

Topical Application of Cream-emulsified CD86 siRNA Ameliorates Allergic Skin Disease by Targeting Cutaneous Dendritic Cells

Patcharee Ritprajak¹, Masaaki Hashiguchi¹ and Miyuki Azuma¹

¹Department of Molecular Immunology, Graduate School, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan

Induction of the co-stimulatory molecule CD86 on dendritic cells (DCs) in the peripheral tissues is a critical event in triggering antigen-specific immune responses. In this study, we propose a new small interfering RNA (siRNA)-based therapy using cream-emulsified CD86 siRNA, targeting DCs for murine contact hypersensitivity (CH) and atopic dermatitis (AD)-like disease. Topical application of CD86 siRNA efficiently inhibited CH and markedly decreased the numbers of infiltrating CD86⁺ or major histocompatibility complex (MHC) class II⁺ cells in murine ear skin. The total number of cells, the percentage of hapten-carrying DCs, and their CD86 expression in the regional lymph nodes (RLNs) also significantly decreased. These results suggest that the silencing of CD86 in local DCs inhibits the recruitment and migration of DCs into the skin and RLNs, respectively, resulting in reduced antigen-specific local inflammation. The therapeutic efficacy of the CD86 siRNA was confirmed in AD-prone NC/Nga mice. Treatment produced marked amelioration in the clinical manifestations of AD and reduced the antigen-specific production of interleukin-4 (IL-4) and serum immunoglobulin E (IgE) and IgG1. Our results suggest that the targeting of cutaneous DCs by CD86 siRNA may be a promising strategy in the treatment of allergic skin disease.

Received 5 December 2007; accepted 7 April 2008; advance online publication 6 May 2008. doi:10.1038/mt.2008.91

INTRODUCTION

The outcome of interactions between naive T cells and antigen-carrying dendritic cells (DCs) in secondary lymphoid organs depends on the maturation and activation status of the DC. The array of co-signaling molecules expressed on DCs greatly affects their function and the type of immune response induced.¹ CD86 is a crucial co-stimulatory ligand on DCs, and blockade of the CD86 pathway interferes with antigen-specific T-cell responses in murine Th2-mediated allergic disease models.^{2–4} CD86 shares two functionally opposing receptors, CD28 and CD152, with another ligand, CD80. CD86 is induced earlier and expressed at higher levels on antigen-presenting cells than CD80 is; however,

CD80 binds CD152 with a higher affinity than CD86 does.⁵ The differential expression patterns and binding affinities of CD80 and CD86 result in variability between the two receptors in controlling immune responses. Nevertheless, the CD86–CD28 co-stimulatory pathway seems to contribute dominantly to the activation of antigen-specific T cells by antigen-presenting cells,⁶ although CD80 is able to compensate the function of CD86 in conditions wherein CD86 is lacking.⁷ Preferential involvement of CD86 expression and function in skin inflammatory allergic disease has been reported in humans^{8–10} and in mice.^{11–16} Specifically, blockade of the CD86 pathway interferes with antigen-specific T-cell responses in murine Th2-mediated allergic disease models.^{2–4}

RNA interference is a naturally occurring phenomenon of RNA-mediated gene silencing, and is highly conserved among multicellular organisms mediated by small-interfering double-stranded RNAs.^{17–19} RNA interference is a powerful method for silencing specific genes and has great potential for therapeutic application.²⁰ However, the limited stability of double-stranded RNA and the absence of a reliable delivery method are major hurdles in the development of RNA interference-based therapeutics.

In this study, we report the development of a small interfering RNA (siRNA)-based therapy for allergic skin disease, targeting CD86, based on three unique features of DCs: (i) cutaneous DCs are found in the outer layers of the skin and therefore a special delivery system may not be required; (ii) CD86 is induced on cutaneous DCs *in situ* by exposure to antigen, and therefore it may be a suitable target for gene silencing; and (iii) DCs migrate from the periphery to the regional lymph nodes (RLNs), and therefore siRNA-targeted DCs may modulate immune responses in the RLNs without systemic delivery of the siRNA.

RESULTS

Efficacy of CD86 gene silencing *in vitro*

We obtained six different siRNAs and examined their gene-silencing efficiencies by co-transfecting the CD86 expression vector and each siRNA into 293T cells. The cell surface expression of CD86 in all of the transfected cells with #1–6 of the CD86 siRNA duplex showed obvious decreases when compared with the control siRNA (Figure 1a). Among these siRNAs, #6 CD86 siRNA showed almost complete inhibition of CD86 expression. Next we

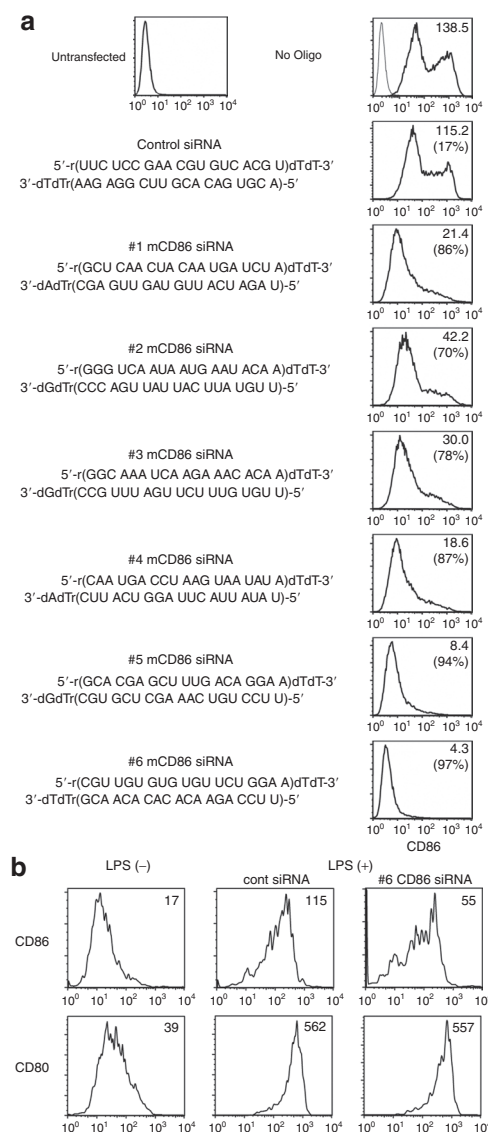


Figure 1 Silencing of CD86 by co-transfection with CD86 small interfering RNA (siRNA) *in vitro*. **(a)** 293T cells were co-transfected with the CD86 expression vector and either the nonsilencing control or CD86 siRNA oligos (#1–6). After 48 hours, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD86 monoclonal antibody (mAb), and analyzed using flow cytometry. The mean fluorescence intensity (MFI) values are shown in the upper right corners. The percentage reduction in CD86 expression was calculated using the following formula: $100 - (\text{MFI of No-oligo sample} - \text{MFI of siRNA sample}) / \text{MFI of No-oligo sample} \times 100$. The sequences of the nonsilencing control and CD86 siRNA oligos (#1–6) are shown. **(b)** Immature bone marrow-derived dendritic cells (BM-DCs) were transfected with either control or #6 CD86 siRNA oligo in the presence of lipopolysaccharide (LPS). After 24 hours, the cells were stained with FITC anti-CD86 mAb, phycoerythrin anti-CD80 mAb, allophycocyanin anti-CD11c mAb, and biotinylated anti-MHC class II mAb, followed by PerCP-Cy5.5-streptavidin. An electronic gate was placed on the CD11c^{high}MHC class II^{high} cells and the expression levels of CD80 and CD86 were displayed as histograms. The MFI values are shown in the upper right corners. MHC, major histocompatibility complex.

confirmed the CD86 gene silencing effect using bone marrow-derived DCs (BM-DCs). The mean fluorescence intensity of CD86 on mature BM-DCs, which are identified as CD11c^{high} major histocompatibility complex (MHC) class II^{high}, was markedly decreased

by the #6 CD86 siRNA, whereas the induced expressions of other B7 family molecules, namely, CD80 (**Figure 1b**), B7-DC, and B7-H1, an adhesion molecule CD54, and a potent DC-derived cytokine interleukin-12 (IL-12) (data not shown) were not affected. These data suggest that the #6 CD86 siRNA induces CD86-specific gene silencing in DCs *in vitro*.

Topical application of CD86 siRNA at the elicitation phase inhibits contact hypersensitivity

We have demonstrated earlier that treatment with an anti-CD86 monoclonal antibody (mAb) at the time of sensitization dramatically inhibits contact hypersensitivity (CH), while a similar treatment at the time of challenge results in partial inhibition.¹² In addition, CD86 was induced on DCs in the dermis and draining LNs 24 hours after challenge.¹² In order to develop an siRNA-based treatment, we investigated the effects of CD86 siRNA on the elicitation phase of CH. Antisense oligonucleotides formulated as cream-based ointments have been shown to accumulate in the epidermis and dermis;^{21,22} Atelocollagen/siRNA complex has earlier been demonstrated to be quite stable and capable of being efficiently transduced into cells.²³ We tested both methods, and the cream-based ointment proved to be more effective (**Figure 2a**). We therefore decided to use cream-emulsified siRNA as topical application. We did not observe any differences in results between the group treated with cream-based ointment alone and the one treated with cream-emulsified nonsilencing control siRNA (**Figure 2b**). However, treatment with 2.5 μg of #6 CD86 siRNA significantly reduced ear swelling 24 and 48 hours after the challenge, whereas similar treatment with #5 CD86 siRNA did not show a clear effect. The titration experiments revealed that a higher dose (5 μg) of #5 siRNA did inhibit the ear swelling, and that the effects of both #5 and #6 siRNA were dose-dependent (**Figure 2c**). We therefore decided to use #6 siRNA in the subsequent studies. Next, we evaluated the efficiency of CD86 siRNA by comparing the results with those of mAb treatment. The degree of swelling observed after intraperitoneal injection of an anti-CD86 mAb was similar to that observed after topical treatment with #6 CD86 siRNA (**Figure 2d**). These results demonstrate that siRNA targeting of CD86 efficiently inhibits hapten-induced skin inflammation *in vivo*.

CD86 siRNA inhibits infiltration of CD86⁺ and MHC class II⁺ cells

In order to further investigate the effects of CD86 siRNA, we performed immunohistological analyses of ear skin 24 hours after challenge. CD86 was barely detectable in the epidermis and only marginally detectable in the dermis of intact ear skin (**Figure 3a**). This is consistent with our earlier observations.¹² In contrast, in the control siRNA-treated ear, CD86⁺ cells were abundant in the epidermis and even more so in the dermis, and the majority of infiltrating cells expressed high levels of CD86 (**Figure 3b**). Conversely, there was an obvious reduction in edema and cellular infiltration in CD86 siRNA-treated ear skin (**Figure 3c and f**), as were the number of CD86⁺ dermal infiltrating cells and the expression levels of CD86 (**Figure 3c**). Similar results were obtained by staining with anti-MHC class II mAb (**Figure 3d–f**). When challenged, a number of MHC class II⁺ cells infiltrated the

control siRNA-treated ear, whereas markedly fewer of these cells were recruited to the dermis. Furthermore, these cells expressed less of MHC class II. Most of the CD86⁺ and MHC class II⁺ infiltrating cells appeared to be DCs, as judged on the basis of their location and morphology, thereby indicating that the CD86 siRNA efficiently inhibits hapten-induced skin inflammation by reducing both the recruitment of cutaneous DCs and the expression of CD86 and MHC class II.

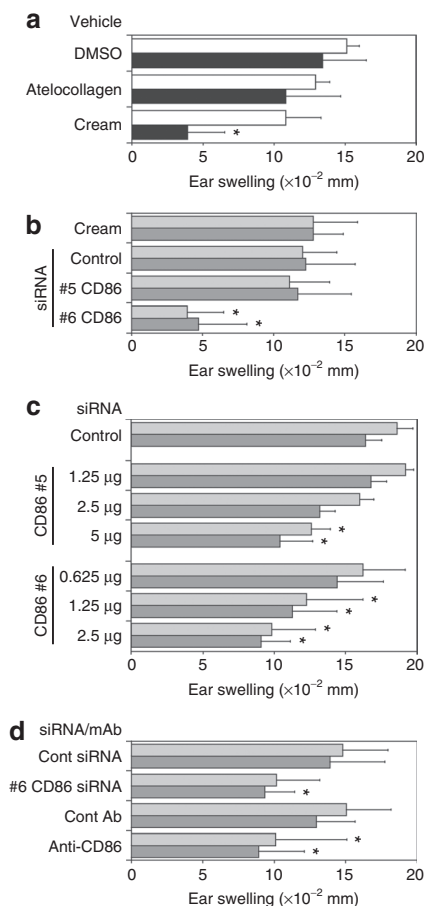


Figure 2 Efficient suppression of contact hypersensitivity (CH) by topical application of cream-emulsified CD86 small interfering RNA (siRNA). CH against dinitrofluorobenzene (DNFB) was induced as described in Methods. On day 5, each siRNA oligo was applied topically to the skin of the right ear; 1 hour later, the same ear was challenged with DNFB. Differences in ear thickness before and after the challenge were recorded. **(a)** Nonsilencing control (open bars) or #6 CD86 siRNA (closed bars) mixed with dimethyl sulfoxide (DMSO), atelocollagen, or cream-based ointment was applied. Ear swelling was measured 24 hours after the challenge. Each group consisted of five mice. **(b)** Nonsilencing control, #5 or #6 CD86 siRNA (2.5 μ g) mixed with a cream-based ointment was applied. As a control, the cream-based ointment was mixed with sterile water. Each group consisted of five or ten mice. The data shown are representative results from two or three experiments. **(c)** Graded doses of #5 and #6 CD86 siRNA mixed with cream-based ointment were applied. **(d)** Nonsilencing control or #6 CD86 siRNA (2.5 μ g) mixed with cream-based ointment was applied. Either control rat IgG or anti-CD86 monoclonal antibody (mAb) (200 μ g/mouse) was administered intraperitoneally 2 hours before the challenge. The values shown are the mean $\pm$ SD for the differences in ear thickness before, 24 hours after (light gray), and 48 hours after (dark gray) the challenge. Values statistically different from the nonsilencing control siRNA or control IgG group are noted (* $P < 0.05$).

CD86 siRNA inhibits induction of CD86 in Langerhans cells and dermal DCs at an earlier time-point

We next examined the ear skin at an earlier time-point (4 hours after the challenge). The number of infiltrating cells in the dermis was clearly increased in the control siRNA-painted ear skin, but the infiltrating cells were reduced in the CD86 siRNA-painted ear skin (**Figure 4**). CD86 expression in both the epidermis and the dermis was clearly upregulated following hapten challenge in the control siRNA-painted skin (**Figure 4b**). However, the upregulation of CD86 was markedly suppressed in the CD86 siRNA-painted ear (**Figure 4c**). We could not distinguish differences in either MHC class II expression or ear swelling at this time-point (data not shown). Six hours after the hapten challenge, differences in CD86 expression and CD86⁺ cell numbers were more obvious in the skin of the ear, and clear differences in MHC class II expression and MHC class II⁺ cell numbers were observed in the dermis (data not shown). We performed quantitative real-time PCR analyses for CD86, CCR7 (a LN-homing chemokine receptor

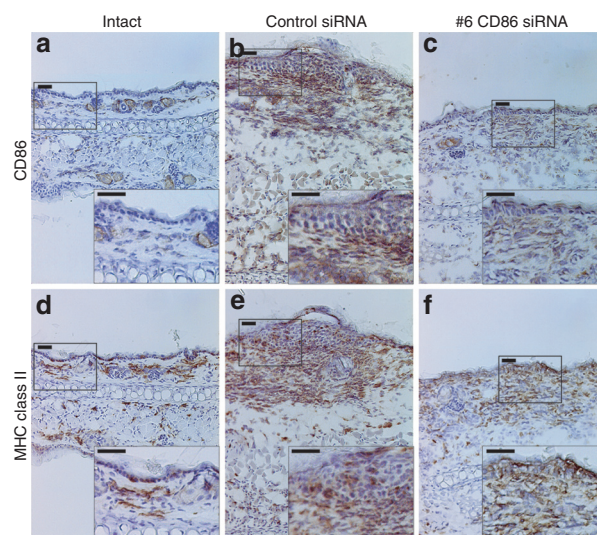


Figure 3 Reduced CD86⁺ and major histocompatibility complex (MHC) class II⁺ cells in the CD86 small interfering RNA (siRNA)-painted ear skin. Ear tissues from **(a, d)** intact mice, and from sensitized and challenged mice treated with either **(b, e)** nonsilencing control or **(c, f)** #6 CD86 siRNA 24 hours after challenge, were stained with **(a, b, c)** anti-CD86 monoclonal antibody (mAb) or **(d, e, f)** anti-MHC class II mAb. Bars = 25 μ m.

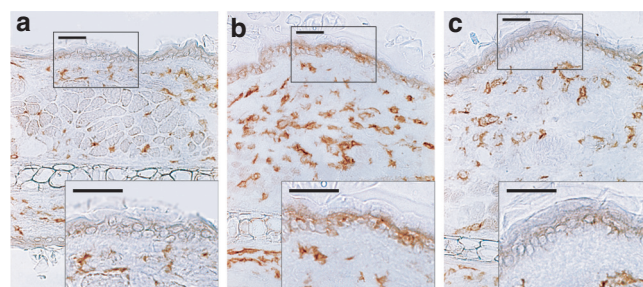


Figure 4 Reduced CD86 expression in the CD86 small interfering RNA-painted ear skin at an early time-point. Ear tissues from **(a)** intact mice, and from sensitized and challenged mice treated with either **(b)** control or **(c)** #6 CD86 siRNA 24 hours after challenge, were stained with anti-CD86 monoclonal antibody. Bars = 25 μ m.

which is upregulated on DCs when they mature),²⁴ and CCR5 (a skin-homing chemokine receptor which is expressed on recruiting immature DCs).²⁵ The expressions of CD86, CCR5, and CCR7 mRNA were strongly enhanced in the ear skin that had been challenged as compared with the ear skin that had been left unchallenged (Figure 5). Six hours after the challenge the level of CD86 mRNA in the #6 CD86 siRNA-painted ears was found to be markedly inhibited. At this time-point, mRNA levels for CCR7 and CCR5 were also observed to be dramatically inhibited. These data indicate that the cream-emulsified CD86 siRNA treatment effectively inhibits the induction of CD86 in cutaneous DCs and subsequently suppresses the new recruitment of CD86 and/or MHC class II-inducible skin-homing cells.

Topical treatment of CD86 siRNA inhibits migration of DCs to the RLNs

The reduction of CCR7 expression in the skin of the ears suggests an inhibition in migration of cutaneous DCs to RLNs. In order to trace the hapten-carrying DCs,²⁶ we used fluorescein isothiocyanate (FITC)-induced CH as a Th2-dominant CH model.²⁷ Inhibition of FITC-induced CH was consistently observed (Figure 6a). We next examined the numbers of DCs and antigen-carrying DCs in the RLNs 24 hours after challenge, given that the number of DCs peaked between 24 and 48 hours after the skin was painted.²⁶ The total cell numbers increased because of the exposure to antigen (Table 1), but this increase was significantly inhibited by the CD86 siRNA treatment. The percentage of CD11c⁺ cells also increased on account of exposure to antigen, and the CD86 siRNA had no clear effect on this increase. RLNs contain both resident and migrating DCs. Hapten-carrying skin-derived DCs were identified as CD11c⁺FITC⁺ cells. The percentage of FITC⁺ cells among the CD11c⁺ cells as well as the numbers of FITC⁺CD11c⁺ cells increased dramatically following the challenge, and this increase was significantly inhibited by CD86

siRNA treatment (Figure 6b and Table 1). These results suggest that the siRNA inhibits the migration of hapten-carrying DCs from the skin of the ear. The mean fluorescence intensity of CD86 on the CD11c⁺FITC⁺ cells was enhanced by the challenge, whereas

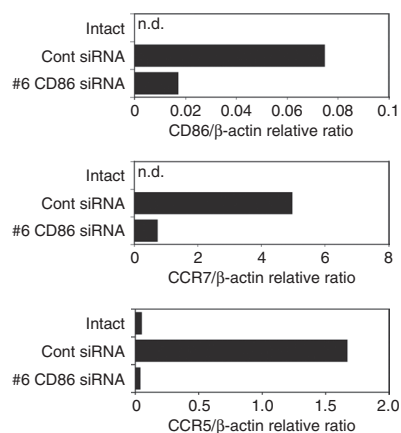


Figure 5 Reduced mRNA expression of CD86, CCR7, and CCR5 in the CD86 small interfering RNA (siRNA)-painted ear skin. At 6 hours after challenge, quantitative real-time PCR analyses were performed for determining CD86, CCR5, and CCR7 expressions in the ear skins from intact-skin mice, and from sensitized and challenged mice treated with either control or #6 CD86 siRNA. Total RNA from each group of two mice was isolated. Ear skins from intact-skin mice were used for comparison. Data shown are representative of two independent experiments. n.d., not detectable (CD86 or CCR7).

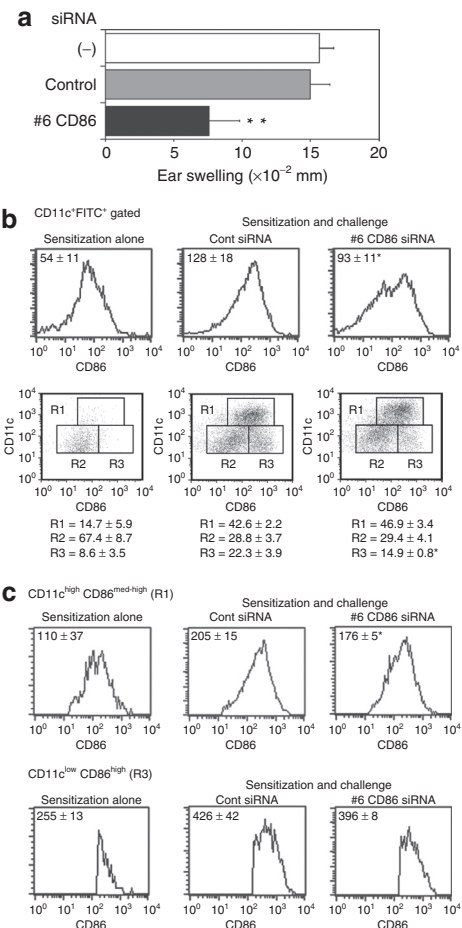


Figure 6 Reductions in dendritic cell migration and CD86 expression in the regional lymph nodes (RLNs). Contact hypersensitivity against fluorescein isothiocyanate (FITC) was induced as described in Materials and Methods. Nonsilencing control or #6 CD86 small interfering RNA (siRNA) (2.5 μg) mixed with a cream-based ointment was applied, and the skin was challenged with FITC. (a) Ear swelling was measured 24 hours after the challenge. Sterile water mixed with the cream-based ointment was used as a control. The data are representative of three independent experiments ($n = 5$) and significantly different from the nonsilencing control siRNA group (** $P < 0.01$). (b and c) Cervical LNs were obtained from the sensitized mice that had been left unchallenged as well as from the nonsilencing control or #6 CD86 siRNA-treated sensitized and challenged mice 24 hours after the challenge. The LN cells were stained with phycoerythrin anti-CD86 monoclonal antibody (mAb) and biotinylated anti-CD11c mAb, or an appropriate control immunoglobulin, followed by allophycocyanin-streptavidin, and analyzed using flow cytometry. Based on a fluorescence-activated cell sorting analysis using forward scatter and CD11c staining, an electronic gate was set for the CD11c⁺ lymphocytes, and then the logical gate was set for the FITC⁺ cells. The data are displayed as histograms for CD86, and dotted plots for CD86 and CD11c. The values in the upper left corners represent the mean fluorescence intensity of CD86 staining. Regions R1 (CD11c^{high}CD86^{med-high}), R2 (CD11c^{low}CD86^{low}), and R3 (CD11c^{low}CD86^{high}) were defined on the basis of the levels of CD86 and CD11c expression. The values below each panel indicate the proportions of CD11c⁺FITC⁺ cells in each region. The data shown are representative of three independent experiments using groups of three mice, and are significantly different from those of the nonsilencing control siRNA group (* $P < 0.05$).

Table 1 Changes in regional LNs induced by CD86 siRNA treatment

Treatment		siRNA	Total cell number (×10 ⁶)	CD11c ⁺	FITC ⁺ within CD11c ⁺ cells (%)	FITC ⁺ CD11c ⁺ cell number (×10 ⁶)
Sensitization	Challenge					
+	–	–	48 ± 1.4	3.9 ± 0.9	13.4 ± 4.8	0.3 ± 0.1
+	+	cont siRNA	85 ± 4.3*	5.2 ± 0.8	35.1 ± 4.4*	1.6 ± 0.4*
+	+	CD86 siRNA	60 ± 8.7**	6.9 ± 0.8	17.2 ± 3.8**	0.7 ± 0.2**

Abbreviations: mAb, monoclonal antibody; siRNA, small interfering RNA.

Cervical LN cells from the sensitized and unchallenged mice or the sensitized and challenged mice treated with control and CD86 siRNA were analyzed. Cells were stained with anti-CD11c mAb and the percentages of CD11c⁺ or FITC⁺ cells were assessed as described in Figure 6. The values are mean ± SD from each group of three mice. Data shown are representative of three individual experiments. Values were significantly different from the control unchallenged group (**P* < 0.05) and from the control siRNA-treated group (***P* < 0.05).

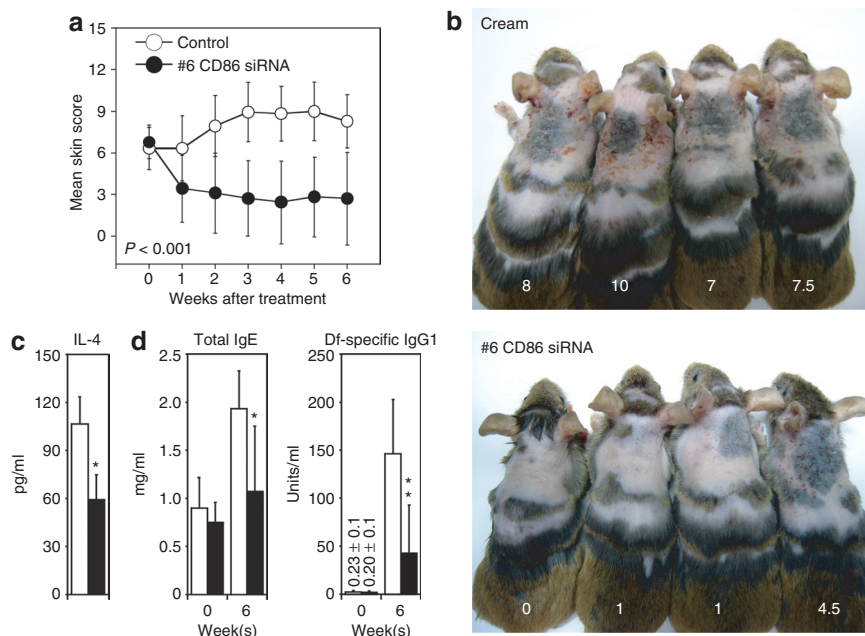


Figure 7 Amelioration of atopic dermatitis (AD) in NC/Nga mice by topical treatment using CD86 small interfering RNA (siRNA). AD was induced by repeated exposure to *Dermatophagoides farinae* (Df) antigen as described in Materials and Methods. siRNA treatment was initiated 2 weeks after the initial application of antigen. At this point, the total skin score of the ear and dorsal skin in all the mice was >5. Either # 6 CD86 siRNA (closed symbols or columns) or water (open symbols or columns) mixed with a cream-based ointment was administered every day for 6 weeks. **(a)** Changes in the mean skin score. **(b)** Photographs taken after 6 weeks of treatment. The values below indicate the individual total skin scores. **(c)** Interleukin-4 (IL-4) produced against the Df antigen by the lymph node (LN) cells. Regional LN (RLN) cells were stimulated with 10 µg/ml Df extract after 6 weeks of treatment, and the level of IL-4 was measured using enzyme-linked immunosorbent assay after 72 hours of culture. **(d)** Serum levels of total immunoglobulin E (IgE) and Df-specific IgG1. The Df-specific IgG1 titer of the standard serum was defined as 200 U and the levels in individual samples are expressed as unit values. The values were significantly different from the group treated with the cream-based ointment alone (**P* < 0.05, ***P* < 0.01).

it was significantly reduced in the CD86 siRNA-treated mice (Figure 6b). After Fluorescence-activated cell sorting analysis with CD11c and CD86 staining, the CD11c⁺ cells were classified into three groups on the basis of differences in the expressions of CD11c and CD86: CD11c^{high}CD86^{med-high} (R1); CD11c^{low}CD86^{low} (R2); and CD11c^{low}CD86^{high} (R3). R2 was the dominant subset in the sensitized mice that had not been subjected to the challenge. However, a hapten challenge increased the proportions of R1 and R3 cells, thereby suggesting that most migratory DCs from the skin were included in these two fractions. Among the three groups of CD11c⁺ cells, the R3 group expressed the highest level of CD86, and CD86 siRNA treatment significantly decreased the prevalence of R3 cells. CD86 expression in both the R1 and R3 groups was enhanced by the challenge, whereas it was inhibited by CD86 siRNA treatment (Figure 6c). Collectively, these results

suggest that topical painting of CD86 siRNA successfully silences the CD86 gene in epidermal Langerhans cells and dermal DCs, and sequentially reduces the recruitment and migration of dermal DCs into the dermis and the RLNs. These changes may lead to a reduction in hapten-specific T-cell responses, resulting in inhibition of CH responses in the skin.

Topical application of CD86 siRNA ameliorates atopic dermatitis in NC/Nga mice

Elevated CD86 has been reported in atopic skin lesions in humans^{9,10,28} and in mice.^{16,29} We therefore examined the therapeutic effects of topically applied CD86 siRNA on the atopic dermatitis (AD)-like lesions of NC/Nga mice. Two weeks after repeated application of mite antigen in order to induce atopic skin lesions, the mice were treated daily with cream-emulsified CD86 siRNA for

6 weeks. The mean skin scores in the control group peaked after 3 weeks and were maintained until the end of the observation period (Figure 7a). In contrast, 1 week after initiating siRNA treatment, the mean skin scores were significantly reduced, and this inhibitory effect was maintained for an additional 5 weeks despite repeated exposure to the antigen. Figure 7b shows the skin lesions from four representative animals 6 weeks after treatment. After 6 weeks, IL-4 production against mite antigen was observed to be significantly inhibited in the CD86 siRNA-treated group (Figure 7c); the serum titers of total IgE and *Dermatophagoides farinae* (Df)-specific IgG1 were also clearly inhibited when compared with those of the control group (Figure 7d). These results indicate that the CD86 siRNA successfully ameliorates the clinical manifestations of AD by inhibiting antigen-specific Th2 responses.

DISCUSSION

In this study, we have developed a new therapeutic approach for treating allergic skin disease using cream-emulsified CD86 siRNA to target cutaneous DCs. This is the first report of successful *in vivo* application of an siRNA-based ointment.

Topical CD86 siRNA treatment in CH initially inhibited CD86 induction in residential epidermal Langerhans cells and dermal DCs at the early time-points. An initial reduction in CD86 on resident DCs may affect their ability to become activated and/or mature, and to migrate. In general, antigen-stimulated DCs secrete various chemokines that are required for the recruitment of DC precursors into the dermis from the blood circulation.³⁰ In addition, antigen-stimulated DCs may enhance the expression of MHC class II and other co-stimulatory molecules, including CD86. The silencing of the CD86 in DCs may dampen this ability and reduce chemokine secretion, resulting in the inhibition of DC precursor recruitment in the dermis. In fact, the mRNA expression of a skin-homing receptor CCR5 at an early time-point (6 hours) was markedly inhibited (Figure 5), and the recruitment of CD86⁺ or MHC class II⁺ cells in the dermis was clearly inhibited at late time-points (6–24 hours) (Figure 3). This crucial secondary effect was therefore inhibited by topical CD86 siRNA treatment. It has been shown that newly recruited DCs (and DC precursors) that mature on-site may play a major role in skin inflammation.^{30–33}

In addition to the events at the local site, CD86 gene silencing in DCs reduced the homing of antigen-captured cutaneous DCs into the RLNs. Our results demonstrate that mRNA expression of CCR7 (a potent LN-homing receptor) as well as the actual numbers of hapten-carrying CD11c⁺ DCs were dramatically inhibited (Figure 5 and Table 1). These results indicate that CD86 siRNA treatment suppresses LN-migration of antigen-captured cutaneous DCs. Some of cutaneous DCs in the CD86 siRNA-treated skin may migrate to RLNs, but their expression of CD86 in RLNs is also inhibited (Figure 6). The antigen-presenting capacity of the CD86-reduced DCs may be impaired, and the quantitative and qualitative changes in the antigen-presenting DCs may greatly suppress overall antigen-specific T-cell responses in the RLNs. The fact that the total cell numbers in the RLNs were markedly decreased indicates the reduction in antigen-specific cellular responses. In the AD model, we also observed that antigen-specific IL-4 and IgG1 production were significantly inhibited by the CD86 siRNA treatment. Our results suggest that the topical application of CD86

siRNA in the periphery could induce the inhibition of antigen-specific T-cell responses in the RLNs. Finally, locally reduced effector T-cell homing and effector cytokine secretion may further affect inflammatory responses in the skin. Collectively, our results suggest that topical CD86 siRNA treatment downregulates DC function quantitatively and qualitatively, resulting in impaired innate and adaptive immunity both locally and in the RLNs.

Several DNA- or siRNA-based vaccine therapies that target DCs have been tested,^{34–37} and most of these have been found to enhance the immunopotency of DCs. DCs may be the most suitable targets because they affect immune responses both positively and negatively. In our approach, we negatively modulated the properties of DCs by applying CD86 gene silencing. Similarly, it has been shown that the successful transfection with CD86 siRNA into BM-DCs reduces their antigen-presenting capacity *in vitro*, and the transfer of the CD86-silenced DCs prolongs cardiac allograft survival *in vivo*.^{38,39} For delivery of naked siRNAs into DCs *in vivo*, intradermal injections using a gene gun³⁵ and LFA 1-targeted fusion proteins⁴⁰ have been used. However, such methods may irritate inflamed skin, and repeated treatments may not be possible. Therefore we have devised a topical solution that contains the siRNA mixed with an anti-irritant cream used in infants. Topical application is a long-standing, convenient, and economical method of drug delivery. The cream included water, mineral oil, glyceryl monostearate, and propylene glycol. While the activity of each ingredient in the cream remains to be elucidated, oil-in-water emulsions stabilize siRNAs.⁴¹ In order to investigate the mechanism through which cream-emulsified siRNA reaches cutaneous DCs, we applied Alexa 488 fluorochrome-conjugated #6 CD86 siRNA and traced the fluorescence in the ear skin sections using fluorescence microscopy. Consistent with the results observed in topically applied oligonucleotides,²² strong fluorescence signals were seen in hair follicles at 2 hours after application in addition to those on the epidermal surface, and these signals were persistent at 24 hours (Supplementary Figure S1). Additionally, we found the recruitment of abundant MHC class II⁺ cells around the hair follicles after hapten challenge (at 4 and 6 hours), whereas only few MHC class II⁺ cells were located in this region of the intact (unchallenged) ear (Supplementary Figure S2). These observations suggest that dermal DCs migrate to hair follicles and take up CD86 siRNA as well as antigens. It is also possible that epidermal Langerhans cells may actively reach siRNA on the skin through the tight junction as reported in the case of gut epithelium.⁴² In order to examine target cell specificity, we tried to use a similar method to silence lamin A/C genes that are expressed in all epidermal keratinocytes. No clear reduction in lamin A/C mRNA was seen after the treatment with cream-emulsified lamin A/C siRNA (data not shown), thereby suggesting that cutaneous DCs, but not keratinocytes, may actively reach siRNA. Further studies are required for examining the exact mechanisms of transport of cream-emulsified siRNA into DCs.

Human clinical trials of systemically administrated CTLA4Ig (abatacept) and LEA29Y (balatacept) are currently underway;⁴³ however, the systemic administration of this type of CD28 antagonist may suppress protective immunity against infectious agents. In this context, topical formulations that target local DCs may have the advantage of inducing specific inhibition against cutaneous

antigens. Our strategy can be expanded to include the genes for additional co-stimulatory molecules and such target tissues as the nasal, oral, alveolar, and gastrointestinal mucosae. Overall, our findings will aid the development of siRNA-based ointments for the treatment of patients suffering from allergic or intractable inflammatory skin disease.

MATERIALS AND METHODS

Mice. Female 6-week-old BALB/c and NC/Nga mice were purchased from SLC (Hamamatsu, Japan), and maintained under specific pathogen-free and conventional conditions, respectively. All procedures were reviewed and approved by the Animal Care and Use Committee of Tokyo Medical and Dental University.

Preparation of siRNAs. All siRNA oligonucleotides for mouse CD86 were designed and synthesized by Qiagen 2-for-silencing siRNA duplexes (Qiagen, Valencia, CA). The sequences of the sense and antisense oligos are shown in Figure 1a. Each pair of sense and antisense siRNAs was denatured, annealed in accordance with the manufacturer's instructions, and then diluted to a final concentration of 20 μ mol/l. Nonsilencing control siRNA was also obtained from Qiagen. The synthetic siRNAs used in the other experiments were obtained from JBioS (Asaka, Saitama, Japan).

siRNA transfection in vitro. 293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Wako, Japan) supplemented with 10% fetal bovine serum (Invitrogen, St. Louis, MO) and gentamycin (Sigma, Steinheim, Germany). The cells were co-transfected with 0.2 μ g of the pcDNA3/CD86 expression vector and 40 pmol of either the control or each of the CD86 siRNA duplexes with Lipofectamine 2000 (Invitrogen, St. Louis, MO) diluted in Opti-minimal essential medium (Invitrogen, St. Louis, MO), in accordance with the manufacturer's instructions. Cell surface expression of CD86 at 48 hours after transfection was analyzed using flow cytometry. BM-DCs were generated as described earlier,⁴⁴ with some modifications. Briefly, isolated BM cells were cultured with GM-CSF and IL-4 (10 ng/ml each; eBioscience, San Diego, CA) for 6 days. The immature BM-DCs were collected (2×10^5 cells/well) and transfected with siRNA duplex in the presence of a maturation stimulus, lipopolysaccharide (10 μ g/ml; Sigma, Steinheim, Germany), as described earlier. After 24 hours, the expression levels of CD80 and CD86 were analyzed by co-staining with anti-CD11c and anti-MHC class II mAbs.

Induction of CH and treatment with siRNA or mAbs. CH to dinitrofluorobenzene or FITC was induced as described earlier.^{12,45} Briefly, 20 μ l of 0.5% dinitrofluorobenzene (Sigma, Steinheim, Germany) dissolved in acetone:olive oil (4:1) was painted onto shaved abdominal skin of the mice on days 0 and 1. On day 5, 20 μ l of 0.2% dinitrofluorobenzene was applied to the left ear. Ear thickness was measured before and 24 and 48 hours after the challenge. For the induction of CH against FITC, the mice were sensitized by painting 400 μ l of 0.5% FITC dissolved in dibutyl phthalate:acetone (1:1) on their shaved abdomens on day 0, and then challenged with 20 μ l of 0.5% FITC on day 5.

As vehicles for siRNA delivery, dimethyl sulfoxide (Sigma, Steinheim, Germany), Atelocollagen (Koken, Tokyo, Japan) and a cream-based ointment (Johnson's baby lotion, no fragrance; Johnson & Johnson, New Brunswick, NJ) were tested. The cream-based ointment and either dimethyl sulfoxide, or Atelocollagen diluted in saline (at a final concentration of 0.5%) (total volume of 7.5 μ l) were mixed with 12.5 μ l of siRNA duplex. A total volume of 20 μ l of the siRNA-containing mixture was painted on the ear skin 1 hour before the challenge. For the neutralizing mAb experiments, the mice received intraperitoneal injections of anti-CD86 mAb (200 μ g/mouse, PO3, rat IgG2b) or control rat IgG (Cappel, Solon, OH) 2 hours before the challenge, as described earlier.¹²

Immunohistochemistry. Ear tissues from intact, or sensitized and challenged mice treated with the siRNAs, were surgically removed, embedded in Tissue-Tek (Sakura, Tokyo, Japan), frozen, and stored at -80°C until use. Cryostat sections (5–6 μ m thick) were fixed in absolute acetone and subjected to enzymatic immunohistochemistry. After blocking, the sections were first incubated with mAbs against CD86 (PO3) and MHC class II (M5/114, rat IgG2b) or control rat IgG, and then with biotinylated anti-rat IgG (Vector Laboratories, Burlingame, CA). The ABC peroxidase system (Vectastain Elite Universal ABC Kit; Vector Laboratories, Burlingame, CA) was used in accordance with the manufacturer's instructions. All the incubation steps were performed in a microwave processor (MI-77; Azumaya, Tokyo, Japan) as suggested by the manufacturer. The sections were visualized with the substrate diaminobenzidine (Merck, Darmstadt, Germany) and counterstained with hematoxylin. Digital images were obtained using an inverted microscope and camera system (IX71 and Pro600ES-D; Olympus, Tokyo, Japan).

Quantitative real-time PCR. Total RNA was extracted from pooled ear skins of two mice using Isogen (Nipongene, Toyama, Japan). First-strand cDNA was synthesized using oligo(dT) primer and Superscript III reverse transcriptase (Invitrogen, St. Louis, MO). Real-time PCR was performed using the LightCycler instrument and the DNA Master SYBR green I kit (Roche Diagnostics, Basel, Switzerland). The primer sequences are β -actin, 5'-AGG GAA ATC GTG CGT GAC AT-3' and 5'-AAC CGC TCG TTG CCA ATA GT-3'; CD86, 5'-GAC CGT TGT GTG TGT TCT GG-3' and 5'-GAT GAG CAG CAT CAC AAG GA-3'; CCR5, 5'-GCA GGC AAC AGA GAC TCT TG-3' and 5'-GAC TGA GCT TGC ACG ATC AG-3'; and CCR7, 5'-AGA CCA CCA CAA CCT TCT CC-3' and 5'-ACT GGC CAG AAT TGC TCT TC-3'. The data were evaluated using LightCycler software (Roche Diagnostics, Basel, Switzerland) and presented as the relative ratio of the respective molecule/ β -actin.

Isolation of LN cells. In the CH model, cervical LNs were collected 24 hours after FITC challenge. Cervical and axillary LNs were collected from the NC/Nga mice 6 weeks after siRNA treatment. LN cells were isolated using the collagenase method so as to ensure the release of interdigitating DCs.

Flow cytometry. mAbs against CD11c (HL3), CD86 (PO3), CD80 (10A1), and MHC class II (M5/114) were used. All FITC-, phycoerythrin-, allophycocyanin-, peridinin-chlorophyll-protein complex-carbocyanin 5.5-conjugated and biotinylated mAbs were obtained from either eBiosciences (San Diego, CA) or BD-Pharmingen (San Diego, CA). For the biotinylated mAb, allophycocyanin-streptavidin was used. Stained cells were analyzed on a FACSCalibur using the CellQuest software (BD Biosciences, San Jose, CA).

Induction of AD in NC/Nga mice. AD-like lesions were induced in NC/Nga mice by repeated application of mite antigen, as described by Inoue *et al.*⁴⁶ Briefly, 20 μ g of mite antigen (mite extract Df; LSL, Tokyo, Japan) was applied to the shaved upper back and ears of the mice three times a week. A cream-based hair remover (Kanebo, Tokyo, Japan) was used as needed.

Evaluation of skin lesions. The skin lesions were assessed once a week by two persons who were unaware of each mouse's treatment status. Scoring was performed as described earlier,⁴⁷ with a minor modification. The extent of severity of erythema/hemorrhage, excoriation/erosion, and scaling/dryness at the two sites (the ears and dorsal skin) were scored as 0 (no symptom), 1 (mild), 2 (moderate), or 3 (severe), and the sum of these scores was defined as the total skin score (range from 0 to 18).

Treatment of AD with siRNA. The siRNA treatment began 2 weeks after the initial application of mite antigen. At this time-point, all the mice exhibited AD-like skin lesions and were assigned a skin score >5 . A total of 40 μ l of CD86 siRNA (5 μ g) in a cream-based ointment was topically applied every day to the dorsal side of the ears and shaved back skin of the mice. In

the preliminary experiments, there was no difference between the groups treated with control siRNA and cream-based ointment alone. We therefore present the results from the cream-based ointment alone as a control.

Measurement of serum IgE and IgG1. Sera were collected once every 2 weeks before and after treatment, and the total IgE was measured using enzyme-linked immunosorbent assay as described earlier.⁴⁸ Df extract (10 µg/ml) was used for antigen coating and the amount of Df-specific IgG1 was measured as described earlier.⁴⁸

Assessment of antigen-specific IL-4 production. LN cells (4×10^5 cells/well) were cultured with or without 10 µg/ml of Df antigen. The supernatants were collected after 72 hours and IL-4 production was determined using enzyme-linked immunosorbent assay.⁴⁸

Statistics. Statistical analyses were performed using either the Mann-Whitney *U*-test or one-way analysis of variance. Values of $P < 0.05$ or $P < 0.01$ were considered significant.

ACKNOWLEDGMENTS

The authors thank Ryo Inoue (Hokkaido University) for helpful advice in murine atopic disease model. We also thank Hiroaki Miyajima (Juntendo University School of Medicine) for providing mAbs. This study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

SUPPLEMENTARY MATERIAL

Figure S1. Trace of fluorochrome labeled CD86 siRNA.

Figure S2. Recruitment of MHC class II⁺ cells around the hair follicles.

REFERENCES

- Banchereau, J, Briere, F, Caux, C, Davoust, J, Lebecque, S, Liu, YJ *et al.* (2000). Immunobiology of dendritic cells. *Annu Rev Immunol* **18**: 767–811.
- Tsuyuki, S, Tsuyuki, J, Einsle, K, Kopf, M and Coyle, AJ (1997). Costimulation through B7-2 (CD86) is required for the induction of a lung mucosal T helper cell 2 (Th2) immune response and altered airway responsiveness. *J Exp Med* **185**: 1671–1679.
- Larché, M, Till, SJ, Haselden, BM, North, J, Barkans, J, Corrigan, CJ *et al.* (1998). Costimulation through CD86 is involved in airway antigen-presenting cell and T cell responses to allergen in atopic asthmatics. *J Immunol* **161**: 6375–6382.
- Haczku, A, Takeda, K, Redai, I, Hamelmann, E, Cieslewicz, G, Joetham, A *et al.* (1999). Anti-CD86 (B7.2) treatment abolishes allergic airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* **159**: 1638–1643.
- Coyle, AJ and Gutierrez-Ramos, JC (2001). The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. *Nat Immunol* **2**: 203–209.
- Hathcock, KS, Laszlo, G, Pucillo, C, Linsley, P and Hodes, RJ (1994). Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J Exp Med* **180**: 631–640.
- Borriello, F, Sethna, MP, Boyd, SD, Schweitzer, AN, Tivol, EA, Jacoby, D *et al.* (1997). B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* **6**: 303–313.
- Yokozeki, H, Katayama, I, Ohki, O, Arimura, M, Takayama, K, Matsunaga, T *et al.* (1997). Interferon-gamma differentially regulates CD80 (B7-1) and CD86 (B7-2/B70) expression on human Langerhans cells. *Br J Dermatol* **136**: 831–837.
- Ohki, O, Yokozeki, H, Katayama, I, Umeda, T, Azuma, M, Okumura, K *et al.* (1997). Functional CD86 (B7-2/B70) is predominantly expressed on Langerhans cells in atopic dermatitis. *Br J Dermatol* **136**: 838–845.
- Jirapongsananurak, O, Hofer, MF, Trumble, AE, Norris, DA and Leung, DY (1998). Enhanced expression of B7.2 (CD86) in patients with atopic dermatitis: a potential role in the modulation of IgE synthesis. *J Immunol* **160**: 4622–4627.
- Kawamura, T and Furue, M (1995). Comparative analysis of B7-1 and B7-2 expression in Langerhans cells: differential regulation by T helper type 1 and T helper type 2 cytokines. *Eur J Immunol* **25**: 1913–1917.
- Nuriya, S, Yagita, H, Okumura, K and Azuma, M (1996). The differential role of CD86 and CD80 co-stimulatory molecules in the induction and the effector phases of contact hypersensitivity. *Int Immunol* **8**: 917–926.
- Reiser, H and Schneeberger, EE (1996). Expression and function of B7-1 and B7-2 in hapten-induced contact sensitivity. *Eur J Immunol* **26**: 880–885.
- Katayama, I, Matsunaga, T, Yokozeki, H and Nishioka, K (1997). Blockade of costimulatory molecules B7-1 (CD80) and B7-2 (CD86) down-regulates induction of contact sensitivity by haptened epidermal cells. *Br J Dermatol* **136**: 846–852.
- Xu, H, Heeger, PS and Fairchild, RL (1997). Distinct roles for B7-1 and B7-2 determinants during priming of effector CD8⁺ Tc1 and regulatory CD4⁺ Th2 cells for contact hypersensitivity. *J Immunol* **159**: 4217–4226.
- Santa, K, Watanabe, K, Nakada, T, Kato, H, Habu, S and Kubota, S (2003). Enhanced expression of B7.2 (CD86) by percutaneous sensitization with house dust mite antigen. *Immunol Lett* **85**: 5–12.
- Fire, A, Xu, S, Montgomery, MK, Kostas, SA, Driver, SE and Mello, CC (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**: 806–811.
- Elbashir, SM, Harborth, J, Lendeckel, W, Yalcin, A, Weber, K and Tuschl, T (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**: 494–498.
- Scherr, M, Morgan, MA and Eder, M (2003). Gene silencing mediated by small interfering RNAs in mammalian cells. *Curr Med Chem* **10**: 245–256.
- de Fougerolles, A, Vornlocher, HP, Maragane, J and Lieberman, J (2007). Interfering with disease: a progress report on siRNA-based therapeutics. *Nat Rev Drug Discov* **6**: 443–453.
- Mehta, RC, Stecker, KK, Cooper, SR, Templin, MV, Tsai, YJ, Condon, TP *et al.* (2000). Intercellular adhesion molecule-1 suppression in skin by topical delivery of anti-sense oligonucleotides. *J Invest Dermatol* **115**: 805–812.
- Dokka, S, Cooper, SR, Kelly, S, Hardee, GE and Karras, JG (2005). Dermal delivery of topically applied oligonucleotides via follicular transport in mouse skin. *J Invest Dermatol* **124**: 971–975.
- Minakuchi, Y, Takeshita, F, Kosaka, N, Sasaki, H, Yamamoto, Y, Kouno, M *et al.* (2004). Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing *in vitro* and *in vivo*. *Nucleic Acids Res* **32**: e109.
- Randolph, GJ, Angeli, V and Swartz, MA (2005). Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol* **5**: 617–628.
- Fernandez, EJ and Lolis, E (2002). Structure, function, and inhibition of chemokines. *Annu Rev Pharmacol Toxicol* **42**: 469–499.
- Macatonia, SE, Edwards, AJ and Knight, SC (1986). Dendritic cells and the initiation of contact sensitivity to fluorescein isothiocyanate. *Immunology* **59**: 509–514.
- Tang, A, Judge, TA, Nickoloff, BJ and Turka, LA (1996). Suppression of murine allergic contact dermatitis by CTLA4Ig. Tolerance induction of Th2 responses requires additional blockade of CD40-ligand. *J Immunol* **157**: 117–125.
- Schuller, E, Teichmann, B, Haberkost, J, Moderer, M, Bieber, T and Wollenberg, A (2001). *In situ* expression of the costimulatory molecules CD80 and CD86 on langerhans cells and inflammatory dendritic epidermal cells (IDEC) in atopic dermatitis. *Arch Dermatol Res* **293**: 448–454.
- Horiuchi, Y, Bae, S, Katayama, I and Nishioka, K (2004). Therapeutic effects of streptococcal preparation OK-432 on atopic dermatitis-like lesions in NC/Nga mice: possible shift from a Th2- to Th1-predominance. *J Dermatol Sci* **35**: 187–197.
- Jakob, T, Ring, J and Udey, MC (2001). Multistep navigation of Langerhans/dendritic cells in and out of the skin. *J Allergy Clin Immunol* **108**: 688–696.
- Weinlich, G, Heine, M, Stössel, H, Zanella, M, Stoitner, P, Ortner, U *et al.* (1998). Entry into afferent lymphatics and maturation *in situ* of migrating murine cutaneous dendritic cells. *J Invest Dermatol* **110**: 441–448.
- Randolph, GJ, Beaulieu, S, Lebecque, S, Steinman, RM and Muller, WA (1998). Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science* **282**: 480–483.
- Ginhoux, F, Tacke, F, Angeli, V, Bogunovic, M, Loubeau, M, Dai, XM *et al.* (2006). Langerhans cells arise from monocytes *in vivo*. *Nat Immunol* **7**: 265–273.
- Condon, C, Watkins, SC, Celluzzi, CM, Thompson, K and Falo, LD Jr. (1996). DNA-based immunization by *in vivo* transfection of dendritic cells. *Nat Med* **2**: 1122–1128.
- Kim, TW, Lee, JH, He, L, Boyd, DA, Hardwick, JM, Hung, CF *et al.* (2005). Modification of professional antigen-presenting cells with small interfering RNA *in vivo* to enhance cancer vaccine potency. *Cancer Res* **65**: 309–316.
- Kim, DH and Rossi, JJ (2007). Strategies for silencing human disease using RNA interference. *Nat Rev Genet* **8**: 173–184.
- Mao, CP, Hung, CF and Wu, TC (2007). Immunotherapeutic strategies employing RNA interference technology for the control of cancers. *J Biomed Sci* **14**: 15–29.
- Gu, X, Xiang, J, Yao, Y and Chen, Z (2006). Effects of RNA interference on CD80 and CD86 expression in bone marrow-derived murine dendritic cells. *Scand J Immunol* **64**: 588–594.
- Xiang, J, Gu, X, Zhou, Y, Gong, X, Qian, S and Chen, Z (2007). Administration of dendritic cells modified by RNA interference prolongs cardiac allograft survival. *Microsurgery* **27**: 320–323.
- Peer, D, Zhu, P, Carman, CV, Lieberman, J and Shimaoka, M (2007). Selective gene silencing in activated leukocytes by targeting siRNAs to the integrin lymphocyte function-associated antigen-1. *Proc Natl Acad Sci USA* **104**: 4095–4100.
- Williams, R, Peisajovich, SG, Miller, OJ, Magdassi, S, Tawfik, DS and Griffiths, AD (2006). Amplification of complex gene libraries by emulsion PCR. *Nat Methods* **3**: 545–550.
- Rescigno, M, Urbano, M, Valzasina, B, Francolini, M, Rotta, G, Bonasio, R *et al.* (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* **2**: 361–367.
- Bluestone, JA, St. Clair, EW and Turka, LA (2006). CTLA4Ig: bridging the basic immunology with clinical application. *Immunity* **24**: 233–238.
- Inaba, K, Inaba, M, Romani, N, Aya, H, Deguchi, M, Ikehara, S *et al.* (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* **176**: 1693–1702.
- Nuriya, S, Enomoto, S and Azuma, M (2001). The role of CTLA-4 in murine contact hypersensitivity. *J Invest Dermatol* **116**: 764–768.
- Inoue, R, Nishio, A, Fukushima, Y and Ushida, K (2007). Oral treatment with probiotic *Lactobacillus johnsonii* NCC533 (La1) for a specific part of the weaning period prevents the development of atopic dermatitis induced after maturation in model mice, NC/Nga. *Br J Dermatol* **156**: 499–509.
- Matsuda, H, Watanabe, N, Geba, GP, Sperl, J, Tsudzuki, M, Hiroi, J *et al.* (1997). Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* **9**: 461–466.
- Nozawa, K, Ohata, J, Sakurai, J, Hashimoto, H, Miyajima, H, Yagita, H *et al.* (2001). Preferential blockade of CD8⁺ T cell responses by administration of anti-CD137 ligand monoclonal antibody results in differential effect on development of murine acute and chronic graft-versus-host diseases. *J Immunol* **167**: 4981–4986.

Figure S1

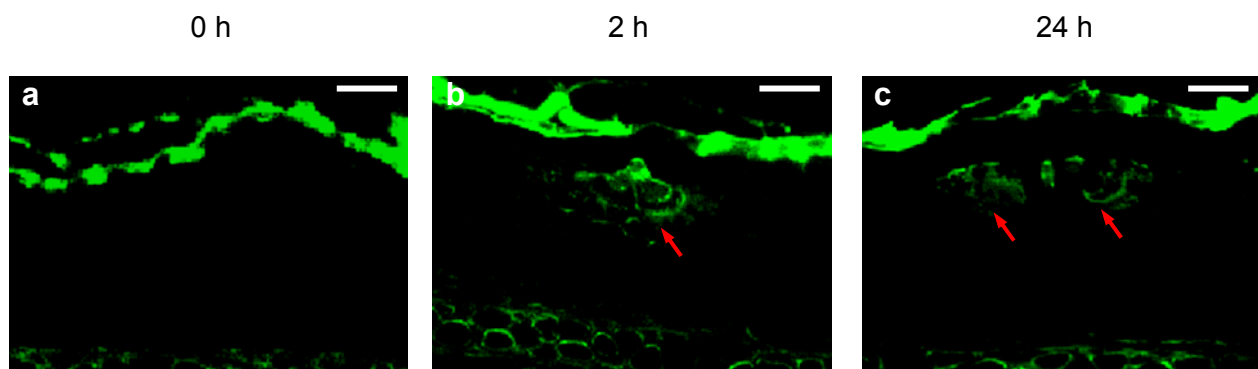


Figure S2

