

Immunostimulatory DNA mediates inhibition of eosinophilic inflammation and airway hyperreactivity independent of natural killer cells in vivo

David H. Broide, MB, ChB, Greg Stachnick, BS, Diego Castaneda, BS,
Jyothi Nayar, BS, Marina Miller, MD, PhD, Jae Cho, MD, PhD,
Monica Rodriguez, BS, Mark Roman, PhD, and Eyal Raz, MD *San Diego, Calif*

Background: Immunostimulatory DNA sequences (ISS) inhibit eosinophilic inflammation and airway hyperreactivity in mouse models of asthma. In vitro ISS activate natural killer (NK) cells to secrete IFN- γ , and this cytokine is hypothesized to contribute to the antiallergic effect of ISS in vivo.

Objective: We investigated whether ISS activation of NK cells is important in mediating the reduction in airway hyperreactivity and the antieosinophilic effect of ISS in vivo.

Methods: We assessed whether ISS modulated the development of eosinophilic airway inflammation and airway hyperreactivity to methacholine in ovalbumin (OVA)-sensitized and OVA allergen-challenged mice pretreated with an antibody to deplete NK cells.

Results: Mice sensitized and challenged with OVA had significant bronchoalveolar lavage and lung eosinophilia, as well as airway hyperresponsiveness. ISS induced significant inhibition of bronchoalveolar lavage and lung eosinophilia, as well as airway hyperresponsiveness, in OVA-sensitized mice pretreated before OVA challenge with an NK cell-depleting antibody (NK⁻ mice), as well as in mice pretreated with a control non-NK cell-depleting antibody (NK⁺ mice). The NK cell-depleting antibody inhibited ISS-induced IFN- γ production by spleen cells.

Conclusion: These studies demonstrate that depletion of NK cells has no significant effect on ISS-mediated inhibition of airway eosinophilia and airway hyperresponsiveness in vivo, suggesting that non-NK cells and cytokines other than IFN- γ derived from NK cells mediate the majority of the ISS-inhibitory effect on eosinophilic inflammation and airway hyperresponsiveness in vivo. (*J Allergy Clin Immunol* 2001;108:759-63.)

Key words: Asthma, eosinophil, natural killer cell, IFN- γ

The ability of immunostimulatory DNA sequences (ISS) containing a CpG motif to inhibit allergen-induced eosinophilic airway inflammation and airway hyperreactivity

Abbreviations used

BAL: Bronchoalveolar lavage
ISS: Immunostimulatory DNA sequences
NK: Natural killer
ODN: Oligodeoxynucleotide
OVA: Ovalbumin
Penh: Enhanced pause

tivity in mouse models of asthma in vivo has been demonstrated in our¹⁻³ and other laboratories.⁴⁻⁷ The cellular mechanism of action of ISS in vivo is as yet not well understood. In vitro studies demonstrate that ISS activate cells of the innate immune system (natural killer [NK] cells, macrophages, and dendritic cells)⁸⁻¹² to produce cytokines, such as IFN- γ ^{8-10,12-16} and IL-12,^{10,13-15} which promote T_H1 responses to antigens. Indeed, the administration of ISS in vivo is associated with an induction of T_H1 (IFN- γ and IL-12) and an inhibition of T_H2 (IL-5 and IL-4) responses.¹⁻⁷ The downregulation of the T_H2 response is not due to a direct effect of ISS on T_H2 lymphocytes but rather to an indirect effect of ISS activating cells of the innate immune system to modulate T_H2 lymphocyte responses.¹

The importance of NK cells in mediating ISS-induced inhibition of allergic responses is suggested from in vitro studies demonstrating that ISS induce NK cells to express IFN- γ ,^{8-10,12} in vivo studies demonstrating that IFN- γ inhibits eosinophilic inflammation in mice,¹⁷⁻¹⁹ and recent studies demonstrating that CpG-containing ISS are not able to inhibit eosinophilic inflammation in IFN- γ -deficient mice,⁵ suggesting that IFN- γ (and, by implication, NK cells) is important in mediating the in vivo effect of ISS. Thus these studies suggest that in the absence of NK cells, ISS would not induce IFN- γ , and there would be no resultant inhibition of eosinophilic inflammation. Although these studies suggest an important role for NK cells and IFN- γ in mediating the inhibitory effect of ISS on eosinophilic inflammation in vivo, other studies with ISS and IFN- γ -deficient mice have not demonstrated a crucial role for IFN- γ in mediating the ISS effect in vivo.²⁰ In this study we have sought to determine the role of NK cells in mediating the

From the Department of Medicine, University of California, San Diego.
Supported by National Institutes of Health grants AI 33977 and AI 38425 to David H. Broide and AI 40682 to Eyal Raz.

Received for publication February 7, 2001; revised July 5, 2001; accepted for publication July 14, 2001.

Reprint requests: David H. Broide, MB, ChB, University of California, San Diego, 9500 Gilman Dr, La Jolla, CA 92093-0635.

Copyright © 2001 by Mosby, Inc.

0091-6749/2001 \$35.00 + 0 1/83/118795

doi:10.1067/mai.2001.118795

inhibitory effect of ISS on eosinophilic inflammation and airway hyperreactivity in vivo by using neutralizing antibodies to deplete NK cells in ISS-treated mice challenged with allergen.

METHODS

Oligonucleotides

Endotoxin-free (<1 ng/mg DNA) phosphorothioate ISS-oligodeoxynucleotide (ODN; 5'-TGACTGTGAACGTTTCGAGATGA-3') or phosphorothioate mutated ODN (M-ODN; 5'-TGACTGTGAAGGTTAGAGATGA-3'; Trilink), as previously described,¹ were used in the in vivo and in vitro experiments described below.

Mice

Female C57BL/6 mice (The Jackson Laboratory) were used at 8 to 10 weeks of age. All animal experimental protocols were approved by the University of California, San Diego, Animal Subjects Committee.

Mouse model of eosinophilic pulmonary inflammation

Pulmonary eosinophilia in mice was induced as previously described in this laboratory.^{21,22} In brief, C57BL/6 mice were sensitized by means of intraperitoneal injection of 50 µg of ovalbumin (OVA) per 1 mg of alhydrogel (Aldrich Chemical Co) in 0.9% sterile saline solution on days 0 and 12. Nonsensitized mice received 1 mg of alhydrogel in 0.9% saline. On day 24, mice (n = 4 mice per group) were exposed 3 times for 30 minutes (at 1-hour intervals) to an aerosol of OVA (10 mg/mL) in 0.9% saline; nonsensitized control mice received saline only. The aerosolized OVA protocol was then repeated on days 26, 28, and 30. Three hours after the last aeroallergen challenge, mice were killed by means of CO₂ asphyxiation.

Administration of ISS-ODN

ISS-ODN or M-ODN were injected intraperitoneally (100 µg in 100 µL of sterile, endotoxin-free PBS) once on day 23 before the first OVA inhalation challenge. We have previously demonstrated that this dose and timing of ISS administration inhibits subsequent OVA aerosol-induced eosinophilic inflammation.¹

Bronchoalveolar lavage eosinophils

Bronchoalveolar lavage (BAL) cells from mice were recovered by means of lavage with 1 mL of PBS administered through a tracheal catheter.¹ Total and differential leukocyte counts were performed after brief acetone fixation and staining of the BAL cells with May-Grünwald-Giemsa stains. The percentage of eosinophils, neutrophils, and mononuclear cells present on each slide were assessed by counting a minimum of 300 cells in random high-power fields with a light microscope (magnification 40×).

Lung tissue eosinophils

Total lung eosinophils per high-power field were enumerated in cryosectioned lung tissues by means of detection of eosinophil peroxidase with the peroxidase substrate DAB (Vector Labs) and microscopic examination, as previously described in this laboratory.¹

Peripheral blood eosinophils

Blood was collected from the carotid artery,¹ and red blood cells were lysed with a 1:10 solution of 100 mmol/L potassium carbonate and 1.5 mol/L ammonium chloride. The remaining cells were cytopun (3 minutes at 500 rpm) onto microscope slides and air-dried. Eosinophil counts were performed as described above.

Bone marrow eosinophils

Bone marrow cells were flushed from femurs with 1 mL of PBS, cytopun onto microscope slides, and stained with Wright-Giemsa or DAB for cell differential counts.¹

Determination of airway responsiveness to methacholine

Airway responsiveness was assessed on day 30 after the final OVA inhalation by using a single-chamber whole-body plethysmograph obtained from Buxco, as previously described.^{1,22} From the box-pressure signals, the phases of the respiratory cycle, and tidal volumes, enhanced pause (Penh) can be calculated. Penh represents a function of the proportion of maximal expiratory to maximal inspiratory box-pressure signals and the timing of respiration. Studies have found a close correlation between Penh as a noninvasive measure of airway responsiveness and pulmonary resistance measured by means of conventional 2-chamber plethysmography in ventilated mice.²³ The Penh values measured during each 3-minute sequence were averaged and are expressed for each concentration of nebulized methacholine. To determine the effect of ISS and NK-cell depletion on airway responsiveness in OVA-sensitized mice, 100 µg of either ISS-ODN or M-ODN was injected intraperitoneally on day 23, twenty-four hours after the mice had received the first dose of the NK cell-depleting or control antibody on day 22.

In vivo depletion of NK cells

Mice were depleted of NK cells by using an anti-NK cell IgG_{2a} antibody PK136 (Pharmigen) or a species- and isotype-matched control antibody (30 µg/kg administered intravenously on day 22 and repeated on day 26). This anti-NK cell antibody protocol has been shown to deplete greater than 95% of NK cells for at least 4 days after a single administration of antibody.²⁴

FACS analysis of NK cells

Spleen cells from NK cell-depleted (NK⁻) or control non-NK cell-depleted (NK⁺) mice were incubated for 30 minutes with the anti-NK cell primary IgM antibody 3A4 (10 µg/mL, Pharmigen) or a species- and isotype-matched control antibody. The cells were then washed and incubated with an FITC-labeled secondary antibody. The percentage of NK cells was assessed with a FACSCalibur instrument (Becton Dickinson) with Cell Quest software. A minimum of 10,000 cells were analyzed for each determination.

Cytokine assays

Splenocytes (5×10^6 /mL) from mice (treated in vivo with the anti-NK cell antibody PK136 or a species- and isotype-matched IgG_{2a} antibody) were incubated in vitro with ISS (10 µg/mL) at 37°C for 2 hours. The ISS-stimulated NK⁺ and NK⁻ spleen cells were then incubated (5×10^5 cells/well) in triplicate for an additional 48 hours. Spleenocyte supernatants (48 hours after stimulation) were assayed in duplicate to determine the level of IFN-γ by using an ELISA with a sensitivity of 2 pg/mL (Pharmigen).

Statistics

Results in different groups were compared by means of ANOVA with the nonparametric Kruskal-Wallis test, followed by posttesting with the Dunn multiple comparison of means. A statistical software package (In Stat) was used for the analysis. *P* values of less than .05 were considered statistically significant. All results are given as means ± SEM.

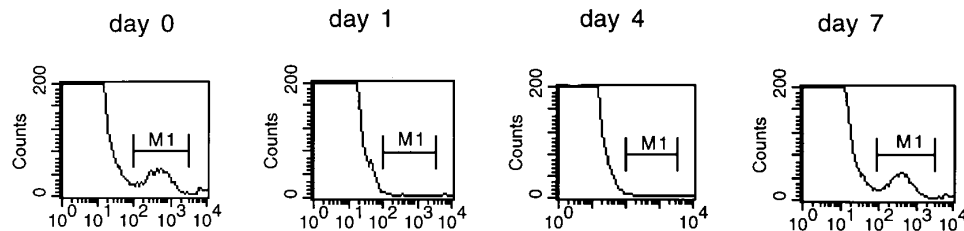


FIG 1. NK-cell FACS analysis. The number of NK cells present in mouse spleen before (day 0) and at varying time points after (days 1, 4, and 7) administration of an NK cell-depleting antibody was assessed by means of FACS analysis gating for NK cells (M1).

RESULTS

Depletion of NK cells in vivo

FACS studies demonstrated greater than 95% depletion of NK cells by 24 hours after administration of the NK cell-depleting antibody (5.20% NK cells before antibody administration vs 0.26% NK cells after antibody administration; Fig 1), with depletion of NK cells persisting for at least 4 days (0.25% NK cells at day 4 after antibody administration) and NK levels returning to pre-depletion levels by day 7 (5.84% NK cells at day 7 after antibody administration). Because we needed to ensure depletion of NK cells for the final 6 days of the ISS experiment, we administered 2 doses of the anti-NK cell antibody 4 days apart. A control IgG_{2a} antibody did not deplete NK cells.

We also performed a functional analysis of the ability of NK cells from NK⁺ mice and NK⁻ mice to generate IFN- γ . Spleen cells, which contain NK cells, generate IFN- γ in vitro in response to ISS stimulation in the absence of antigen stimulation. NK-cell depletion of spleen cells inhibited the ability of spleen cells to generate IFN- γ in vitro in response to ISS (NK⁺ cells, 260 ± 20 pg/mL IFN- γ , vs NK⁻ cells, <2 pg/mL IFN- γ [the sensitivity of the assay]; $P < .01$; $n = 4$). Control NK cells in spleens that had received neither ISS nor anti-NK antibody do not generate detectable levels of IFN- γ (<2 pg/mL). The demonstration that NK cell-depleted mouse spleen cells do not generate IFN- γ in response to ISS provides additional evidence that we have adequately depleted NK cells in vivo.

Effect of depletion of NK cells on ISS-mediated inhibition of eosinophilic airway inflammation

Sensitization and OVA-allergen challenge of wild-type mice ($n = 5$) induced significant BAL eosinophilia ($68.8\% \pm 3.7\%$ BAL eosinophils) compared with that seen in mice not sensitized or challenged with OVA ($0.6\% \pm 0.1\%$ BAL eosinophils, $P < .001$) or compared with mice immunized with OVA and challenged with PBS diluent ($1.5\% \pm 0.4\%$ BAL eosinophils, $P < .001$). As previously demonstrated,¹ ISS significantly inhibited pulmonary eosinophilia in OVA-challenged mice by 85% ($P < .001$, $n = 5$; Fig 2). ISS treatment inhibited BAL eosinophilia as effectively in NK⁻ mice (84% inhibition vs non-ISS-treated OVA-challenged mice, $P < .001$) as

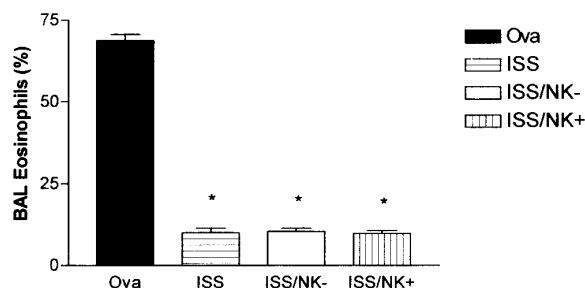


FIG 2. Effect of ISS and NK-cell depletion on BAL eosinophils. There was a significant inhibition in the number of BAL eosinophils in OVA-challenged mice that received ISS, as well as OVA-challenged mice that received ISS plus NK-depleting (ISS/NK⁻) or control (ISS/NK⁺) antibody ($n = 5$). * $P < .001$ versus OVA-challenged mice.

in NK⁺ mice immunized and challenged with OVA (86% inhibition vs non-ISS-treated OVA-challenged mice; $P < .001$; $n = 5$; Fig 2).

Analysis of the number of lung tissue eosinophils also demonstrated that NK-cell depletion had minimal influence on the inhibitory effect of ISS on lung tissue eosinophils. Sensitization and OVA allergen challenge of wild-type mice ($n = 5$) induced significant lung eosinophilia (82.3 ± 8.3 lung eosinophils per high-power field) compared with that seen in mice not sensitized to or challenged with OVA (2.4 ± 0.7 lung eosinophils per high-power field, $P < .001$) or compared with mice immunized with OVA and challenged with PBS diluent (5.3 ± 1.1 lung eosinophils per high-power field, $P < .001$). ISS treatment inhibited lung tissue eosinophilia as effectively in NK⁻ mice (75% inhibition vs non-ISS-treated OVA-challenged mice, $P < .001$) as in NK⁺ mice immunized and challenged with OVA (72% inhibition vs non-ISS-treated OVA-challenged mice, $P < .001$).

Effect of depletion of NK cells on ISS-mediated inhibition of blood and bone marrow eosinophilia

We have previously demonstrated that ISS administration to OVA-challenged mice inhibits trafficking of eosinophils to the lung by inhibiting the bone marrow production of eosinophils, as well as the number of peripheral blood eosinophils.¹ ISS treatment inhibited

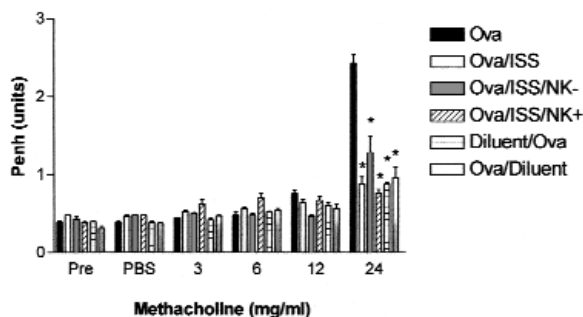


FIG 3. Effect of NK-cell depletion on ISS-mediated inhibition of airway hyperreactivity to methacholine. There was a significant inhibition in methacholine responsiveness (24 mg/mL) in OVA-challenged mice that had received ISS, as well as in OVA-challenged mice that received ISS plus NK-depleting (ISS/NK⁻) or control (ISS/NK⁺) antibody (n = 5). * $P < .001$ versus OVA-challenged mice.

bone marrow eosinophilia as effectively in NK⁻ mice (52% inhibition vs non-ISS-treated OVA-challenged mice, $P < .01$) as in NK⁺ mice immunized and challenged with OVA (59% inhibition vs non-ISS-treated OVA-challenged mice, $P < .01$).

Similarly, ISS treatment inhibited blood eosinophilia as effectively in NK⁻ mice (74% inhibition vs non-ISS-treated OVA-challenged mice, $P < .01$) as in NK⁺ mice immunized and challenged with OVA (75% inhibition vs non-ISS-treated OVA-challenged mice, $P < .01$).

Effect of depletion of NK cells on ISS-mediated inhibition of airway hyperreactivity to methacholine

Airway responsiveness to methacholine was significantly increased in mice after OVA sensitization and OVA challenge. Mice sensitized to OVA without inhalation challenge or mice challenged with OVA without sensitization showed minimal change in Penh in response to methacholine (Fig 3). As previously reported, ISS significantly inhibited airway responsiveness to methacholine in OVA-sensitized and OVA-challenged mice at the 24 mg/mL methacholine dose by 66% (Fig 3). NK-cell depletion had no influence on the inhibitory effect of ISS on airway responsiveness to concentrations of 3, 6, and 12 mg/mL methacholine but did have a slight, although not statistically significant, effect at the 24 mg/mL methacholine concentration (Fig 3). Thus at 24 mg/mL methacholine, ISS treatment inhibited airway responsiveness in NK⁻ mice by 49% compared with 66% inhibition in NK⁺ mice immunized and challenged with OVA (Fig 3).

DISCUSSION

NK cells are cytotoxic, T-cell receptor-negative, surface Ig-negative, large granular lymphocytes that comprise approximately 5% to 15% of peripheral blood lymphocytes and about 5% of spleen cells.²⁵ NK cells have the capacity to produce several cytokines, including IFN- γ .^{8-10,12} In primary cell-mediated immune responses, NK

cells would be the first to respond to IL-12 by producing IFN- γ (before antigen-activated T lymphocytes produce IFN- γ).²⁴ We¹ and others⁴⁻⁶ have postulated that this early production of IFN- γ by NK cells would account for the early ability of ISS to inhibit eosinophilic inflammation within 2 days of ISS administration. In vitro studies have demonstrated that ISS induction of IFN- γ by NK cells is indirect and dependent on ISS induction of macrophages to generate IL-12, which subsequently activates NK cells to generate IFN- γ .¹⁵ Highly purified NK cells do not generate IFN- γ in response to ISS, whereas addition of macrophages to the NK cells restores the ability of NK cells to generate IFN- γ in response to ISS.¹⁵ Anti-IL-12 or anti-IL-10 treatment of NK cell-macrophage cocultures inhibits NK-cell IFN- γ generation.¹⁵

On the basis of these in vitro studies, we hypothesized that NK cells would be important in mediating the in vivo effect of ISS. However, depletion of NK cells in vivo did not affect the ability of ISS to inhibit eosinophilic inflammation and airway hyperreactivity. These studies suggest that although ISS can activate NK cells to generate IFN- γ , this is not the predominant cellular mechanism of the antieosinophilic action of ISS in vivo. ISS can also activate macrophages and dendritic cells, which may play a more important role compared with NK cells in mediating the in vivo effects of ISS. For example, recent studies have demonstrated an important role for IL-6 in inhibiting eosinophilic inflammation and airway hyperreactivity.²⁶ ISS are important stimuli for IL-6 production by macrophages,^{27,28} which could mediate this antieosinophilic effect independent of NK cells. Similarly, studies have demonstrated that IL-12 (a cytokine induced by ISS-activated macrophages)^{10,13-15} can inhibit allergen-induced eosinophil influx^{29,30} in both an IFN- γ -dependent and an IFN- γ -independent pathway.²⁹ Overall, this study suggests that NK cells and NK cell-derived cytokines play only a minor in vivo role in mediating the antieosinophilic effect of ISS.

One potential technical concern in interpreting our studies of depleting NK cells in vivo is the demonstration that NK cells play an important role in mediating eosinophilic inflammation in mouse models of asthma.^{31,32} However, these studies demonstrate that NK-cell depletion only affects the level of eosinophilic inflammation if NK cells are depleted during the immunization phase.³¹ Although NK-cell depletion during the immunization phase inhibits OVA-specific IgE responses,³¹ this effect on IgE is unlikely to influence airway hyperresponsiveness because airway hyperresponsiveness has been shown to be independent of IgE in mice.^{33,34} In contrast, NK-cell depletion during the challenge period (as in this study) has no effect on the magnitude of pulmonary eosinophilia, OVA-specific IgE, and cytokines in BAL fluid.³¹ Thus our timing of the depletion of NK cells (after the immunization phase and during the challenge period) would not affect the important influence of NK cells on the acquired immune response to allergens.³¹

The cellular mechanism of action of ISS in vivo may thus be more complex than that initially hypothesized on

the basis of results from in vitro studies. Although ISS induce a strong T_H1 response, the antiallergic effect of ISS may not be mediated by this mechanism in vivo. In support of this hypothesis are studies demonstrating that adoptive transfer of antigen-specific T_H1 cells does not protect against eosinophilic airway inflammation in a mouse model of asthma^{35,36} and studies demonstrating that ISS can mediate their antiallergic effects in vivo, independent of the T_H1 cytokines IFN- γ and IL-12.²⁰ Thus the ability of ISS to induce a T_H1 response may not be essential to their inhibitory effects on allergic inflammation.

In summary, this study has demonstrated that ISS activation of NK cells does not play a significant role in mediating the beneficial effect of ISS on airway eosinophilic inflammation and airway hyperreactivity. Further studies are needed to define the cellular pathway or pathways activated by ISS that mediate the majority of the antiallergic effect in vivo.

We thank Lanesha Hill for expert secretarial support during the preparation of this manuscript.

REFERENCES

- Broide D, Schwarze J, Tighe H, Gifford T, Nguyen MD, Malek S, et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 1998;161:7054-62.
- Broide D, Raz E. DNA-Based immunization for asthma. *Int Arch Allergy Immunol* 1999;118:453-6.
- Broide D, Stachnick G, Castaneda D, Nayar J, Miller M, Cho JY, et al. Systemic administration of immunostimulatory DNA sequences mediate reversible inhibition of Th2 responses in a mouse model of asthma. *J Clin Immunol* 2001;21:163-70.
- Kline JN, Waldschmidt TJ, Businga TR, Lemish JE, Weinstock JV, Thorne PS, et al. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 1998;160:2555-9.
- Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 1999;162:6284-93.
- Shirotta H, Sano K, Kikuchi T, Tamura G, Shirato K. Regulation of murine airway eosinophilia and Th2 cells by antigen-conjugated CpG oligodeoxynucleotides as a novel antigen-specific immunomodulator. *J Immunol* 2000;164:5575-82.
- Serebinsky D, Teper AA, Huang C-K, Lee S-Y, Zhang T-F, Schofield BH, et al. CpG oligonucleotides can reverse Th2 associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. *J Immunol* 2000;165:5906-12.
- Tokunaga T, Yano O, Kuramoto E, Kimura Y, Yamamoto T, Kataoka T, et al. Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. *Microbiol Immunol* 1992;36:55-66.
- Ballas ZK, Rasmussen WL, Krieg AM. Induction of natural killer activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol* 1996;157:1840-5.
- Chace JH, Hooker NA, Mildenstein KL, Krieg AM, Cowdery JS. Bacterial DNA-induced NK cell IFN- γ production is dependent on macrophage secretion of IL-12. *Clin Immunol Immunopath* 1997;84:185-93.
- Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995;374:546-9.
- Yamamoto S, Yamamoto T, Kataoka T, Kuramoto E, Yano O, Tokunaga T. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J Immunol* 1992;148:4072-6.
- Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med* 1997;3:849-54.
- Sato YM, Tighe H, Lee D, Corr M, Nguyen M-D, Silverman GJ, et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 1996;273:352-4.
- Halpern MD, Kurlander R, Pisetsky DS. Bacterial DNA induces murine interferon- γ production by stimulation of interleukin-12 and tumor necrosis factor- α . *Cell Immunol* 1996;167:72-8.
- Yamamoto T, Yamamoto S, Kataoka T, Komuro K, Kohase M, Tokunaga T. Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes in vitro. *Jpn J Cancer Res* 1994;85:775-9.
- Nakajima H, Iwamoto I, Yoshida S. Aerosolized recombinant interferon- γ prevents antigen-induced eosinophil recruitment in mouse trachea. *Am Rev Respir Dis* 1993;148:1102-4.
- Iwamoto I, Nakajima H, Endo H, Yoshida S. Interferon γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4+ T cells. *J Exp Med* 1993;177:573-6.
- Li XM, Chopra RK, Chou TY, Schofield BH, Wills-Karp M, Huang SK. Mucosa IFN- γ gene transfer inhibits pulmonary allergic responses in mice. *J Immunol* 1996;157:3216-9.
- Kline JN, Krieg AM, Waldschmidt TJ, Ballas ZK, Jain V, Businga TR. CpG oligodeoxynucleotides do not require Th1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. *J Allergy Clin Immunol* 1999;104:1258-64.
- Broide DH, Campbell K, Gifford T, Sriramarao P. Inhibition of eosinophilic inflammation in allergen-challenged, IL-1 receptor type 1-deficient mice is associated with reduced eosinophil rolling and adhesion on vascular endothelium. *Blood* 2000;95:263-9.
- Broide DH, Stachnick G, Castaneda D, Nayar J, Sriramarao P. Inhibition of eosinophilic inflammation in allergen challenged TNF receptor p55/p75 and TNF receptor p55 deficient mice. *Am J Respir Cell Mol Biol* 2001;24:303-11.
- Hamelman E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Gelfand EW. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156:766-75.
- Wang M, Ellison CA, Gartner JG, HayGlass KT. Natural killer cell depletion fails to influence initial CD4 T cell commitment in vivo in exogenous antigen-stimulated cytokine and antibody responses. *J Immunol* 1998;160:1098-105.
- Trinchieri G. Natural killer cells wear different hats: effector cells of innate resistance and regulatory cells of adaptive immunity and of hematopoiesis. *Semin Immunol* 1995;7:83-8.
- Wang J, Homer RJ, Chen Q, Elias JA. Endogenous and exogenous IL-6 inhibit aeroallergen induced Th2 inflammation. *J Immunol* 2000;165:4051-61.
- Yi AK, Klinman DM, Martin TL, Matson S, Krieg AM. Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J Immunol* 1996;157:5394-402.
- VanHeyningen TK, Collins HL, Russell DG. IL-6 produced by macrophages infected with *Mycobacterium* species suppresses T cell responses. *J Immunol* 1997;158:330-7.
- Brusselle GG, Kips JC, Peleman RA, Joos GF, Devos RR, Tavernier JH, et al. Role of interferon- γ in the inhibition of the allergic airway inflammation caused by IL-12. *Am J Respir Cell Mol Biol* 1997;17:767-71.
- Keane-Myers A, Wysocka M, Trinchieri G, Wills-Karp M. Resistance to antigen induced airway hyperresponsiveness requires endogenous production of IL-12. *J Immunol* 1998;161:919-26.
- Korsgren M, Persson CGA, Sundler F, Bjerke T, Hansson T, Chambers BJ, et al. Natural killer cells determine development of allergen induced eosinophilic airway inflammation in mice. *J Exp Med* 1999;189:553-62.
- Walker C, Checkel J, Cammisuli S, Leibson PJ, Gleich GJ. IL-5 production by NK cells contributes to eosinophil infiltration in a mouse model of allergic inflammation. *J Immunol* 1998;161:1962-9.
- Hamelmann E, Takeda K, Haczku A, Cieslewicz G, Shultz L, Hamid Q, et al. Interleukin (IL)-5 but not immunoglobulin E reconstitutes airway inflammation and airway hyperresponsiveness in IL-4-deficient mice. *Am J Respir Cell Mol Biol* 2000;23:327-34.
- Hamelmann E, Takeda K, Schwarze J, Vella AT, Irvin CG, Gelfand EW. Development of eosinophilic airway inflammation and airway hyperresponsiveness requires interleukin-5 but not immunoglobulin E or B lymphocytes. *Am J Respir Cell Mol Biol* 1999;21:480-9.
- Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 1999;103:175-83.
- Randolph DA, Stephens R, Carruthers CJ, Chaplin DD. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J Clin Invest* 1999;104:1021-9.