-4 and IL-5 secretion in the vicinity of the lungs (30). The finding that STAT4-deficient mice are not responsive to shHgM12 Ab treatment is consistent with this hypothesis. However, the possibility that STAT4 is an important signaling intermediary for a B7-DC ligand also remains open to question.

A recent report documents the ability of bone marrow-derived DC to secrete IFN-γ in response to IL-12 and skew the T cell response toward a Th1 polarity (31). However, we have only detected very small increases in IFN-γ production in these mice. Spleen cells from Ab-treated mice display a profoundly altered cytokine secretion pattern, suggesting that systemic Ab treatment alters the outcome of subsequent intranasal Ag challenge. Remarkably, no inflammation in the lungs occurs. The up-regulation of IL-10 production by splenocytes suggests the possibility that systemic treatment with shHgM12 Ab may induce immunomodulatory regulatory T cells that suppress the effector responses in the lungs. A recent report demonstrates the ability of DCs to stimulate T regulatory cells in a costimulation-dependent fashion (32). The details of this kind of response still need to be established.

The possible involvement of PD-1, an inhibitory receptor for PD-L2 expressed by activated T cells, in immunomodulatory effects of shHgM12 Ab treatment is not known. It remains possible that administration of the IgM Ab blocks interactions between DC and T cells, altering the course of T cell activation. The view that the Ab acts directly on DC in vitro (12) (S. Radhakrishnan and L. R. Pease, unpublished observations). In a very recent study, activation of transcription (Stat)3 and Stat4.


A more recent study by Schipper et al. (2001) also showed that abrogates IFN-γ production in these mice. It is not clear if STAT4 is an important signaling intermediary for a B7-DC ligand. It remains open to question.

References

Although it remains to be seen whether systemic treatment of human patients with this Ab can alter the course of asthmatic responses, this is an exciting possibility.


Letter of Retraction


In the course of investigating suspicious patterns of experimental results in the laboratory, a systematic and in-depth study of key findings in this article was carried out using blinded protocols. In these repeat studies, no evidence was found to support our original conclusions that B7-DC XAb modulates dendritic cell functions. We do not believe our failure to reproduce our earlier findings is the result of a technical problem. A member of the B7-DC XAb investigative team, Dr. Suresh Radhakrishnan, who was involved in or had access to all the work on this subject, was found in a formal investigation to have engaged in scientific misconduct in unpublished experiments involving the B7-DC XAb reagent. This finding of misconduct and our inability to reproduce key findings using blinded protocols has undermined our confidence in our published report. We seek, therefore, to retract this body of work.

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