研究成果の刊行に関する一覧表（雑誌）

発表者氏名	論文タイトル名	発表雑誌	巻号	ページ	出版年
Abe R, Yoshioka N, Murata J, Fujita Y, Shimizu H	Granulysin as a marker for early diagnosis of the Stevens-Johnson syndrome	**Ann Intern Med**	151	514-515	2009
Hamasaka A, Abe R, Koyama Y, Yoshioka N, Fujita Y, Hoshina D, Sasaki M, Hirasawa T, Onodera S, Ohshima S, Leng L, Bucala R, Nishihira J, Shimizu T, Shimizu H	DNA vaccination against macrophage migration inhibitory factor improves atopic dermatitis in murine models	**J Allergy Clin Immunol**	124	90-99	2009
Ando S, Abe R, Sasaki M, Murata J, Inokuma D, Shimizu H	Bone marrow-derived cells are not the origin of the cancer stem cells in ultraviolet-induced skin cancer	**Am J Pathol**	174	595-601	2009
Shinkuma S, Abe R, Nishimura M, Natsuga K, Fujita Y, Nomura T, Nishie W, Shimizu H	Secondary syphilis mimicking warts in an HIV-positive patient	**Sex Transm Infect**	85	484	2009
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Honda A, Abe R, Yoshihisa Y, Makino T, Matsunaga K, Nishihira J, Shimizu H, Shimizu T	Deficient deletion of apoptotic cells by macrophage migration inhibitory factor (MIF) overexpression accelerates photocarcinogenesis	**Carcinogenesis**	30	1597-1605	2009
Arita K, Abe R, Baba K, McGrath JA, Akiyama M, Shimizu H	A novel OSMR mutation in familial primary localized cutaneous amyloidosis in a Japanese family	**J Dermatol Sci**	55	64-65	2009
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Qiao H, Shibaki A, Long HA, Wang G, Li Q, Nishie W, Abe R, Akiyama M, Shimizu H, McMillan JR.	Collagen XVII participates in keratinocyte adhesion to collagen IV, and in p38MAPK-dependent migration and cell signaling	**J Invest Dermatol**	129	2288-2295	2009
Wu C, Mino K , Akimoto H, Kawabata M, Nakamura K, Ozaki M, Ohmiya Y.	In vivo far-red luminescence imaging of a biomarker based on BRET from Cypridina bioluminescence to an organic dye	**Proc Nat Acad Sci USA**	106 (37)	15599-15603	2009

Ogino T, Ozaki M, Hosako M, Omori M, Okada S, Matsukawa A.	Activation of c-Jun N-terminal kinase is essential for oxidative stress-induced Jurkat cell apoptosis by monochloramine	**Leuk Res**	33	151-158	2009
Akimoto H, Kwon HJ, Ozaki M, Yasuda K, Honma K, Ohmiya Y	In vivo bioluminescence imaging of bone marrow-derived cells in brain inflammation	**Biochemical and Biophysical Research Communications**	380	844-849	2009
Ikeda O, Ozaki M, Murata S, Matsuo R, Nakano Y, Watanabe M, Hisakura K, Myronovych A, Ohkohchi N	Autonomic regulation of liver regeneration after partial hepatectomy in mice	**J Surg Res**	52	218-223	2009
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Horie Y, Meguro A, Ota M, Kitaichi N, Katsuyama Y, Takemoto Y, Namba K, Yoshida K, Song YW, Park KS, Lee, EB, Inoko H, Mizuki N, Ohno S.	Association of TLR4 polymorphism with Behcet disease in Korean population	**Rheumatology**	48	638-642	2009
Horie Y, Kitaichi N, Katsuyama Y, Yoshida K, Miura T, Ota M, Asukata Y, Inoko H, Mizuki N, Ishida S, Ohno S.	Evaluation of PTPN22 polymorphisms and Vogt-Koyanagi-Harada disease in Japanese patients	**Mol Vis**	15	1115-1119	2009
Kitamei H, Kitaichi N, Namba K, Kotake S, Goda C, Kitamura M, Miyazaki A, Ohno S	Clinical features of intraocular inflammation in Hokkaido, Japan	**Acta Ophthalmol**	87	424-428	2009

Ⅳ. 研究成果の刊行物・別冊

eradication treatment will probably give a huge advantage in terms of social health, especially in high-risk areas.

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Potential Conflicts of Interest: None disclosed.

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CLINICAL OBSERVATIONS

Granulysin as a Marker for Early Diagnosis of the Stevens–Johnson Syndrome

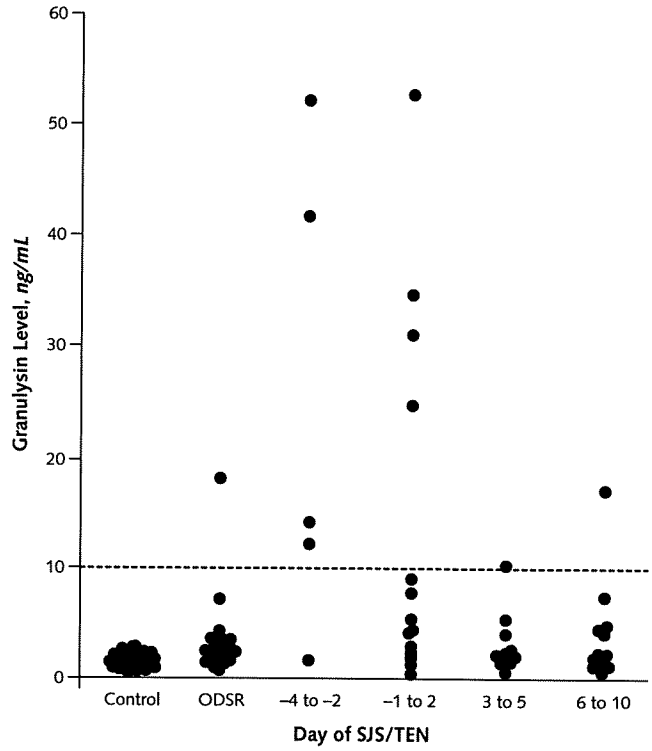
Background: The Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening adverse drug reactions characterized by massive epidermal necrosis. In the early stage, clinical presentations of SJS/TEN are very similar to those of ordinary drug-induced skin reactions (ODSRs); therefore, SJS/TEN is difficult to diagnose and the start of treatment is often delayed, resulting in high mortality rates. Other investigators (1) reported that granulysin is highly expressed in blisters of SJS/TEN and causes disseminated keratinocyte death. Because SJS/TEN progresses and spreads rapidly, the granulysin level should be increased in the serum of patients with active SJS/TEN if it is a key mediator of these diseases.

Objective: To determine whether serum granulysin levels are higher in patients with SJS/TEN than in healthy control participants or those with ODSRs.

Methods: We measured granulysin in the sera of 31 healthy control participants, 24 patients with ODSR, 13 patients with SJS, and 7 patients with TEN by using enzyme-linked immunosorbent assay (2). Disease onset in patients with SJS/TEN was defined as the day (day 1) on which the mucocutaneous or ocular lesion first eroded or ulcerated (3), and we collected sera from these patients from 4 days before to 10 days after ulceration. We used the Tukey–Kramer test to conduct multiple comparisons between groups.

Results: None of the 31 healthy control participants had a granulysin level greater than the upper limit of normal, which was 10 ng/mL (0% elevated; mean, 1.6 ng/mL [SD, 0.6]), and among 24 patients with ODSRs, only 1 patient had an elevated granulysin level (4.2% elevated; mean, 3.5 ng/mL [SD, 3.4]) (**Figure**). We obtained samples from 5 patients with SJS/TEN on day −4 to day −2, and we detected the highest granulysin concentrations (elevated in 80% of patients); mean, 24.8 ng/mL [SD, 21.2]). Granulysin levels were lower in the 14 samples collected on day −1 to day 2 (28.6% elevated; mean, 13.7 ng/mL [SD, 16.0]), and were even lower in the 10 samples collected from day 3 to day 5 (10.0% elevated; mean, 4.2 ng/mL [SD, 3.0]) and in the 13 samples collected from day 6 to day 10 (7.7% elevated; mean, 4.5 ng/mL [SD, 4.5]). When we compared granulysin levels from day −4 to day −2 among patients with SJS/TEN, patients with ODSRs, and healthy control participants, the differences were statistically significant ($P < 0.010$).

***Figure.* Granulysin levels of healthy control participants, patients with ODSRs, and patients with SJS/TEN.**



ODSR = ordinary drug-induced skin reaction; SJS/TEN = Stevens–Johnson syndrome/toxic epidermal necrolysis.

Discussion: Granulysin is cytotoxic for tumor cells, transplant cells, bacteria, fungi, and parasites, in which it damages negatively charged cell membranes because of its positive charge (4). It plays an important role in the host defense against pathogens, and it induces apoptosis of target cells by using a mechanism involving caspases and other pathways (4). Its potency makes it a credible mediator of skin damage in patients with SJS/TEN. Adding to this credibility is a report (1) that granulysin is the most highly expressed cytotoxic molecule in the blisters of patients with SJS/TEN. We show that serum granulysin levels in 4 of 5 patients with SJS/TEN were elevated before skin detachment or mucosal lesions develop. Soluble Fas ligand (sFasL) shares some properties with granulysin: It contributes to keratinocyte death in SJS/TEN (3, 5), and levels are elevated in the sera of patients with SJS/TEN (3). Serum granulysin levels, however, are approximately 100 times higher than those of sFasL on day

−4 to day −2 (23.1 ng/mL [SD, 16.6] vs. 147.76 pg/mL [SD, 104.4]). Therefore, we believe it would be easier to develop bedside granulysin serum measurement, for example, by using immunochromatography, than it would be to develop a similar sFasL measurement. Monitoring serum granulysin might enable early diagnosis of SJS/TEN in patients with cutaneous adverse drug reactions that otherwise could not be distinguished from ODSRs.

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Potential Conflicts of Interest: None disclosed.

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Localized Amyloidosis at the Site of Enfuvirtide Injection

Background: Enfuvirtide is the first of a new class of antiretroviral agents that block fusion of the viral particle with the host target cell. Its safety and antiviral activity have been demonstrated (1, 2). In clinical trials, injection site reactions occurred in 80% to 100% of patients (3). The most common signs and symptoms reported were induration in 94%, erythema in 91%, and subcutaneous nodules and cysts in 70% (4).

Objective: To describe a case of amyloidosis at the injection site of enfuvirtide.

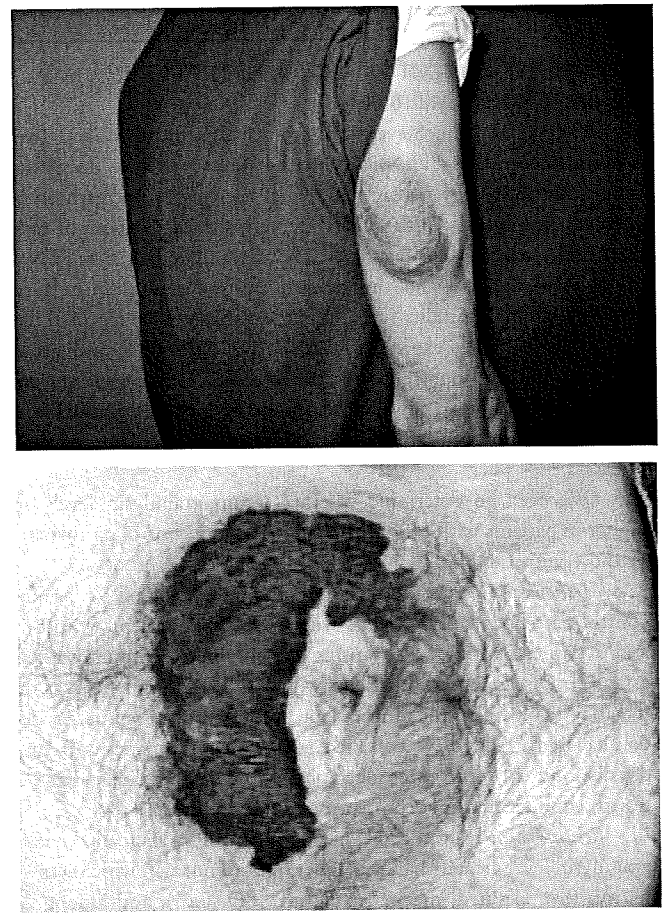
Case Report: The patient was a man aged 47 years who had a history of sexual intercourse with men and extensive treatment for HIV with a triple-class viral resistance profile. He also had long-standing leg pain thought to be secondary to HIV neuropathy and no history of intravenous drug use. There was no history of opportunistic or chronic infections.

Because of a persistently elevated viral load, enfuvirtide by subcutaneous injection was added to his highly active antiretroviral treatment regimen for 41 months; enfuvirtide therapy was then stopped in February 2007 because of intolerable injection site reactions. While he was receiving enfuvirtide, his viral loads were completely suppressed. Eighteen months after enfuvirtide therapy was stopped, large, tender, indurated reactions with fragile epithelial surfaces persisted at all injection sites (**Figure**, *top*). These reactions bled extensively into the subcutaneous tissue with minor trauma (**Figure**, *bottom*). A lesion on the triceps was excised surgically, and the wound healed without complications. Pathologic examination showed extensive deposits of proteinaceous material with intense Congo red staining that was consistent with amyloid. A lesion on the opposite arm was resected and showed similar findings. The patient had a normal leukocyte count and normal hemoglobin, blood urea nitrogen, and creatinine levels and had no evidence of plasma cell dyscrasia and no history of organ dysfunction to suggest systemic amyloidosis.

Discussion: In 7 patients receiving enfuvirtide, biopsy of injection site reactions revealed an inflammatory response consistent with a localized hypersensitivity reaction (5), and other studies (3) have reported similar findings. Other reports (6) have described 3 histologic patterns: an acute urticaria- or vasculitis-like pattern with inflammation of the fat tissue, a subacute pattern with an initial dermal sclerosis, and a long-term scleroderma-like pattern.

In our patient, surgical excision of enfuvirtide injection site reactions revealed subcutaneous nodular amyloidosis. Localized

Figure. **Lesion in right triceps area (*top*) and periumbilical site with spontaneous intradermal and subcutaneous hemorrhage (*bottom*).**



DNA vaccination against macrophage migration inhibitory factor improves atopic dermatitis in murine models

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Background: Atopic dermatitis (AD) is a common chronic inflammatory skin disease. Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that has been implicated in the pathogenesis of AD. Recently, we developed a novel DNA vaccine that generates neutralizing endogenous anti-MIF antibodies.

Objective: This study explores the preventive and therapeutic effects of this MIF-DNA vaccine in mouse models of AD.

Methods: Two different AD model mice (DS-Nh and NC/Nga) received MIF-DNA vaccination to analyze preventive and therapeutic effects, as assessed by clinical skin scores, histologic findings, and serum IgE levels.

Results: In murine models of AD, MIF-DNA vaccination prevented the occurrence of the AD skin phenotype. Furthermore, administration of MIF-DNA vaccine to mice that had already developed AD produced a rapid improvement in AD skin manifestation. There were reduced histologic signs of inflammation and lower serum IgE levels in treated mice compared with those seen in control animals. Finally, passive transfer of IgG from MIF-DNA vaccinated mice to AD mice also produced a significant therapeutic effect. These results demonstrate that MIF-DNA vaccination not only prevents the development of AD but also improves the symptoms of pre-existing AD.

Conclusion: Taken together, the induction of an anti-MIF autoantibody response using MIF-DNA vaccination appears to be a useful approach in the treatment of AD. (J Allergy Clin Immunol 2009;124:90-9.)

Key words: Atopic dermatitis, macrophage migration inhibitory factor, DNA vaccination

Abbreviations used
AD: Atopic dermatitis
MIF: Macrophage migration inhibitory factor
TTX: Tetanus toxin P30 T_H epitope

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease with significant morbidity and an adverse effect on patient well-being.[1] The prevalence of AD has increased 2- to 3-fold during the past 3 decades in industrialized countries, and it presently occurs in 10% to 20% of children and 1% to 3% of adults.[2] AD is thought to result from a dysregulation in the normal interaction between the environment, genes, defects in skin barrier function, and systemic and local immunologic responses.[3] The contribution of the immune response to the pathogenesis of AD has been largely attributed to abnormalities in the adaptive immune system, with key roles played by T_H1/T_H2 cell dysregulation, IgE production, dendritic cell signaling, and mast cell hyperactivity, leading to the pruritic inflammatory dermatosis that characterizes AD.[3]

Macrophage migration inhibitory factor (MIF) is an upstream regulator of the inflammatory response, and it is upregulated in various inflammatory disorders, including AD.[4] We previously reported that serum MIF levels in patients with AD were significantly increased compared with those seen in healthy control subjects and patients without AD.[5] In addition, circulating MIF levels in patients with AD decrease as the clinical features of the disease improve, suggesting that MIF might play a pivotal role in the inflammatory response in these patients.[5,6] Moreover, MIF promotes IL-2 and IL-2 receptor expression and memory T-cell development, and it might influence T_H1/T_H2 cell differentiation responses.[6,7] Based on these observations suggesting that MIF might be a therapeutic target in AD, we hypothesized that inhibition of MIF with neutralizing antibodies might induce beneficial therapeutic effects in patients with AD.

Monoclonal antibodies directed against proinflammatory cytokines, such as TNF-α, have been used for the treatment of rheumatoid arthritis, Crohn disease, and psoriasis,[8-10] and there have been a few reports describing the use of anti–TNF-α mAbs for the treatment of AD.[7,8] The application of mAbs to AD nevertheless might be difficult because of the requirement for frequent injections, the large quantities of immunoglobulin protein required, and the associated costs of production. Moreover, even fully humanized antibodies are potentially immunogenic and might elicit antibody responses, thereby limiting their long-term therapeutic efficacy. These limitations have led to the development of alternative neutralization strategies, including methods that aim to elicit autoantibodies against target proteins,

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such as cytokines or pathogens, by administering them in a naked or partially modified form as therapeutic vaccines.

We recently developed an MIF-DNA vaccine that breaks immunologic tolerance by introducing oligonucleotides encoding a foreign T_H cell epitope into the murine MIF cDNA sequence.[11,12] We demonstrated that this MIF-DNA vaccination elicits production of endogenous anti-MIF antibodies and showed a significant amelioration of symptoms in murine models of rheumatoid arthritis[9] and sepsis.[10]

The present study describes for the first time the preventive and therapeutic effects of this MIF-DNA vaccine in 2 different mouse models of AD.

METHODS

Animals

Six-week-old female BALB/c mice were purchased from Japan Clea (Shizuoka, Japan). Male DS-Nh mice were provided by Aburabi Laboratories, Shionogi and Co, Ltd (Shiga, Japan), and male NC/Nga mice were purchased from SLC (Hamamatsu, Japan). All mice were bred and housed under conventional conditions, and procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

Production of DNA vaccine

We previously reported the design of the MIF/tetanus toxin P30 T_H epitope (TTX) DNA expression plasmid and our analysis of the *in vitro* expression of MIF/TTX by using this plasmid.[11] For the generation of immunologically active MIF antigen, an MIF construct harboring a T_H epitope at its second loop region was designed. For that purpose, the coding region for the second loop of the mouse MIF, amino acids 32 to 37 (GKPAQY), was deleted from the MIF cDNA and substituted with an *Eco*RI site. A complementary DNA coding for the TTX (FNNFTVSFWLRVPKVSASHL) with *Eco*RI sites at both termini was obtained by means of hybridization of partially overlapping oligo DNAs (sense, ggaattcaacaacttcaccgtgagcttctggctgcgcgtgcccaa; antisense, ggaattccaggtggctggcgctcaccttgggcacgcgcagccaga) after polymerization with the Klenow fragment of DNA polymerase. After digestion with *Eco*RI, the cDNA coding for the P30 T_H epitope was inserted into the *Eco*RI site of the MIF expression plasmid lacking the second loop, and a clone with the insert of correct orientation was selected. For vaccination, the plasmid DNA was purified by using standard methods with alkaline lysis followed by 2 rounds of CsCl density gradient ultracentrifugation.

Vaccination protocols

Gene transfer into muscle by means of electroporation was performed as described previously.[11] Briefly, mice were anesthetized with ether and shaved near their hind legs. A pair of electrode needles (5-mm gap and 0.5-mm diameter; NEPA GENE, Chiba, Japan) was then inserted into an anterior tibial muscle, and DNA vaccine (25 μg/25 μL of 0.9% saline) was injected into the portion between the needles. Electrical pulses (50 V, 50 ms, 3 times) were applied (T820 and Optimizer 500; BTX, San Diego, Calif) and followed by another 3 pulses with inverted polarity. The same injection and electroporation was applied to the other tibial muscle. Thus 50 μg of the naked plasmid was injected per mouse into the tibias. A similar vaccination was repeated 3 weeks later.

Evaluation of anti-MIF antibody titer in sera of DNA-vaccinated mice

Anti-MIF titers in plasma were determined by means of direct ELISA. Briefly, individual plasma from vaccinated mice were collected from the tail vein and diluted with 0.1% BSA/PBS/0.05% Tween 20. Small aliquots of diluted plasma (1:200) were added into 96-well flat-bottom plates precoated with recombinant MIF. Anti-MIF antibodies that reacted with the precoated recombinant MIF were detected with goat anti-mouse antibody conjugated with horseradish peroxidase, followed by color development with substrate reagent (Techne, Minneapolis, Minn).

Evaluation of clinical skin severity score

Mice were macroscopically observed and scored by 2 persons blind to the treatment protocol. Before skin conditions were scored, scratching behavior was observed for 2 minutes. A total clinical severity for AD-like lesions was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of 5 signs and symptoms (itch, erythema, edema, excoriation/erosion, and scaling/dryness).[13]

Measurement of IgE and TNF-α levels in sera

Serum total IgE levels were measured by using a sandwich ELISA kit (Yamasa Shouyu, Chiba, Japan). Serum MIF levels were assayed with ELISA kits for Genetic Lab (Sapporo, Japan). The ELISA procedures were conducted according to the manufacturer's instructions. The concentration of TNF-α was determined by using the BD Cytometric Bead Array (BD PharMingen, San Jose, Calif). Flow cytometric analysis was carried out with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif).

Real-time PCR analysis

Total RNA was extracted from dorsal skin to quantify cytokine mRNA expression levels in dermatitis lesions. RNA samples were analyzed with the ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, Calif). Primers and probes specific for IL-1β, IL-4, IL-6, and IFN-γ were obtained from the TaqMan gene expression assay (Applied Biosystems). Differences between the mean cycle threshold (CT) values of cytokines and those of β-actin (Applied Biosystems) were calculated as

$$\Delta CT_{sample} = CT_{cytokine} - CT_{\beta-actin},$$

and those of ΔCT for the normal adult skin were calculated as

$$\Delta CT_{calibrator} = CT_{cytokine} - CT_{\beta-actin}.$$

Final results for fetal skin sample/adult skin (as percentages) were determined as $2-(\Delta CT_{sample} - \Delta CT_{calibrator})$.

Histologic analysis

Six-micrometer-thick sections of dorsal skin were stained with hematoxylin and eosin, acidic toluidine blue (pH 4.0) for mast cells, and direct fast scarlet for eosinophils. Cells between the epithelium and panniculus carnosus were counted at a magnification of ×400 and were expressed as the total number of cells in 5 fields.

Treatment of neutralizing MIF mAbs

Neutralizing anti-MIF mAb (NIH-III.D9) was previously described.[14] Neutralizing MIF mAbs (50 μg) or control IgG (50 μg) were injected intravenously into 15-week-old NC/Nga mice with dermatitis twice a week for 3 weeks.

Adoptive transfer of autoantibodies elicited by DNA vaccines

IgG was purified from the sera of control pCAGGS plasmid– or MIF/TTX-vaccinated DS mice at 6 weeks after the vaccination by using the protein A Antibody Purification Kit (Amersham Biosciences, Piscataway, NJ). The purified IgG was tested for its ability to suppress ongoing dermatitis in an adoptive transfer experiment. DS-Nh mice with developing dermatitis were separated at 15 weeks of age into 3 equally sick groups of 3 mice each. Every 3 days, these animals were administered 50 μg per mouse of purified IgG from control pCAGGS plasmid–vaccinated DS-Nh mice, purified IgG from MIF/TTX-vaccinated DS-Nh mice, or an equal volume of PBS.

RESULTS

MIF/TTX vaccination prevents the onset of AD in DS-Nh mice

DS-Nh mice housed under conventional conditions but not in a specific pathogen free environment spontaneously exhibit AD-like skin symptoms, including erythema, edema, excoriation, erosion, dry skin, and desquamation.[15-17] Early skin symptoms appear around 9 weeks of age and continue to worsen until age 25 weeks. An increase in total serum IgE levels is detected at approximately 17 weeks of age and after the development of skin lesions.[15-17]

We first examined the potential protective effect of the MIF/TTX vaccine on dermatitis development by treating 9-week-old DS-Nh mice before the development of skin eruptions. The clinical features of the control pCAGGS plasmid–vaccinated mice were similar to those of untreated mice. At 18 weeks of age, or 9 weeks after the vaccination, both groups of mice showed severe erythema, erosions, and dry skin (Fig 1, *A*). By contrast, the MIF/TTX-vaccinated mice exhibited almost no eruptions (Fig 1, *B*). The clinical skin score of MIF/TTX-vaccinated mice was low until 21 weeks of age (Fig 1, *C*), which is a time at which the MIF/TTX-vaccinated mice showed high serum levels of anti-MIF antibodies (Fig 1, *D*). Furthermore, in the MIF/TTX-vaccinated mice the serum level of IgE was significantly decreased and the serum MIF level was only slightly decreased when compared with those seen in the control vaccinated mice (Fig 1, *E* and *F*). In addition, cytokine expression in affected skin lesions was analyzed by using real-time PCR. The T_H2 cytokine IL-4 was very slightly downregulated, and the T_H1 cytokine IFN-γ was slightly upregulated. Of note, the expression of the proinflammatory cytokines IL-1β and IL-6 was significantly suppressed in MIF-vaccinated mice compared with that seen in control mice (Fig 1, *G*). Therefore the inhibition of MIF in the atopic model mice appears to result primarily in the suppression of inflammation rather than affecting the T_H1/T_H2 cytokine balance.

Improvement of clinical skin condition with MIF/TTX vaccine also was confirmed by the observation that the lesions of mice vaccinated with MIF/TTX vaccine showed amelioration in hyperkeratosis, acanthosis, dermal edema, and infiltration of the inflammatory cells at 21 weeks when compared with the condition of mice vaccinated with control pCAGGS plasmid (Fig 2, *A*). At the affected skin sites, the numbers of eosinophils and mast cells decreased significantly in the MIF/TTX-vaccinated mice at 21 weeks when compared with those seen in the control vaccinated mice (Fig 2, *B-D*).

These data clearly show that MIF/TTX vaccination can prevent the onset of AD-like dermatitis in DS-Nh mice.

MIF/TTX vaccination improves pre-existing AD

To determine whether the MIF/TTX vaccine has any therapeutic effect in AD, we next vaccinated 15-week-old DS-Nh mice with pre-existing AD and evaluated the progression of skin changes. Mice treated with the control plasmid continued to exhibit severe dermatitis 6 weeks after vaccination (Fig 3, *A*). By contrast, the MIF/TTX vaccination significantly improved dermatitis symptoms (Fig 3, *B*). The clinical skin scores of control-vaccinated mice increased after the vaccination, whereas that of MIF/TTX-vaccinated mice began to decrease at 21 weeks of age (Fig 3, *C*). At this time, the MIF/TTX-vaccinated mice showed high levels of anti-MIF antibodies (Fig 3, *D*). Furthermore, the serum IgE and MIF levels of MIF/TTX-vaccinated mice also were lower than those of control mice at 21 weeks of age (Fig 3, *E* and *F*). In addition, serum TNF-α levels were significantly lower in the MIF/TTX-vaccinated mice when compared with those seen in the control plasmid–vaccinated mice (Fig 3, *G*).

By means of histologic analysis, the lesions of mice vaccinated with MIF/TTX vaccine showed improvement of hyperkeratosis, acanthosis, dermal edema, and infiltration of inflammatory cells at 21 weeks when compared with the control plasmid–vaccinated mice (Fig 4, *A*). In addition, the numbers of eosinophils and mast cells decreased significantly in the MIF/TTX-vaccinated mice at 21 weeks when compared with those seen in the control mice (Fig 4, *B-D*). The serum IgE level of MIF/TTX-vaccinated mice decreased at 21 weeks of age compared with that of control-vaccinated mice (Fig 4, *E*).

These data indicate that MIF/TTX vaccination leads to an improvement in already established dermatitis in the DS-Nh mice.

We further observed that MIF-DNA vaccine improved the manifestation of pre-existing AD in a second model of AD, which develops in the NC/Nga strain.[11,13] We vaccinated 15-week-old NC/Nga mice with dermatitis, and although the control pCAGGS plasmid–vaccinated mice still had severe dermatitis 6 weeks after the vaccination treatment (Fig 5, *A*), the MIF/TTX-vaccinated mice showed significant improvement (Fig 5, *B*). The clinical skin score of control-vaccinated mice increased after the vaccination, whereas that of MIF/TTX-vaccinated mice began to decrease at 21 weeks of age (Fig 5, *C*). At this time, the MIF/TTX-vaccinated mice showed high levels of anti-MIF antibodies (Fig 5, *D*). The serum IgE and MIF levels of MIF/TTX-vaccinated mice decreased at 21 weeks of age (Fig 5, *E* and *F*). These data show that MIF-DNA vaccination improves dermatitis not only in the DS-Nh strain but also in the NC/Nga mouse strains.

To confirm that anti-MIF antibodies suppress AD, we performed an additional therapeutic experiment by using a neutralizing anti-MIF mAb. Anti-MIF mAb (50 μg) or an isotypic control IgG (50 μg) were injected intravenously into 15-week-old NC/Nga mice with dermatitis twice a week for 3 weeks. Anti-MIF mAbs, as well MIF vaccination, significantly improved AD skin manifestations when compared with conditions seen in control IgG–treated mice (Fig 5, *G* and *H*).

Adoptive transfer of autoantibodies elicited by MIF/TTX-DNA vaccine suppressed AD

To better substantiate that the therapeutic action of MIF/TTX-DNA vaccination could be attributed to anti-MIF autoantibodies, we performed adoptive transfer of purified IgG from vaccinated DS-Nh mice into naive DS-Nh mice. The purified IgG was adoptively transferred into the 15-week-old DS-Nh mice that had already demonstrated skin eruptions. As shown in Fig 6, this IgG was effective in ameliorating AD, indicating that the therapeutic effect of MIF/TTX vaccination could be adoptively transferred by immune serum IgG.

DISCUSSION

In the present study we have shown that active vaccination against MIF is a novel preventive and therapeutic approach in 2 murine models of AD. We showed that mice administered a MIF/TTX-DNA vaccine did not experience the cutaneous manifestations of AD. The MIF/TTX-DNA vaccine also improved the symptoms

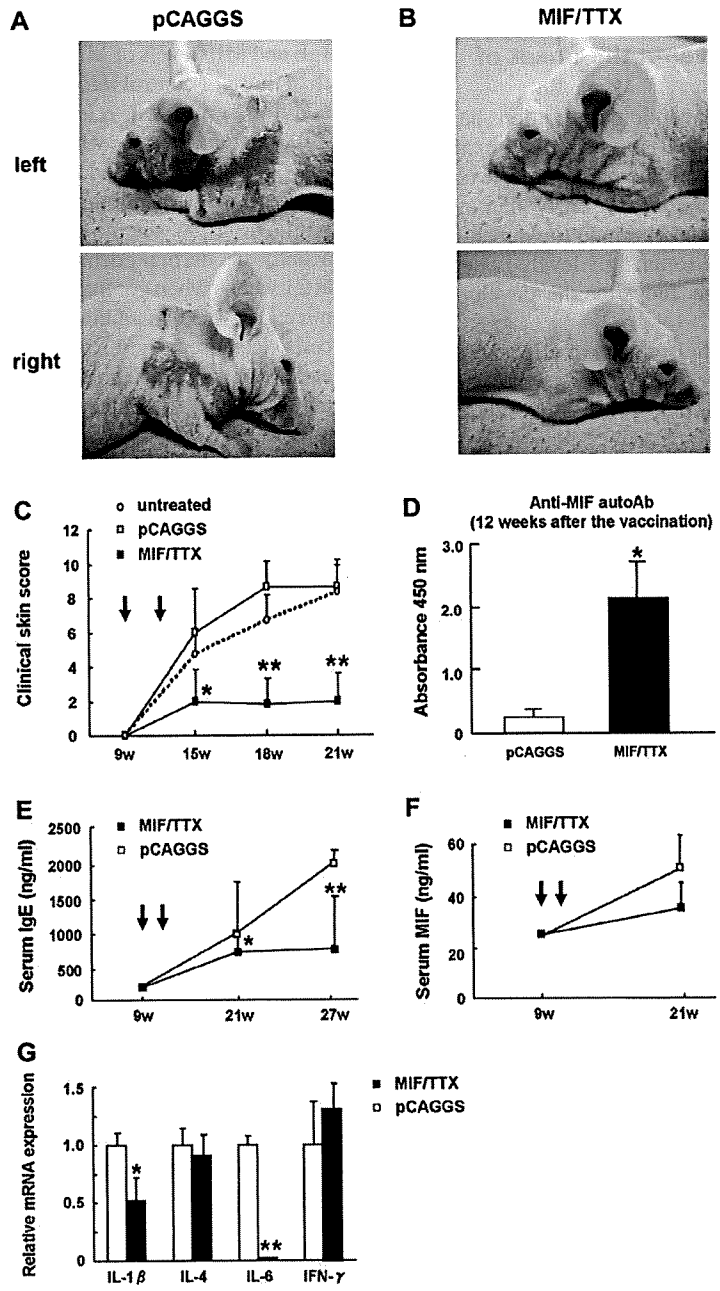

FIG 1. Prevention of the onset of AD by MIF/TTX-DNA vaccine. Nine-week-old DS-Nh mice without skin eruptions were subjected to administration of MIF/TTX or a control plasmid (pCAGGS). Clinical features of 21-week-old DS-Nh mice vaccinated with endotoxin-free pCAGGS **(A)** and MIF/TTX (**B**; 12 weeks after the vaccination) are shown. **C**, The clinical skin score of mice immunized with the MIF/TTX-DNA vaccine *(solid squares)*, immunized with pCAGGS plasmid *(open squares)*, or left untreated *(open circles)*. Results are given as means ± SEs of 5 mice in each group. $*P < .01$ and $**P < .005$ versus pCAGGS at the same time point. **D**, Serum level of anti-MIF autoantibodies *(autoAb)* at 12 weeks after vaccination. Means ± SEs are shown (n = 5). $*P < .01$. **E**, Serum IgE levels of the mice vaccinated with MIF/TTX *(solid squares)* and pCAGGS *(open squares)*. $*P < .01$ and $**P < .005$ for MIF/TTX versus pCAGGS at the same time point. Means ± SEs of 5 mice in each group are shown. **F**, Serum MIF levels of the mice vaccinated with MIF/TTX *(solid squares)* and pCAGGS *(open squares)*. **G**, Cytokine expression (IL-1β, IL-4, IL-6, and IFN-γ) in affected skin lesions was analyzed by using real-time PCR. $*P < .05$ and $**P < .001$.

of pre-existing AD in 2 different strains of AD-prone mice, the DS-Nh and NC/Nga strains. Finally, we demonstrated that the therapeutic effect of MIF/TTX vaccination could be adoptively transferred by serum IgG that contained MIF autoantibodies.

Proinflammatory cytokines are believed to be important contributors to the pathogenesis of skin inflammation in patients with AD, which might depend on the duration of the skin lesion. Patients with acute AD typically have a systemic T_H2 response

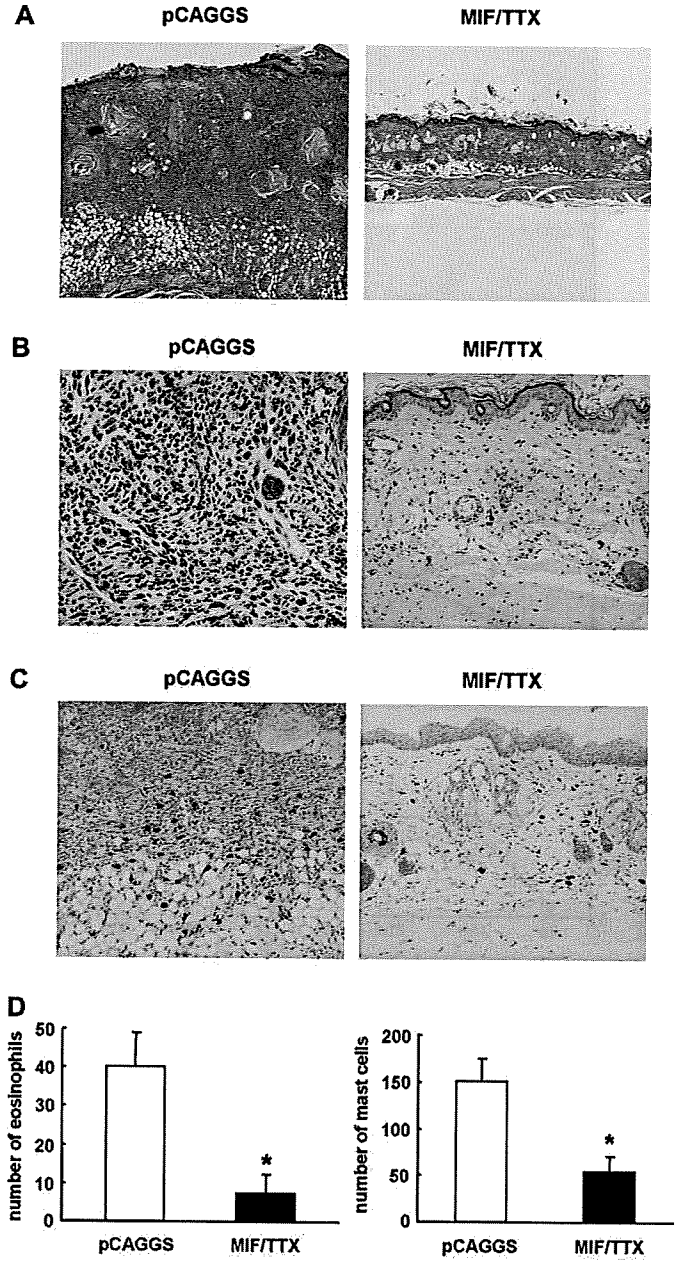

FIG 2. Histologic analysis of AD in DS-Nh mice vaccinated with MIF/TTX or pCAGGS plasmid before disease onset. Nine-week-old DS-Nh mice without eruptions were administered MIF/TTX or control plasmid. Specimens were collected from the dorsal skin 6 weeks after the first vaccination and stained with hematoxylin and eosin (**A**, original magnification ×40), direct fast scarlet for eosinophils (**B**, original magnification ×200), or toluidine blue for mast cells (**C**, original magnification ×200). **D**, The number of eosinophils and mast cells in 5 high-power fields from 4 individual skin specimens were enumerated by means of microscopy. Means ± SEs of 4 mice are shown. *$P < .001$ for MIF/TTX versus pCAGGS.

with increased serum IgE levels, eosinophilia, and a marked infiltration of T_H2 cells into acute skin lesions. The infiltrating T cells show a predominance of IL-4, IL-5, IL-10, and IL-13 expression.[12,18] In patients with chronic AD, however, there is infiltration of eosinophils and macrophages, and the disease becomes associated with an increase in the expression of IL-12, with a switch to T_H1 cellular responses.[12,18] Chronic AD skin lesions in adults with a prolonged duration of disease have been shown to manifest an increase in the expression of IL-1, IL-5, IL-12, IFN-γ, TNF-α, GM-CSF, and MIF.[12,18] This biphasic T_H1/T_H2 switch in immune response is characterized pathologically by lichenification, epidermal hyperplasia, and dermal fibrosis. MIF regulates the production of various proinflammatory cytokines, including TNF-α, and the inflammatory cytokines in response to stimulation by LPS are known to be suppressed in MIF-deficient mice. We previously reported that MIF-deficient mice have an impaired contact hypersensitivity (CH) response and that immunoneutralization of MIF effectively suppresses CH response[19]; these observations led us to speculate that MIF would be a therapeutic target for AD.

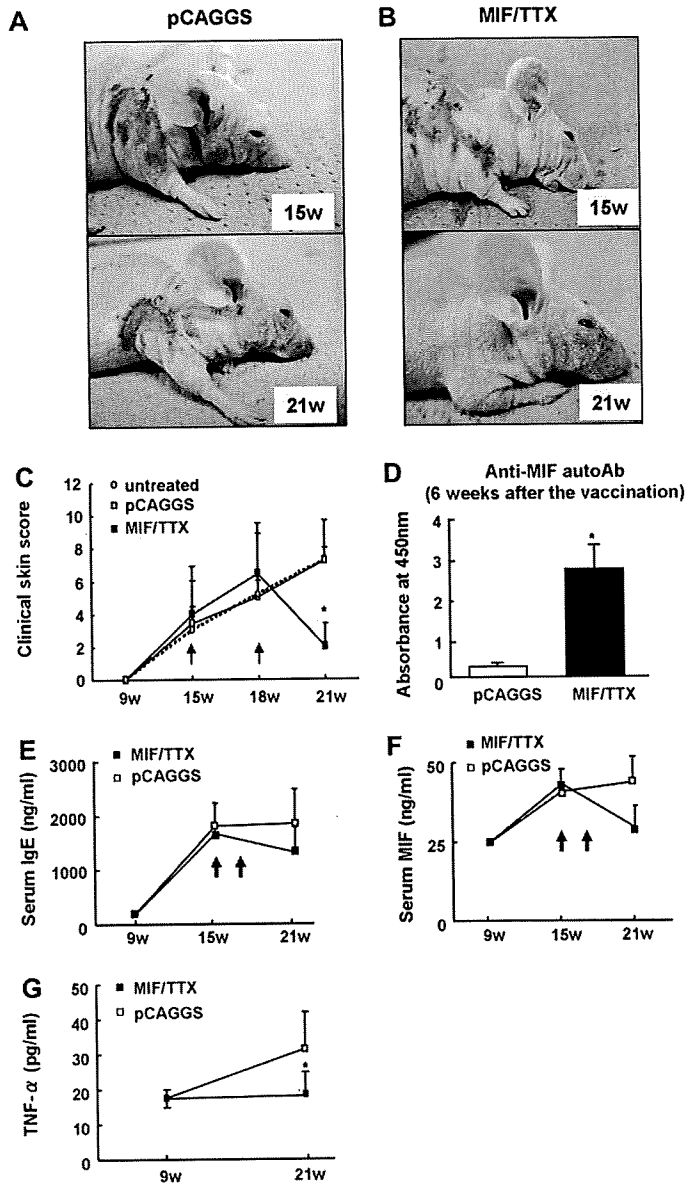

FIG 3. Therapeutic effect of MIF-DNA vaccination in DS-Nh mice with pre-existing AD. Fifteen-week-old DS-Nh mice with ongoing dermatitis were administered MIF/TTX or control plasmid (pCAGGS) or left untreated. **A**, Clinical features of DS-Nh mice vaccinated with control plasmid. **B**, Clinical features of DS-Nh mice vaccinated with MIF/TTX. **C**, Clinical skin scores of mice immunized with MIF/TTX-DNA vaccine *(solid squares)*, control plasmid *(open squares)*, or left untreated *(open circles)*. Means ± SEs of 10 mice per group are shown. $*P < .005$ for MIF/TTX versus pCAGGS. **D**, Serum level of anti-MIF autoantibodies *(autoAb)* at 6 weeks after the vaccination. Means ± SEs are shown (n = 10). $*P < .001$. Serum IgE **(E)** and MIF **(F)** levels of the mice vaccinated with MIF/TTX vaccine *(solid squares)* and control pCAGGS plasmid *(open squares)* are shown. The data shown are for 10 mice per group. **G**, The serum levels of TNF-α were decreased in MIF/TTX-vaccinated mice *(solid squares)* compared with those seen in the control (pCAGGS) plasmid–vaccinated mice *(open squares)*. $*P < .01$ for MIF/TTX versus pCAGGS.

The therapeutic aim of cytokine vaccine therapy is to induce high titers of circulating polyclonal autoantibodies to neutralize the pathologic levels of a particular cytokine. The advantages of this therapy include the potential to maintain high antibody titers, long-term efficacy, and low cost. Monoclonal antibodies directed against TNF-α have been used for the treatment of psoriasis.[10] Jacobi et al[7] recently reported a clinical trial of infliximab monotherapy for 9 patients with moderate or severe AD who showed significant improvement in all clinical parameters; however, this improvement was not sustained by maintenance of the therapy. The authors considered that the development of antichimeric antibodies could explain the lack of a durable response to infliximab maintenance therapy. A cytokine vaccine results in the production of native antibodies, and it might overcome this limitation in anti-cytokine antibody therapy.

It is unknown whether long-term inhibition of MIF activity might be safe in human subjects. A major limitation of an active immunization approach is the inability to control the outcome. However, it should be noted that serum MIF levels in the MIF-DNA–vaccinated mice were maintained at a baseline level

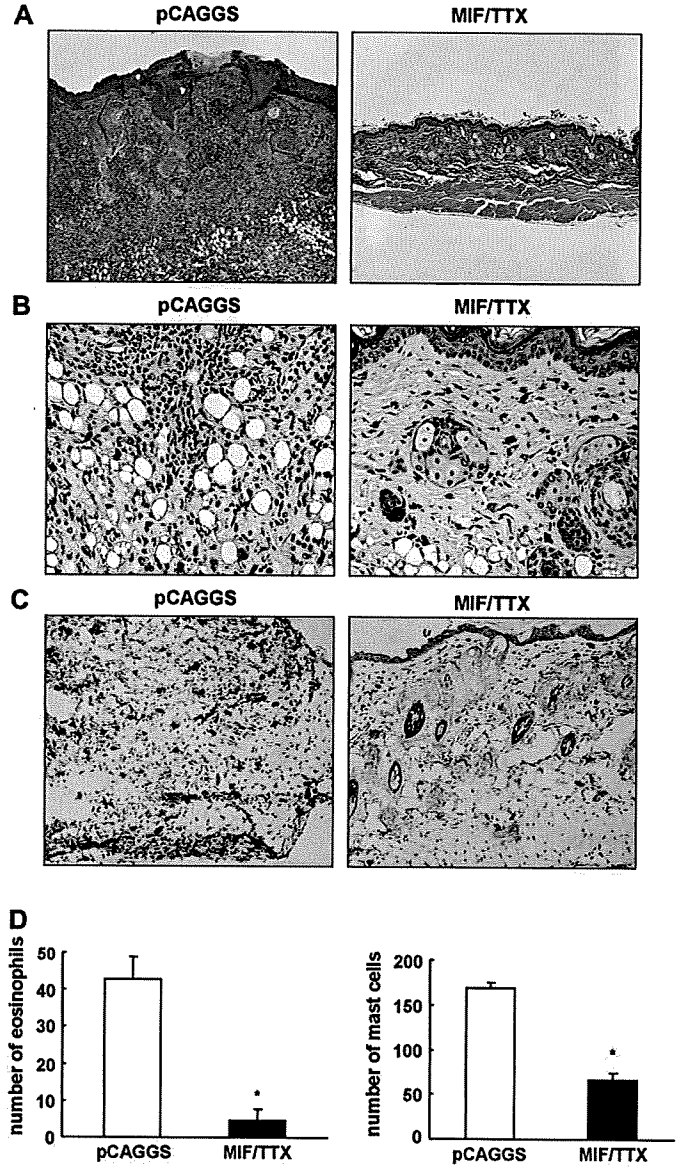

FIG 4. Histologic analysis of AD in DS-Nh mice vaccinated with MIF/TTX or control plasmid after disease onset. Fifteen-week-old DS-Nh mice with ongoing dermatitis were administered MIF/TTX or control (pCAGGS) plasmid. Specimens were collected at 6 weeks after the first vaccination and stained with hematoxylin and eosin (**A**, original magnification ×40), direct fast scarlet for eosinophils (**B**, original magnification ×200), or toluidine blue for mast cells (**C**, original magnification ×200). **D**, The number of eosinophils and mast cells in 5 high-power fields from 4 individual skin specimens were enumerated by means of microscopy. Data represent means ± SEs of 4 mice. *$P < .001$ for MIF/TTX versus pCAGGS.

(Figs 2, *F*, and 4, *F*), despite an anti-MIF antibody level that remained high for 6 weeks of vaccine administration. It is possible that the present protocol of vaccination dose not induce a high enough level of anti-MIF antibody to inhibit serum MIF protein completely. There are reports that autoantibody production induced by vaccine-encoded antigens regress to baseline levels shortly after remission in acute experimental autoimmune encephalomyelitis,[15] whereas in adjuvant-induced arthritis[16] autoantibodies continue to be produced at high titer. It has been considered that targeted DNA vaccines amplify a pre-existing anti–self-regulatory response that by itself is capable of limiting, although not preventing, the emerging autoimmune condition.[15-17,20-22] In addition, we observed that MIF-DNA–vaccinated mice did not show serious side effects, such as evident infections.

Serum IgE levels were significantly decreased when MIF vaccination was used as a preventive agent, whereas the levels were not significantly decreased when it was used as a therapeutic agent. It is known, however, that IgE levels do not parallel the clinical severity of AD in human patients.[19]

The cardinal principles in the treatment of AD are to reduce symptoms, prevent exacerbations, and minimize medication side effects. This approach incorporates the use of emollients, topical corticosteroids, topical calcineurin inhibitors, antihistamines, stress management, and avoidance of allergens or

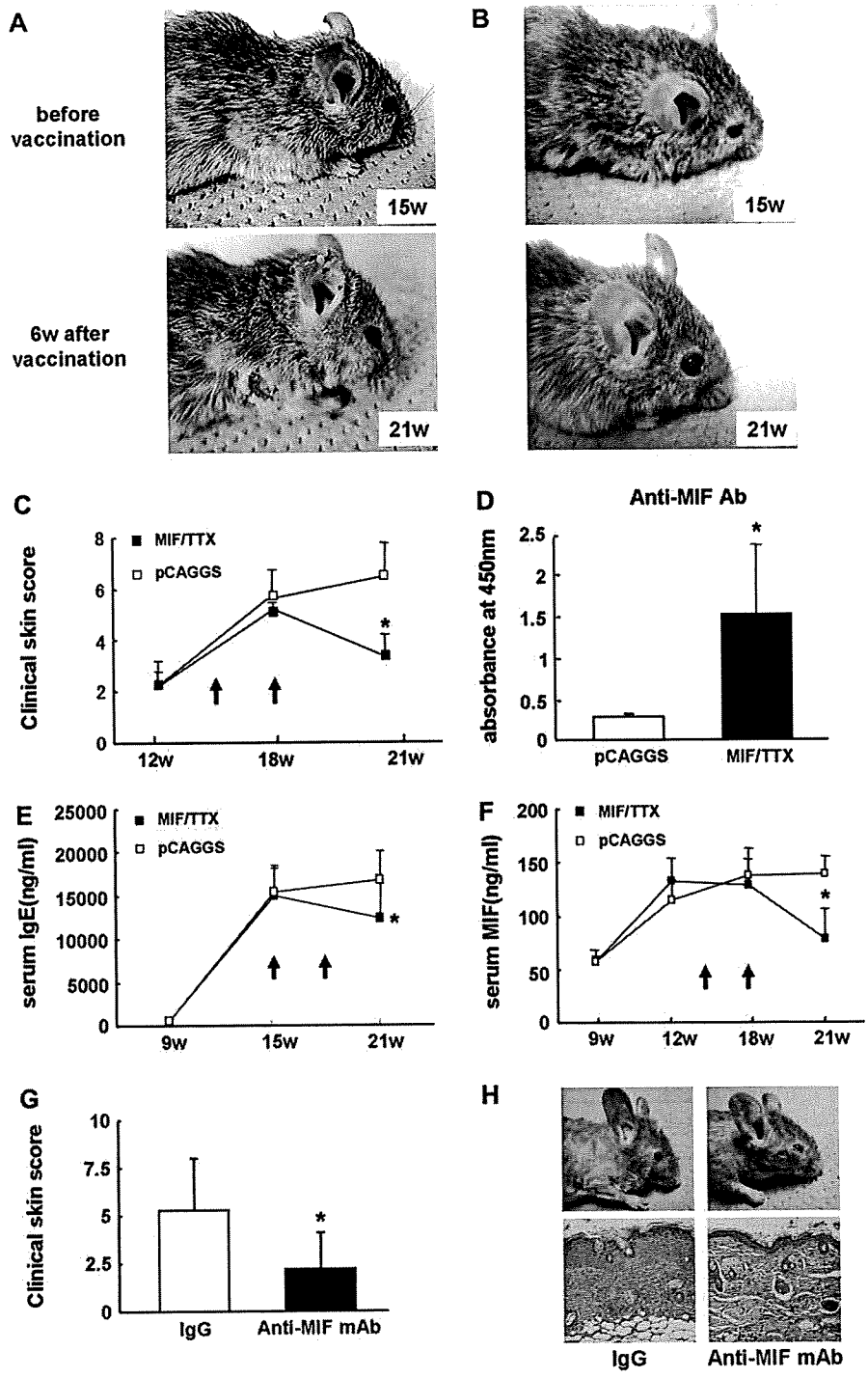

FIG 5. Therapeutic effect of MIF/TTX-DNA in vaccine for pre-existing AD on NC/Nga mice. Fifteen-week-old NC/Nga mice with dermatitis were vaccinated with control pCAGGS plasmid or MIF/TTX. **A**, Clinical features of NC/Nga mice vaccinated with control pCAGGS plasmid. **B**, Clinical features of DS-Nh mice vaccinated with MIF/TTX (*top*, vaccination). **C**, Clinical skin scores of mice vaccinated with MIF/TTX *(solid squares)* and control pCAGGS plasmid *(open squares)*. $*P < .05$ for MIF/TTX versus pCAGGS at the same time point. Each point represents means ± SEs of 10 mice in each group. **D**, Serum level of anti-MIF autoantibodies *(Ab)* at 6 weeks after the vaccination. Means ± SEs are shown (n = 10). $*P < .005$. Serum IgE **(E)** and MIF **(F)** levels of mice vaccinated with MIF/TTX vaccine *(solid squares)* and control pCAGGS plasmid *(open squares)* are shown. Means ± SEs of 10 mice in each group are shown. $*P < .0005$. **G** and **H**, Neutralizing anti-MIF mAbs (50 μg) or control IgG (50 μg) were injected intravenously into 15-week-old NC/Nga mice with dermatitis twice a week for 3 weeks. Fig 5, *G*, shows clinical skin scores ($*P < .05$). In Fig 5, *H*, the *upper panels* show clinical features, and the *lower panels* show histologic images.

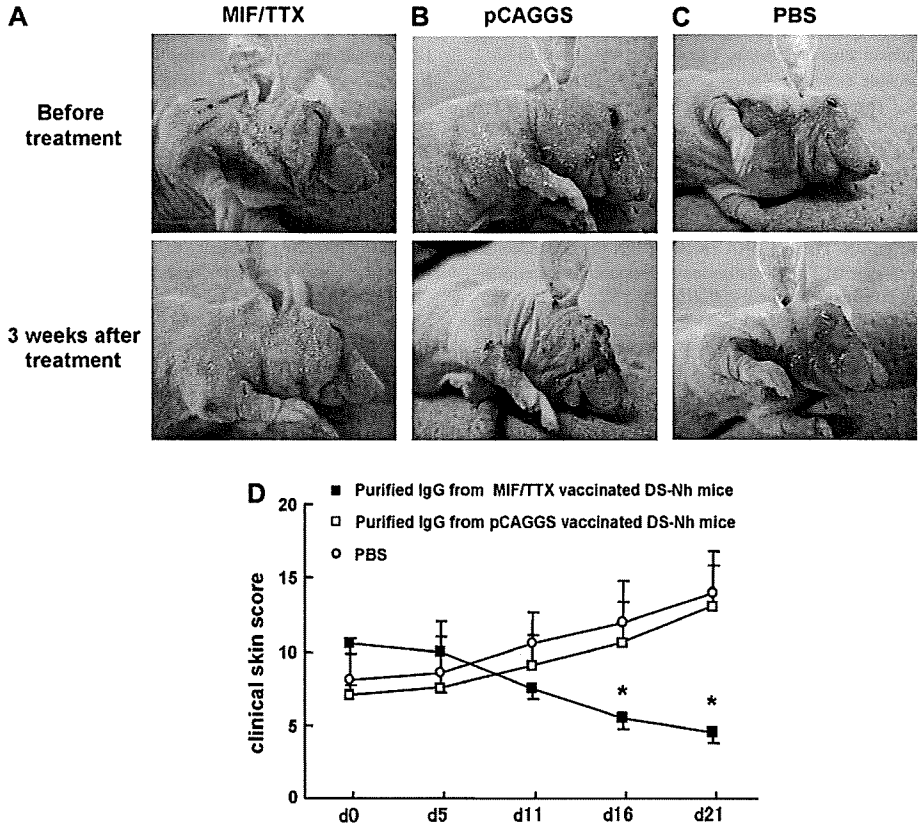

FIG 6. Adoptive transfer of autoantibodies elicited by DNA vaccines. IgG were purified from the sera of control (pCAGGS) plasmid– or MIF/TTX-vaccinated DS mice and adoptively transferred. DS-Nh mice were administered 50 μg per mouse of purified IgG from control plasmid–vaccinated DS-Nh mice, purified IgG from MIF/TTX-vaccinated DS-Nh mice, or PBS. **A-C**, Clinical features at 3 weeks after administration of purified IgG from MIF/TTX-vaccinated mice (Fig 6, *A*), purified IgG from control pCAGGS plasmid–vaccinated mice (Fig 6, *B*), and clinical features after administration of PBS (Fig 6, *C*). **D**, Clinical skin scores of DS-Nh mice administered IgG from MIF/TTX-vaccinated DS-Nh mice *(solid squares)*, IgG from control pCAGGS plasmid–vaccinated DS-Nh mice *(open squares)*, or PBS *(open circles)*. Results are shown as means ± SEs of 3 mice in each group. $*P < .05$ versus pCAGGS at the same time point.

disease triggers.[3] The fact that the treatment of AD mainly depends on the self-application of topical agents often hinders the effective and long-term treatment of the disease. Notwithstanding these conventional treatments, if a patient with severe and refractory AD requires additional therapy, cyclosporine A has been used despite systemic side effects, such as renal toxicity.[23,24] The relapsing and remitting course of AD also places a psychologic, social, and financial burden on patients and their families. New treatment options are needed to prevent the progression of AD to more severe forms of disease and to halt the so-called atopic march toward asthma. An MIF-DNA vaccine approach offers the additional advantage of requiring only a periodic booster injection, and it might allow for the potential resolution of immunopathology in those with chronic refractory disease.

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Clinical implications: MIF-DNA vaccination might be a useful preventive and therapeutic approach for AD.

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Stem Cells, Tissue Engineering and Hematopoietic Elements

Bone Marrow-Derived Cells Are Not the Origin of the Cancer Stem Cells in Ultraviolet-Induced Skin Cancer

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Several lines of evidence have demonstrated that various cancers are derived from cancer stem cells (CSCs), which are thought to originate from either tissue stem or progenitor cells. However, recent studies have suggested that the origin of CSCs could be bone marrow-derived cells (BMDCs); for example, gastric cancer, which follows persistent gastric inflammation, appears to originate from BMDCs. Although our previous research showed the capability of BMDCs to differentiate into epidermal keratinocytes, it has yet to be determined whether skin CSCs originate from BMDCs. To assess the possibility that BMDCs could be the origin of CSCs in skin squamous cell carcinoma (SCC), we used a mouse model of UVB-induced skin SCC. We detected a low percentage of BMDCs in the lesions of epidermal dysplasia (0.59%), SCC *in situ* (0.15%), and SCC (0.03%). Furthermore, we could not find any evidence of clonal BMDC expansion. In SCC lesions, we also found that most of the BMDCs were tumor-infiltrating hematopoietic cells. In addition, BMDCs in the SCC lesions lacked characteristics of epidermal stem cells, including expression of stem cell markers (CD34, high α6 integrin) and the potential retention of BrdU label. These results indicate that BMDCs are not a major source of malignant keratinocytes in UVB-induced SCC. Therefore, we conclude that BMDCs are not the origin of CSCs in UVB-induced SCC. ***(Am J Pathol 2009, 174:595–601; DOI: 10.2353/ajpath.2009.080362)***

Stem cells, which have the capacity to self-renew and to differentiate into the various mature cells that constitute the tissue of organ, are found in many adult tissues including the skin.[1] Stem cells are critical for replenishing and maintaining the balance of cells (homeostasis) within the tissue and reconstituting tissue damaged during injury. Numerous studies have shown that the specific stem cell properties and the characteristics of stem-cell systems (populations of cells that derive from stem cells are organized in a hierarchical manner) are relevant to some forms of human cancer.[2,3] In cancers, cancer stem cells (CSCs) are thought to exist. CSCs, like tissue stem cells, would have a capacity for self-renewal and a proliferative ability with successive expansion potential promoting tumor structure organization Tumor-initiating cells, which are considered to be a population rich in CSCs, have been identified in cancers of the hematopoietic system[4,5] and various organs.[6–10]

Although several lines of evidence indicate that CSCs can arise from tissue stem cells[6,8,11,12] or mutated progenitor cells[13,14] current reports showed that gastric cancer, which follows persistent gastric inflammation because of the infection with *Helicobacter felis* (*H. felis*), appears to originate from bone marrow-derived cells (BMDCs).[15] Indeed, some populations of BMDCs have the potential to differentiate into mature cells of various nonhematopoietic organs including liver,[16] skeletal-muscle,[17] brain,[18] and skin.[19] We also showed that BMDCs and mesenchymal stem cells are able to transdifferentiate into keratinocytes.[20,21] BMDCs with this plasticity are frequently recruited to sites of injured or inflamed tissue, where they differentiate into mature tissue cells to contribute to tissue repair.[22] Results from *H. felis*-induced gastric cancer suggest that BMDCs with plasticity would differentiate into tissue stem or mature cells to reconstitute the damaged tissue, they then covert into CSCs, and contribute to carcinoma formation. Although recent investigations have demonstrated that BMDCs could contribute to cancers of small intestine, colon, lung,[23] larynx, and brain,[24] it is yet to be determined whether cancers originating from BMDCs certainly exist.

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Skin cancer is currently the most common malignancy in humans.[25] The skin has the role to protect our bodies from a wide range of environmental assaults including UVB irradiation, chemical carcinogens, and the entry of viruses and other pathogens. Therefore, epidermal keratinocytes have more opportunity to manifest maturation arrest. Particular epidemiological and scientific evidence has shown that UVB is one of the most important factors affecting skin carcinogenesis in the physical environment.[25,26]

As in the case of BMDC-originated gastric cancer after persistent inflammation with *H. felis* infection, it is presumed that BMDCs, which are recruited to the UVB-damaged epidermis and differentiate into epidermal keratinocytes to reconstitute the damaged skin, could then give rise to the maturation arrest during continuous UVB irradiation, convert into CSCs, and finally propagate to form bone marrow (BM)-derived skin cancer. Such a novel hypothesis, if true, would have profound implications for our present understanding of the pathogenesis of squamous cell carcinoma (SCC).

To investigate the possible role of BMDCs in skin cancer, we used a mouse model of UVB-induced skin SCC and evaluated the number and marker expressions of labeled BMDCs that differentiated into keratinocytes in skin SCC.

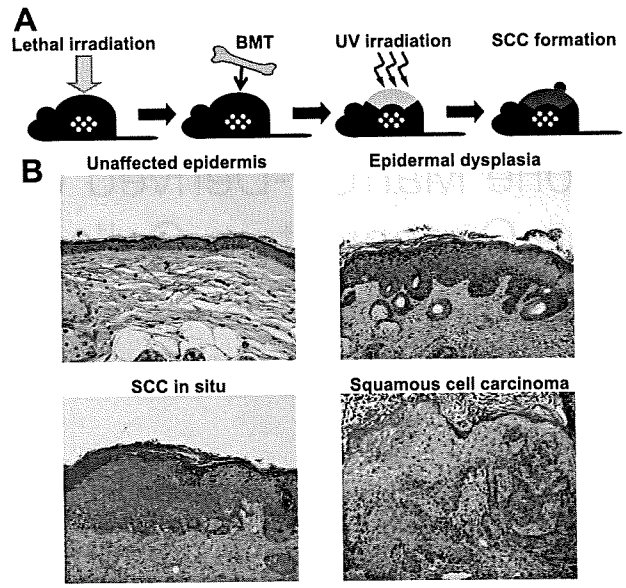

Figure 1. UVB-induced SCC model mice in which BMDCs are labeled with β-Gal enzyme or GFP. **A:** Lethally irradiated mice were transplanted with BM from ROSA26 mice expressing β-Gal enzyme or GFP mice expressing GFP. After confirmation of BM reconstitution, mice were UVB-irradiated. Intermittent UVB irradiation leads mice skin to form SCC. **B:** Tumors were histologically classified as unaffected, dysplasia, SCC *in situ*, or SCC based on tumor architecture, keratinocyte differentiation, and cytological atypia.

Materials and Methods

BM Transplantation

All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol. BM was isolated from the femurs and tibias of male C57BL/6JGtrosa26 (ROSA26) or C57BL/6-TgN(ACTB-EGFP)1Osb/J (GFP) mice (The Jackson Laboratory, Bar Harbor, ME). After lethal irradiation (9 Gy), 1×10^6 BM cells from donor mice in a volume of 200 μl of sterile phosphate-buffered saline were transplanted to recipient C57BL/6 female mice via a single tail vein injection. Hematopoietic reconstitution was subsequently evaluated in peripheral blood 4 weeks after transplantation and more than 94% of BM cells were donor-derived cells.

Induction of UVB Radiation-Induced SCC

UVB-induced carcinogenesis was performed as previously reported (Figure 1A).[27] The UVB light source was a FL20SE30 fluorescent lamp (Clinical Supply, Tokyo, Japan). The UVB irradiation (180 mJ/cm^2) was continued daily for 10 days for tumor initiation to mice (n = 20). One week after the initiation, UVB exposure (180 mJ/cm^2) was performed twice a week until the end of the experiment at 10 months from the last UVB exposure. At 5 months, all irradiated mice (n = 8) had small papules (at least two papules) and erosion. At 10 months, all irradiated mice (n = 6) had tumors (at least three tumors), papules (at least five papules), and ulcer.

Histological Analysis

Mice were sacrificed and tissue was removed, embedded in OCT compound (Sakura, Torrance, CA), snap-frozen or fixed in 4% formalin, and embedded in paraffin. Tumor sections were visualized by routine staining with hematoxylin and eosin (H&E). All of the slides were reviewed twice in blinded manner by three dermatologists, and assessed for tumor architecture, keratinocyte differentiation, cytological atypia, and inflammation. Tumors were classified as dysplasia (typical papilloma), SCC *in situ*, or SCC based on tumor architecture and cytological atypia as described previously.[28] Some lesions exhibiting nonpapillomatous architecture and comprising one to three layers with well-differentiated keratinocytes were classified as normal. Ten samples were analyzed in each normal growth, dysplasia, SCC *in situ*, and SCC. Counts were averaged from eight or nine separate fields in each histological category.

Determination of Enzyme (X-Gal) Activity

Frozen sections (5 μm) were fixed for 30 minutes in 0.2% glutaraldehyde, washed in sodium phosphate buffer containing 0.01% sodium deoxycholate and 0.02% Nonidet P-40 and 1 mmol/L MgCl and incubated for 10 hours at 37°C in a 1-mg/ml X-Gal solution [5-bromo-4-chloro-3-indolyl-β-galactopyranoside: X-Gal, dissolved in dimethyl sulfoxide, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$ $3H_2O$ in 0.1mol/L sodium phosphate buffer] and counterstained with H&E.

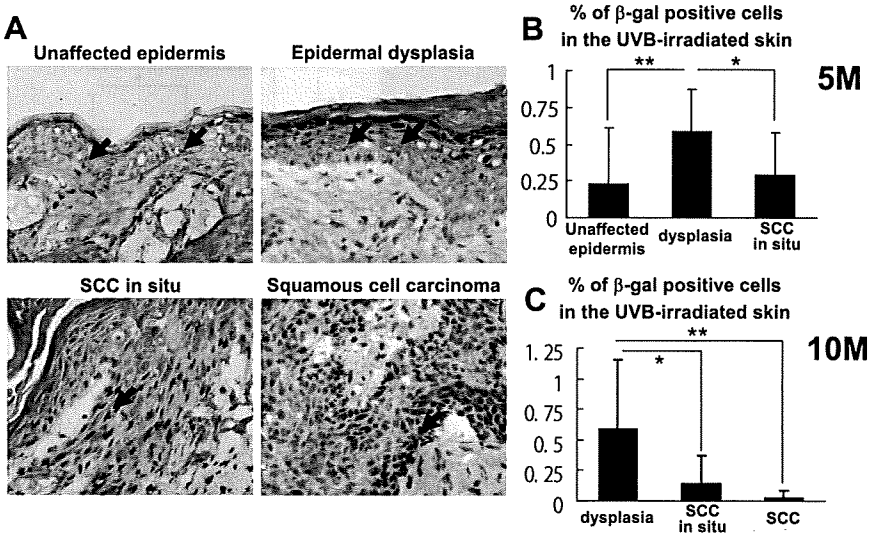

Figure 2. BMDCs in UVB-irradiated mouse skin. **A:** X-Gal-positive cells located within the basal layer in the unaffected epidermis lesions. In the epidermal dysplasia lesions, most X-Gal-positive cells (**arrows**) were found in the suprabasal layers. In the SCC *in situ* lesions, X-Gal-positive cells were found at the inner part of the tumor. In the SCC lesions, X-Gal-positive cells were also found at the inner part of the tumor. **B:** After 5 months of UVB irradiation, the percentage of X-Gal-positive cells was found at 0.15 ± 0.21% in the unaffected epidermis lesions, increased to 0.58 ± 0.25% in the epidermal dysplasia lesions, and decreased to 0.25 ± 0.20% in the SCC *in situ* lesions ($^{*}P < 0.05$, $^{**}P < 0.01$). **C:** After 10 months of UVB irradiation, the percentage of X-Gal-positive cells was 0.59 ± 0.57% in the epidermal dysplasia lesions and 0.15 ± 0.22% in the SCC *in situ* lesions. In the SCC lesions, the percentage of X-Gal-positive cells in the tumor was decreased to 0.03 ± 0.06% ($^{*}P < 0.05$, $^{**}P < 0.01$).

Immunofluorescence

Frozen blocks were prepared and sectioned as described above. Sections were fixed in 4% paraformaldehyde and analyzed for β-galactosidase-expressing cells by using polyclonal antibodies (Cappel, Aurora, OH) and fluorescent secondary antibodies (fluorescein isothiocyanate-labeled goat anti-rabbit antibody; Jackson ImmunoResearch, West Grove, PA). Sections fixed in 4% paraformaldehyde were also analyzed for GFP-expressing cells by using polyclonal antibodies (Molecular Probes, Carlsbad, CA). β-Galactosidase-expressing cells were also stained with antibodies to CD45 (BD Biosciences, San Diego, CA), pan cytokeratins (Progen, Heidelberg, Germany), CD34 (BD Biosciences), or α6 integrin (BD Biosciences). Sections ware viewed with a confocal laser-scanning fluorescence microscope (FV1000; Olympus, Tokyo, Japan).

BrdU Assay

The procedure for BrdU pulse labeling and the subsequent detection were performed as previously reported.[29] In brief, at the time of 9-month UVB irradiation, the tumor-bearing model mice were fed with water containing BrdU (1 mg/ml) for 10 days. Forty-five days after BrdU labeling, the tissues were removed. Frozen sections were fixed with 4% paraformaldehyde or 70% ethanol, stained with antibodies to BrdU (Roche, Penzberg, Germany) and fluorescent second antibodies (tetramethyl-rhodamine isothiocyanate-labeled goat anti-mouse antibody; Southern Biotechnology, Birmingham, AL).

Fluorescence in Situ *Hybridization*

X and Y chromosomes were detected on sections from the UVB-irradiated mice skin using a dual-color detection kit (Cambio, Cambridge, UK) according to the manufacturer's protocol (Cy5 for Y chromosomes and Cy3 for X chromosomes) and immediately viewed with a confocal microscope.

Results

Low Frequency of BMDCs in UVB-Irradiated Skin

To investigate the possible role of BMDCs in UVB-induced skin dysplasia/carcinoma progression, we used a model mouse whose BMDCs are labeled with β-galactosidase (β-Gal) or green fluorescent protein (GFP). Lethally irradiated mice were transplanted with BM from ROSA26 mice or GFP transgenic mice (Figure 1A). After the confirmation of BM reconstitution, mice were irradiated with UVB and developing tumors in mice skin were evaluated histologically. Each section was divided into four categories of unaffected, dysplasia, SCC *in situ*, and SCC (Figure 1B).[28] After 5 months of UVB irradiation, we found the dysplasia lesions and the SCC *in situ* lesions, whereas we found no SCC lesions in irradiated skin. After 10 months of UVB irradiation, the dysplasia lesions and the SCC *in situ* lesions were found to be continuous with the SCC lesions, whereas the unaffected epidermis lesions were completely absent.

To detect the presence of BMDCs in UVB-irradiated mouse skin, X-galactosidase (X-Gal) staining was performed. The numbers of BMDCs were quantified by counting the number of X-Gal-positive cells in the UVB-irradiated mouse skin (Figure 2A). After 5 months of UVB irradiation, even in the unaffected epidermis lesions, some X-Gal-positive cells, indicating BMDCs, were located within the basal layer. In the epidermal dysplasia lesions, some X-Gal-positive cells were also found within the basal layer, but most X-Gal-positive cells were found within the suprabasal layers. In the SCC *in situ* lesions, X-Gal-positive cells were found within the inner parts of the tumor. The percentage of occurrence of X-Gal-positive cells was 0.15% in the unaffected epidermis lesions. Since we previously showed that wounded skin contained BMDCs (0.03%),[20] repeated UVB irradiation might induce BMDC accumulation. The percentage of X-Gal-positive cells in the epidermal dysplasia lesions increased to 0.58%, whereas the percentage of X-Gal-