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β7 Integrin Controls Mast Cell Recruitment, whereas αE Integrin Modulates the Number and Function of $CD8^+$ T Cells in Immune Complex–Mediated Tissue Injury

Daisuke Yamada, Takafumi Kadono, Yuri Masui, Koichi Yanaba, and Shinichi Sato

Immune complex (IC) deposition causes significant tissue injury associated with various autoimmune diseases such as vasculitis. In the cascade of inflammation, cell-to-cell and cell-to-matrix adhesion via adhesion molecules are essential. To assess the role of αE and β7 integrin in IC-mediated tissue injury, peritoneal and cutaneous reverse-passive Arthus reaction was examined in mice lacking αE integrin ($\alpha E^{-/-}$) or β7 integrin ($\beta 7^{-/-}$). Both $\alpha E^{-/-}$ and $\beta 7^{-/-}$ mice exhibited significantly attenuated neutrophil infiltration in the peritoneal and cutaneous Arthus reaction. β7 integrin deficiency, not αE integrin deficiency, significantly reduced the number of mast cells in the peritoneal cavity, which was consistent with the result that mast cells expressed only α4β7 integrin, not αEβ7 integrin. $\alpha E^{-/-}$ mice instead revealed the reduction of $CD8^+$ T cells in the peritoneal cavity, and nearly half of them in wild-type mice expressed αE integrin. These αE^+CD8^+ T cells produced more proinflammatory cytokines than αE^-CD8^+ T cells, and adoptive transfer of αE^+CD8^+ T cell into $\alpha E^{-/-}$ recipients restored cutaneous and peritoneal Arthus reaction. These results suggest that in the peritoneal and cutaneous reverse-passive Arthus reaction, α4β7 integrin is involved in the migration of mast cells for initial IC recognition. αEβ7 integrin, in contrast, contributes by recruiting αE^+CD8^+ T cells, which produce more proinflammatory cytokines than αE^-CD8^+ T cells and amplify IC-mediated inflammation. ***The Journal of Immunology*, 2014, 192: 4112–4121.**

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Tissue injuries and inflammations caused by the formation of immune complexes (ICs) are referred to as type III hypersensitivity reactions, which are strongly associated with the pathogenesis of autoimmune diseases. This IC-mediated tissue injury has been implicated in the pathogenesis of vasculitis syndrome, systemic lupus erythematosus, rheumatoid arthritis, and cryoglobulinemia (1). Arthus reaction is a classical animal model for this IC-mediated tissue injury (2). In the original report, Arthus (2) described that repeated injection of horse serum into a rabbit skin induced edema, hemorrhage, and neutrophil infiltration. The most prevalent murine model currently used is reverse-passive Arthus reaction because of its reproducibility. In reverse-passive Arthus reaction, Ab is injected at the site where the investigator wants the reaction to develop, and the Ag is administered i.v. immediately before or after Ab injection (1). In this model, IC is largely recognized by FcRs on tissue-resident mast cells (3, 4), which is followed by proinflammatory cytokine production such as TNF-α. Such cytokines induce perivascular neutrophil infiltration to cause vascular and tissue damage characteristic of Arthus reaction (5, 6).

Integrins are transmembrane adhesion molecules that mediate cell-to-cell or cell-to-matrix attachment. They are composed of two subunits, α subunit and β subunit. β7 integrin can be paired either with α4 or αE integrin (7). α4β7 Integrin is expressed on lymphocytes (8), macrophages (9), mast cells (10), NK cells (11), and eosinophils (12), and its ligands include mucosal addressin cell adhesion molecule-1 (13, 14) and VCAM-1 (15). The primary role of α4β7 integrin is to mediate migration of lymphocytes to gut-associated lymphatic tissue such as mesenteric lymph nodes, Peyer's patches, and lamina propria (16). αEβ7 integrin is expressed on various hematopoietic cells including subpopulations of $CD8^+$ T cells (17), γδ T cells (18), dendritic cells (DCs) (19), and a certain subset of regulatory T cells (20). αEβ7 integrin is also expressed by the majority of T lymphocytes in mucosal epithelia and mediates their retention to epithelial cells via E-cadherin (21–23). In addition to E-cadherin, αEβ7 integrin is known to have two other ligands. One is a ligand expressed on microvascular endothelial cells (24), whereas the other is expressed on oral and skin keratinocytes (25). Details of these two ligands are yet to be clarified.

As for the functions of α4β7 and αEβ7 integrin, both of these integrins are well known to be involved in mucosal immunity and intestinal diseases. For example, α4β7 integrin is critical for the invasion of alloreactive donor T cells into the gut and the subsequent development of intestinal graft-versus-host disease (GvHD) (26), and αEβ7 integrin–expressing $CD8^+$ donor T cells predominantly infiltrate into the gut epithelium and are responsible for the manifestations of intestinal GvHD (27). Apart from intestinal immunity, contributions of α4β7 or αEβ7 integrin are not clear. Recently, we have reported that α4β7 integrin contributes to contact hypersensitivity responses by regulating T cell migration to inflammatory skin (28). However, their roles in IC-mediated tissue injury such as Arthus reactions remain unclear.

In this study, to clarify the roles of α4β7 and αEβ7 integrin in IC-mediated tissue injury, we examined peritoneal and cutaneous Arthus reactions in mice lacking αE or β7 integrin. The results of this study indicate that α4β7 integrin plays a role in the Arthus reactions by recruiting mast cells, whereas αEβ7 integrin makes a contribution through αE integrin–expressing $CD8^+$ T cells,

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Abbreviations used in this article: $\beta 7^{-/-}$, β7 integrin–deficient; BMMC, bone marrow–derived mast cell; C_T, threshold cycle; DC, dendritic cell; $\alpha E^{-/-}$, αE integrin–deficient; GvHD, graft-versus-host disease; IC, immune complex; KC, keratinocyte-derived chemokine; MIP-2, macrophage inflammatory protein 2; WT, wild-type.

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suggesting a role of α4β7 and αEβ7 integrins in IC-induced inflammation that occurs in the skin and peritoneum other than the gut.

Materials and Methods

Mice

C57BL/6 mice were purchased from SLC Japan (Tokyo, Japan). β7 integrin–deficient ($\beta7^{-/-}$) mice were generated as described (29). αE integrin–deficient ($\alpha E^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were healthy, fertile, and did not display evidence of infection or disease. All mice were backcrossed between 5 and 10 generations onto the C57BL/6 genetic background. Mice used for experiments were 10–14 wk old. All mice were housed in a pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the Animal Experiment Committee of the Graduate School of Medicine of the University of Tokyo guided by the Bioscience Committee of the University of Tokyo.

Reverse-passive Arthus reactions

For cutaneous reverse-passive Arthus reactions, mice anesthetized by inhalation of diethyl ether were shaved on their dorsal skin and wiped with 70% alcohol. Rabbit IgG anti–chicken egg albumin Abs (60 μg/30 μl; Sigma-Aldrich, St. Louis, MO) were injected intradermally with a 29-gauge needle, followed immediately by an i.v. injection of chicken egg albumin (20 mg/kg; Sigma-Aldrich) (5). The intradermal injection of purified polyclonal rabbit IgG (60 μg/30 μl; Sigma-Aldrich) followed by i.v. installation of chicken egg albumin served as a control. The solution of chicken egg albumin contained 0.5% Evans blue dye (Sigma-Aldrich) for the evaluation of edema.

The peritoneal reverse-passive Arthus reaction was initiated by the i.v. injection of chicken egg albumin at 20 mg/kg, followed immediately by the i.p. injection of 800 μg rabbit IgG anti–chicken egg albumin Ab or control-purified rabbit polyclonal IgG in a volume of 400 μl (5). Four or 8 h later, the peritoneum was exposed by a middle abdominal incision, and 5 ml ice-cold PBS containing 0.1% BSA was injected into the peritoneal cavity via a 27-gauge needle. Cells in the recovered lavage fluid were analyzed by flow cytometry to determine the number of various cells.

Quantitation of edema and hemorrhage

Edema was evaluated by measuring the vascular leak 4 h after IC challenge (5). Mice were sacrificed, and the skin containing the injection site was removed at the level of fascia above skeletal muscle and reversed. The diameter of extravascular Evans blue dye on the fascia side of the injection site was measured directly. Evans blue dye binds to serum proteins and thereby can be used to quantify alterations in vascular permeability. The diameter of the major and minor axis of the blue spot was averaged for analysis. The amount of hemorrhage was assessed 8 h after IC challenge by direct macroscopic measurement of the purpuric spot. The diameter of the major and minor axis of the purpuric spot was averaged for analysis.

Histological examination

Tissues were harvested 4 or 8 h after IC challenge using a disposable sterile 6-mm punch biopsy (Maruho, Osaka, Japan) and assessed for tissue damage and number of infiltrating neutrophils and mast cells. Tissues were cut into halves, fixed in 3.5% paraformaldehyde, and then paraffin embedded. Sections (6 μm) were stained using H&E for neutrophil evaluation and toluidine blue for mast cell staining. Neutrophil and mast cell infiltration was evaluated by counting extravascular neutrophils and mast cells in the entire section and averaging the numbers present in 10 serial skin sections from the injection site. Each section was examined independently by three investigators in a blinded manner, and the mean was used for analysis.

Immunohistochemical staining

Before and 4 h after inducing cutaneous reverse-passive Arthus reaction, skin tissues were harvested and stained using predetermined optimal concentrations of anti-F4/80 Ab (clone Cl:A3-1; Serotec, Raleigh, NC) to examine the numbers of macrophages.

Flow cytometric analysis

Isolated peritoneal lavage cells (1.0×10^6) were stained using predetermined optimal concentrations of anti–c-Kit–PerCP/Cy5.5 Ab (CD117, clone 2B8; BioLegend, San Diego, CA) and anti–Gr-1–FITC Ab (clone RB6-8C5; BioLegend) for 20 min at 4°C in the dark. Cells were washed and analyzed on an FACSVerse flow cytometer (BD Pharmingen, San Diego, CA). The numbers of c-Kit–positive mast cells and Gr-1–positive neutrophils were calculated. Other Abs used in the current study included anti–αE integrin–APC Ab (clone 2E7; BioLegend), anti–β7 integrin–allophycocyanin Ab (clone FIB504, BioLegend), anti–CD8-FITC Ab (clone 53-6.7; BD Pharmingen), and anti–CD16/32-PerCP/Cy5.5 Ab (clone 93; BioLegend). Positive and negative populations of cells were determined using unreactive isotype-matched mAbs as controls for background staining. Propidium iodide (final concentration 1 μg/ml) was used to exclude dead cells.

RNA isolation and real-time PCR

Tissues were harvested 4 or 8 h after IC challenge using a disposable, sterile, 6-mm punch biopsy (Maruho) and cut into halves. All skin samples were snap-frozen in liquid nitrogen and stored at −80°C before use. Total RNA was isolated from frozen tissue with RNeasy fibrous tissue kit (Qiagen, Crawley, U.K.) and then reversely transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Cytokine mRNA expression levels were analyzed using a real-time PCR quantification method according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). 18S-rRNA was used as endogenous control to normalize the expression levels of RNA. Relative expression of PCR products was determined using the ΔΔ threshold cycle (C_T) method. Briefly, each set of samples was normalized using the difference in between the target gene and endogenous control (18S-rRNA): $\Delta C_T = (C_{T\ \text{target gene}} - C_{T\ \text{18S-rRNA}})$. Relative mRNA levels were calculated by the expression $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\ \text{sample}} - \Delta C_{T\ \text{calibrator}}$. Each sample was examined in duplicate, and the mean C_T was used in the equation. For real-time RT-PCR analysis of mRNA expression, five to eight mice were used in each group. Primer sequences used in the current study were as follows: IL-6 forward, 5′-GAT GGA TGC TAC CAA ACT GGA T-3′ and IL-6 reverse, 5′-CCA GGT AGC TAT GGT ACT CCA GA-3′; TNF-α forward, 5′-ACC CTC ACA CTC AGA TCA TCT TC-3′ and TNF-α reverse, 5′-TGG TGG TTT GCT ACG ACG T-3′; keratinocyte-derived chemokine (KC) forward, 5′-GGC TGG GAT TCA CCT CAA GAA C-3′ and KC reverse; 5′-TGT GGC TAT GAC TTC GGT TTG G-3′; IFN-γ forward, 5′-TCA AGT GGC ATA GAT GTG GAA GAA-3′ and IFN-γ reverse, 5′-TGG CTC TGC AGG ATT TTC ATG-3′; IL-1β forward, 5′-CTC CAT GAG CTT TGT ACA AGG-3′ and IL-1β reverse, 5′-TGC TGA TGT ACC AGT TGG GG-3′; macrophage inflammatory protein 2 (MIP-2) forward, 5′-ACC AAC CAC CAG GCT ACA G-3′ and MIP-2 reverse, 5′-GCG TCA CAC TCA AGC TCT-3′; and 18S-rRNA forward, 5′-CGC CGC TAG AGG TGA AAT TC-3′ and18S-rRNA reverse, 5′-TTG GCA AAT GCT TTC GCT C-3′.

Cytokine ELISA

Levels of TNF-α and IL-6 in the peritoneal lavage were determined by using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The plates were coated with cytokine-specific Abs and incubated with appropriately diluted peritoneal lavage samples. After incubation with biotinylated cytokine-specific Abs and streptavidin-HRP, the reaction was developed.

Purification of cell subsets and adoptive transfer

Peritoneal lavage cells were used to prepare single-cell suspensions. CD8-positive T cells were isolated by negative selection by using the MACS system ($CD8a^+$ T Cell Isolation Kit II; Miltenyi Biotec, Bergisch Gladbach, Germany). After enriching CD8-positive T cells, αE integrin–positive cells were then isolated by positive selection by using the MACS system with indirect magnetic labeling method. After cell isolation, purity was confirmed by flow cytometry analysis, which revealed the purity to be >80%. For the reconstitution of αE^+CD8^+ T cells in the peritoneal cavity and the dermis, 6×10^4 cells was injected prior to the i.v. Ag injection i.p. and intradermally, respectively.

Preparation of bone marrow–derived mast cells and adoptive transfer

$\beta7^{-/-}$ mice were reconstituted by the injection of cultured bone marrow–-derived mast cells (BMMCs) into the peritoneal cavity, as described (30). In brief, femoral bone marrow cells from wild-type (WT) mice were collected and cultured in vitro for 4 wk in RPMI 1640 complete medium (Sigma-Aldrich) supplemented with 10% FBS in the presence of stem cell factor (10 ng/ml; R&D Systems) and IL-3 (10 ng/ml; Life Technologies, Carlsbad, CA). During culture, medium was refreshed once weekly. After this culture period, mast cells represented >95% of the total cells, as determined by toluidine blue staining and FACS analysis staining c-Kit and FcεR. Subsequently, mast cells were harvested, and 3×10^6 cells in 100 μl PBS was injected into $\beta7^{-/-}$ mice i.p. Reconstituted mice were used in peritoneal reverse-passive Arthus reaction experiments after allowing 5 wk for mast cells to differentiate within the peritoneum (31, 32).

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Ab blocking of α4β7 or αEβ7 integrin

Anti–α4β7 integrin Ab (clone DATK-32; eBioscience, San Diego, CA) or anti–αEβ7 integrin Ab (clone M290; BD Biosciences, San Jose, CA) was used in the blocking study. A total of 40 μg Ab was administered i.v. immediately before IC challenge.

Statistical analysis

The Mann–Whitney *U* test was used for determining the level of significance of differences in sample means, and Bonferroni test was used for multiple comparisons. A p value <0.05 was considered significant.

Results

$\beta7^{-/-}$ and $\alpha E^{-/-}$ mice exhibit impaired peritoneal reverse-passive Arthus reaction

The i.p. injection of Ab with the i.v. injection of Ag elicits a reverse-passive Arthus reaction characterized by leukocyte influx into the peritoneal cavity (1). After 4 h of IC challenge, neutrophil numbers in the peritoneal cavity were significantly reduced in $\beta7^{-/-}$ (78%; $p < 0.01$) and $\alpha E^{-/-}$ mice (64%; $p < 0.01$) compared with WT mice (Fig. 1A). However, after 8 h of IC formation in the peritoneal cavity, neutrophil numbers revealed no significant differences among these three groups (Fig. 1B).

By contrast, mast cell numbers were reduced only in $\beta7^{-/-}$ mice (72%; $p < 0.01$), whereas they were not in $\alpha E^{-/-}$ mice after 4 h of IC challenge (Fig. 1A). Similar results were obtained after 8 h of IC formation (Fig. 1B). Thus, in the peritoneal reverse-passive Arthus reaction, impaired neutrophil infiltration was observed both in $\beta7^{-/-}$ and $\alpha E^{-/-}$ mice, whereas mast cell infiltration was impaired only in $\beta7^{-/-}$ mice.

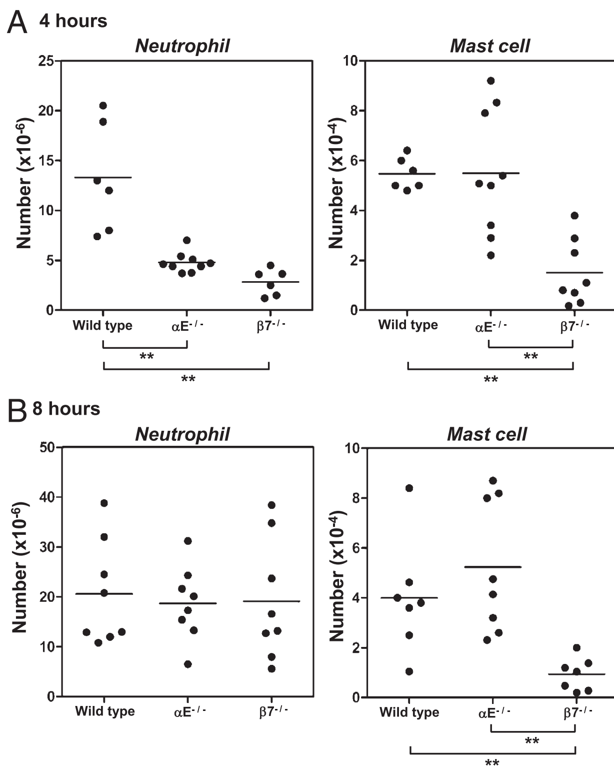

FIGURE 1. Arthus reaction–induced recruitment of neutrophils and mast cells in the peritoneum of $\alpha E^{-/-}$, $\beta7^{-/-}$, and WT mice at 4 (**A**) and 8 h (**B**) after IC challenge. The peritoneal reverse-passive Arthus reaction was induced by the i.v. injection of chicken egg albumin, followed immediately by the i.p. injection of rabbit IgG anti–chicken egg albumin Ab. Live cells in the recovered lavage fluid were counted using a leukocytometer with trypan blue staining. The numbers of c-Kit–positive mast cells and Gr-1–positive neutrophils were analyzed by flow cytometry. Horizontal bars indicate mean values for each group of mice. n = 5–10. **$p < 0.01$.

$\beta7^{-/-}$ and $\alpha E^{-/-}$ mice exhibit impaired cutaneous reverse-passive Arthus reaction

Cutaneous inflammation induced by the Arthus reaction can be separated into two distinct responses: edema, which reaches a maximum at 3 to 4 h after IC challenge, and hemorrhage, which peaks in intensity at 8 h (33). To assess the role of β7 and αE integrin in the cutaneous Arthus reaction, edema and hemorrhage were evaluated 4 and 8 h after IC challenge in $\beta7^{-/-}$, $\alpha E^{-/-}$, and WT mice. The diameter of edema assessed by Evans blue dye in the extravascular space was not significantly different among these groups (Fig. 2A, 2C). By contrast, hemorrhage that was macroscopically quantitated after 8 h by measuring the size of the purpuric spot was reduced both in $\beta7^{-/-}$ and $\alpha E^{-/-}$ mice compared with WT mice ($p < 0.01$; Fig. 2B, 2C). Thus, loss of either β7 or αE integrin reduced the later response of hemorrhage, but not the earlier response of edema.

Leukocyte infiltration in the cutaneous reverse-passive Arthus reaction

Extravascular neutrophils were assessed in skin tissue sections after 4 and 8 h of IC formation in $\beta7^{-/-}$, $\alpha E^{-/-}$, and WT mice (Fig. 3A, 3C). Before IC challenge, there were no significant differences in cutaneous neutrophil numbers between mutant and WT mice. After 4 h of IC challenge, neutrophil numbers were significantly reduced both in $\beta7^{-/-}$ (62%; $p < 0.001$) and $\alpha E^{-/-}$ mice (20%; $p < 0.05$) compared with WT mice. Similar results were obtained after 8 h of IC challenge (Fig. 3A, 3C).

Mast cell numbers were also analyzed in skin tissue sections stained with toluidine blue (Fig. 3B, 3C). Before IC challenge, skin mast cell numbers did not differ significantly between mutant and WT mice. After 4 h of IC challenge, mast cell numbers were significantly reduced in $\beta7^{-/-}$ mice (51%; $p < 0.001$). $\alpha E^{-/-}$ mice also tended to show reduced mast cell numbers (29%; p = 0.07). After 8 h of IC challenge, both $\beta7^{-/-}$ (58%; $p < 0.001$) and $\alpha E^{-/-}$ mice (25%; $p < 0.05$) showed reduced mast cell numbers. We also examined the numbers of macrophages in the skin tissue before and 4 h after the IC challenge. The numbers of macrophages were not significantly different among WT, $\alpha E^{-/-}$, and $\beta7^{-/-}$ mice at both time points (Fig. 3D).

Thus, loss of either β7 or αE integrin reduced infiltrating neutrophils and mast cells, whereas the number of macrophages was similar. Although the reduction of infiltrating neutrophils and mast cells occurred as early as 4 h after the IC challenge, macroscopically only hemorrhage after 8 h, not edema after 4 h, exhibited significant differences. These results suggest that the reduction of infiltrating cells occurs prior to the macroscopic changes.

Proinflammatory cytokine levels in the peritoneal reverse-passive Arthus reaction

IC-induced inflammation in the skin and peritoneum is associated with the production and release of proinflammatory cytokines (5, 6). We examined IL-6 and TNF-α levels in peritoneal lavage samples after 4 h of IC formation by ELISA (Fig. 4A). Both IL-6 and TNF-α levels in the peritoneal lavage were significantly reduced in $\beta7^{-/-}$ mice (59%; $p < 0.05$), and $\alpha E^{-/-}$ mice showed a tendency to have lower levels of these cytokines. Thus, $\beta7^{-/-}$ mice, in which both mast cells and neutrophils were reduced, contained significantly less IL-6 and TNF-α in the peritoneal lavage, whereas $\alpha E^{-/-}$ mice, in which only neutrophils were reduced, showed only a tendency.

Cytokine and chemokine mRNA expression in the cutaneous reverse-passive Arthus reaction

We next examined the expression levels of IL-6, KC, and TNF-α in the skin tissue after 8 h of IC formation by real-time PCR

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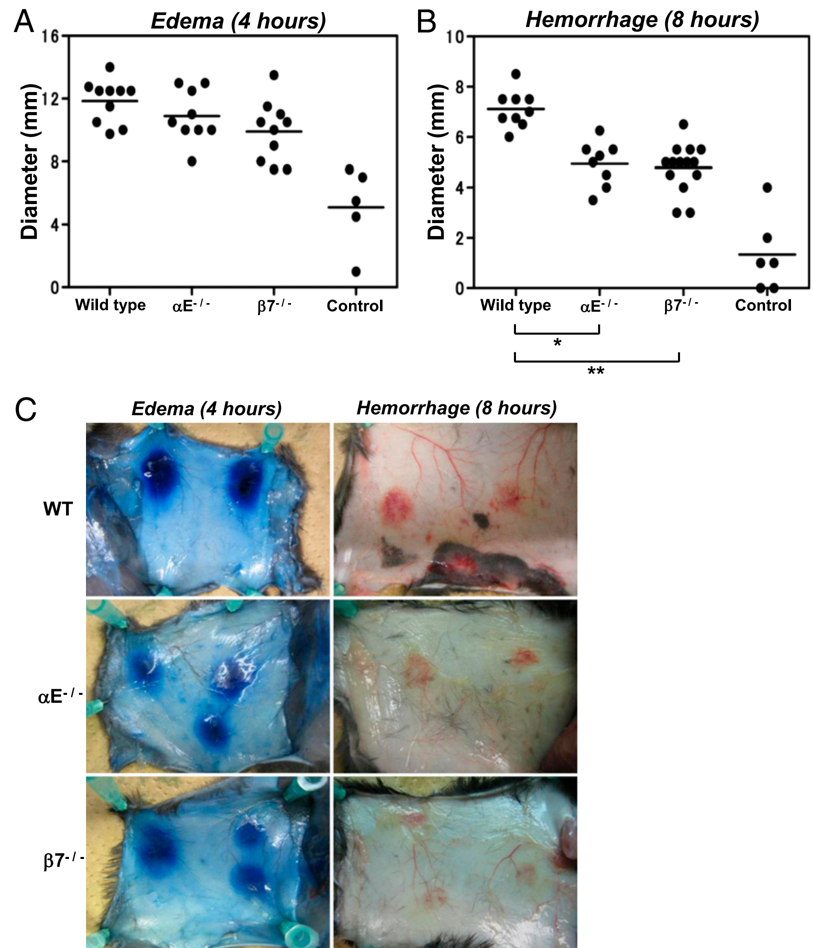

FIGURE 2. The effect of αE and β7 integrin deficiency on edema and hemorrhage in the cutaneous reverse-passive Arthus reaction. Mice were injected intradermally with rabbit IgG anti–chicken egg albumin Ab, followed by systemic chicken egg albumin. For the evaluation of edema, 0.5% Evans blue dye was added in the solution of Ag. After 4 or 8 h, dorsal skins were assessed from $\alpha E^{-/-}$, $\beta 7^{-/-}$, and WT mice. (**A**) Edema was evaluated as the diameter of extravasated Evans blue spot. WT mice that received an intradermal injection of polyclonal rabbit IgG followed by i.v. installation of chicken egg albumin served as controls. (**B**) Hemorrhage after 8 h was assessed as the diameter of the purpuric spot. (**C**) Representative macroscopic findings of the cutaneous reverse-passive Arthus reaction. Edema after 4 h and hemorrhage after 8 h are shown. $^{*}p < 0.05$, $^{**}p < 0.01$.

(Fig. 4B). KC is a major neutrophil chomoattractant in mice, and its local production is closely related to the recruitment of leukocytes (34). IL-6 levels were significantly reduced in both of the mutant mice relative to WT mice. KC was significantly reduced in $\beta 7^{-/-}$ mice. Although it did not reach the significance, $\alpha E^{-/-}$ mice showed a tendency to have lower expression levels of KC ($p = 0.12$). TNF-α levels were similar among these three groups (data not shown). Thus, $\beta 7^{-/-}$ mice, which exhibited attenuated hemorrhage with less infiltration of both mast cells and neutrophils in the cutaneous Arthus reaction, showed reduced expression of both IL-6 and KC, whereas in $\alpha E^{-/-}$ mice, which showed milder phenotype, only IL-6 levels were significantly reduced.

β7 and αE integrin expression on neutrophil or mast cell

In the peritoneal reverse-passive Arthus reaction, neutrophil infiltration was reduced both in $\beta 7^{-/-}$ and $\alpha E^{-/-}$ mice, although mast cell infiltration was decreased only in $\beta 7^{-/-}$ mice (Fig. 1A). When we examined the expression of these integrins on neutrophils and mast cells by flow cytometry analysis, β7 integrin was expressed only on mast cells and not neutrophils, and αE integrin was not expressed either on mast cells or neutrophils (Fig. 5A).

Reconstitution of BMMCs restored the peritoneal reverse-passive Arthus reaction in $\beta 7^{-/-}$ mice

To directly evaluate the involvement of mast cells in $\beta 7^{-/-}$ mice, cultured BMMCs were injected i.p. into $\beta 7^{-/-}$ mice. Five weeks after the injection, mice were challenged with IC. The adoptively transferred BMMCs significantly increased neutrophil infiltration to the level comparable to that of WT mice (Fig. 5B). Thus, the reduced neutrophil infiltration in $\beta 7^{-/-}$ mice is attributable to the reduced mast cells.

The number of $CD8^{+}$ T cells is reduced in $\alpha E^{-/-}$ mice in the peritoneal reverse-passive Arthus reaction

αE integrin is known to be expressed on various hematopoietic cells including intraepithelial lymphocytes, subpopulations of $CD8^{+}$ T cells (17), γδ T cells (18), and DCs (19). As neutrophils did not express αE integrin, we assumed that some αE integrin–expressing cell might be associated in the peritoneal reverse-passive Arthus reaction. Therefore, we compared the number of $CD8^{+}$ T cells, γδ T cells, and DCs between $\alpha E^{-/-}$ and WT mice at an earlier time point. After 2 h of IC challenge, the number of $CD8^{+}$ T cells was significantly reduced in $\alpha E^{-/-}$ mice compared with WT mice (43%, $p < 0.05$; Fig. 6A), whereas the number of DCs and γδ T cells was not significantly different (data not shown).

We next examined the expression of αE integrin on $CD8^{+}$ T cells in the peritoneal cavity and found that nearly half of $CD8^{+}$ T cells expressed αE integrin (Fig. 6B). In the Arthus reaction, IC recognition by FcγRs plays a central role in initiating the inflammation (33). Therefore, we examined the expression of FcγRs (CD16/32) on $\alpha E^{+}CD8^{+}$ T cells by flow cytometry. However, $\alpha E^{+}CD8^{+}$ T cells did not express FcγRs (Fig. 6C), suggesting IC is unlikely to stimulate these $\alpha E^{+}CD8^{+}$ T cells directly.

$\alpha E^{+}CD8^{+}$ T cells produce larger amount of proinflammatory cytokines than $\alpha E^{-}CD8^{+}$ T cells

We next compared the cytokine expressions between $\alpha E^{+}CD8^{+}$ T cells and $\alpha E^{-}CD8^{+}$ T cells because $\alpha E^{+}CD8^{+}$ T cell activity is

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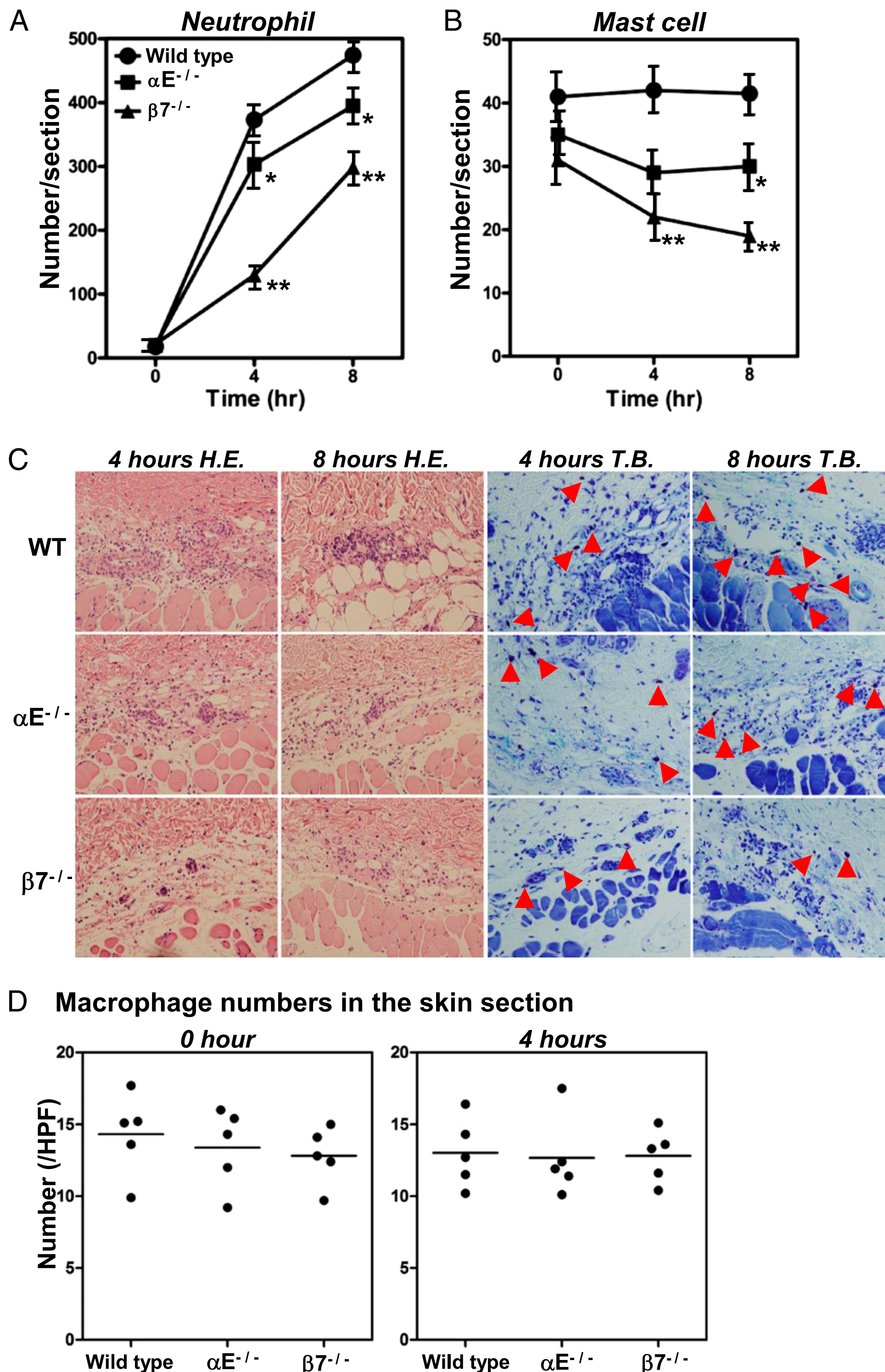

FIGURE 3. Arthus reaction–induced recruitment of neutrophils (**A**) and mast cells (**B**) in the skin of $\alpha E^{-/-}$, $\beta 7^{-/-}$, and WT mice at 4 and 8 h after IC challenge. Numbers of neutrophils and mast cells per skin section were determined by counting in H&E- and toluidine blue–stained skin sections, respectively. All values represent the mean ± SEM of results obtained from 5–10 mice in each group. (**C**) Histological tissue sections showing neutrophil and mast cell infiltration in the skin of WT, $\alpha E^{-/-}$, and $\beta 7^{-/-}$ mice at 4 and 8 h after IC challenge. Mast cells (arrowheads) were detected as cells with metachromatic staining of granules in toluidine blue–stained sections. Original magnification ×200. (**D**) Macrophage numbers in the skin section before and 4 h after the cutaneous Arthus reaction. The number of F4/80-positive cells in 10 high-powered fields (HPF) was counted and then averaged in each mouse. $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$. H.E., H&E; T.B., toluidine blue.

known to be enhanced through the interaction between αE integrin and its ligand (35). To that end, $\alpha E^{+}CD8^{+}$ and $\alpha E^{-}CD8^{+}$ T cells were separated using the MACS system, and then mRNA expression was evaluated using quantitative real-time PCR. The expression levels of proinflammatory cytokines including IL-6, TNF-α, and IL-1β were significantly greater in $\alpha E^{+}CD8^{+}$ T cells than $\alpha E^{-}CD8^{+}$ T cells (Fig. 6D), whereas the expression levels of IFN-γ were significantly lower in $\alpha E^{+}CD8^{+}$ T cells

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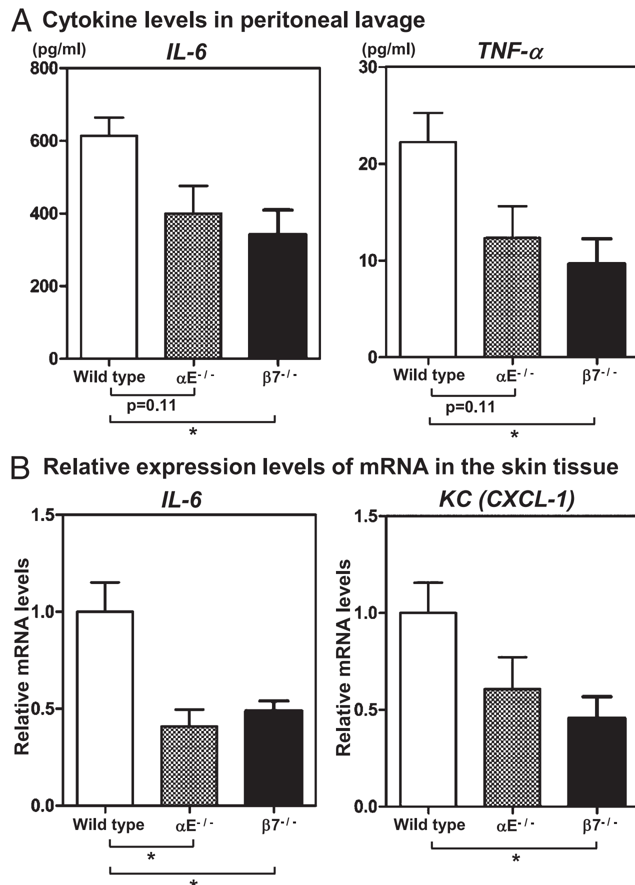

FIGURE 4. Cytokine and chemokine quantification. (**A**) Peritoneal reverse-passive Arthus reaction–induced IL-6 and TNF-α production in $\alpha E^{-/-}$, $\beta 7^{-/-}$, and WT mice at 4 h after IC challenge. IL-6 and TNF-α levels in the peritoneal lavage samples were determined by ELISA. (**B**) Cutaneous reverse-passive Arthus reaction–induced mRNA expression of IL-6 and KC in the skin from $\alpha E^{-/-}$, $\beta 7^{-/-}$, and WT mice at 8 h after IC challenge. Total RNA was isolated from frozen skin tissues, reverse transcribed into cDNA, and then amplified using primers. Relative mRNA levels of IL-6 and KC were measured by real-time PCR and normalized relative to that of 18S-rRNA as an endogenous control. All values represent the mean + SEM of results obtained from five to eight mice in each group. $*p < 0.05$.

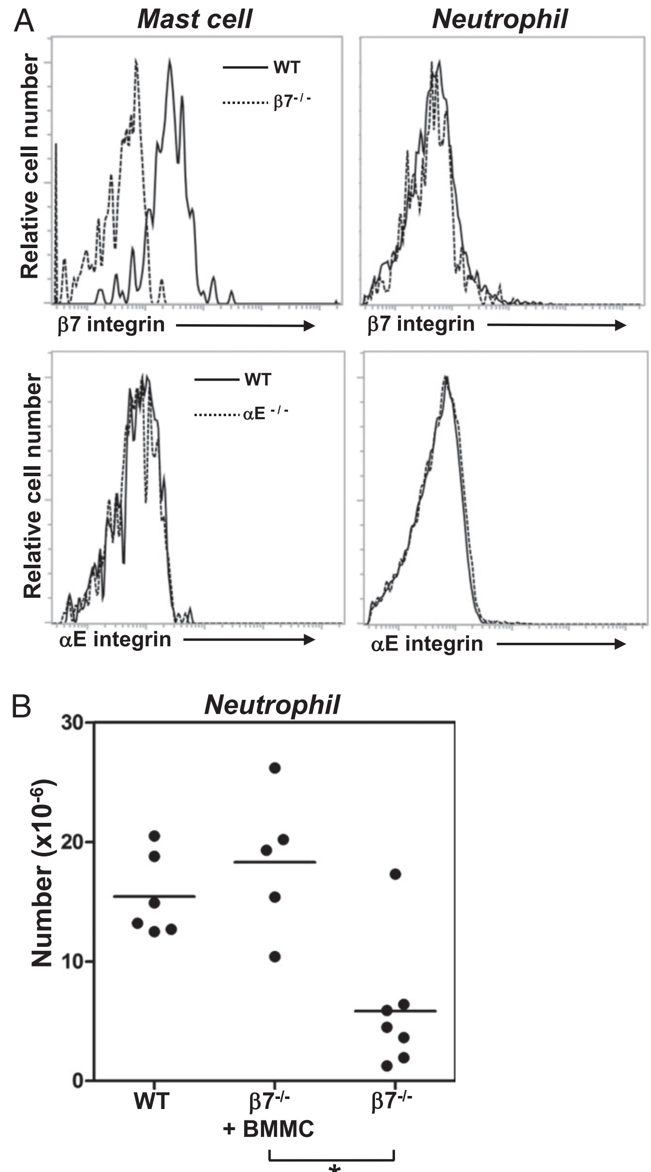

FIGURE 5. (**A**) Expression of β7 integrin and αE integrin on peritoneal mast cells and neutrophils. The dotted lines represent control staining obtained from mutant mice. (**B**) Arthus reaction–induced recruitment of neutrophils in the peritoneal lavage after reconstitution of BMMCs. Horizontal bars indicate mean values for each group of mice. $*p < 0.05$.

(Fig. 6D). The expression levels of MIP-2 tended to be higher in $\alpha E^{+}CD8^{+}$ T cells ($p = 0.06$), and no significant difference was found regarding the expression levels of KC. Thus, $\alpha E^{+}CD8^{+}$ T cells expressed more proinflammatory cytokines relevant to Arthus reaction than $\alpha E^{-}CD8^{+}$ T cells.

Adoptive transfer of αE^{+} $CD8^{+}$ T cells augmented peritoneal and cutaneous reverse-passive Arthus reaction

To directly evaluate the functional difference between $\alpha E^{+}CD8^{+}$ and $\alpha E^{-}CD8^{+}$ T cells in the Arthus reaction, $\alpha E^{-/-}$ mice were adoptively transferred either with $\alpha E^{+}CD8^{+}$ or $\alpha E^{-}CD8^{+}$ T cells from WT mice and then were challenged with IC i.p. The adoptively transferred $\alpha E^{+}CD8^{+}$ T cells significantly increased neutrophil infiltration in the pritoneum ($p < 0.05$), whereas $\alpha E^{-}CD8^{+}$ T cells had no such effect (Fig. 7A). We next examined the role of $\alpha E^{+}CD8^{+}$ T cells in cutaneous Arthus reaction as well. Immediately after the intradermal injection of $\alpha E^{+}CD8^{+}$ or $\alpha E^{-}CD8^{+}$ T cells, cutaneous reverse-passive Arthus reaction was induced. As shown in Fig. 7B, hemorrhage after 8 h was restored by the reconstitution of $\alpha E^{+}CD8^{+}$ T cells, whereas $\alpha E^{-}CD8^{+}$ T cells did not show such effect.

We next adoptively transferred $\alpha E^{+}CD8^{+}$ T cells into $\beta 7^{-/-}$ mice. We found that $\alpha E^{+}CD8^{+}$ T cells augmented the reaction in $\beta 7^{-/-}$ mice, whereas $\alpha E^{-}CD8^{+}$ T cells did not (Fig 7C).

Thus, αE^{+} $CD8^{+}$ T cells are suggested to play important roles both in peritoneal and cutaneous Arthus reaction, and αE^{+} $CD8^{+}$ T cells are able to increase neutrophil infiltration even when β7 integrin is deficient and mast cell number is low.

The blockade of αEβ7 or α4β7 integrin using Ab attenuated the peritoneal reverse-passive Arthus reaction

We used a blocking Ab to examine the function of αEβ7 or α4β7 integrin in the peritoneal reverse-passive Arthus reaction. WT mice were i.v. injected with either anti–α4β7 or anti–αEβ7 integrin–neutralizing Ab. Immediately after the injection, Arthus reaction was induced. Both mast cell and neutrophil numbers were reduced by blocking α4β7 integrin (Fig. 8A, 8C). Similarly, administration of anti–αEβ7 integrin Ab attenuated the infiltration of

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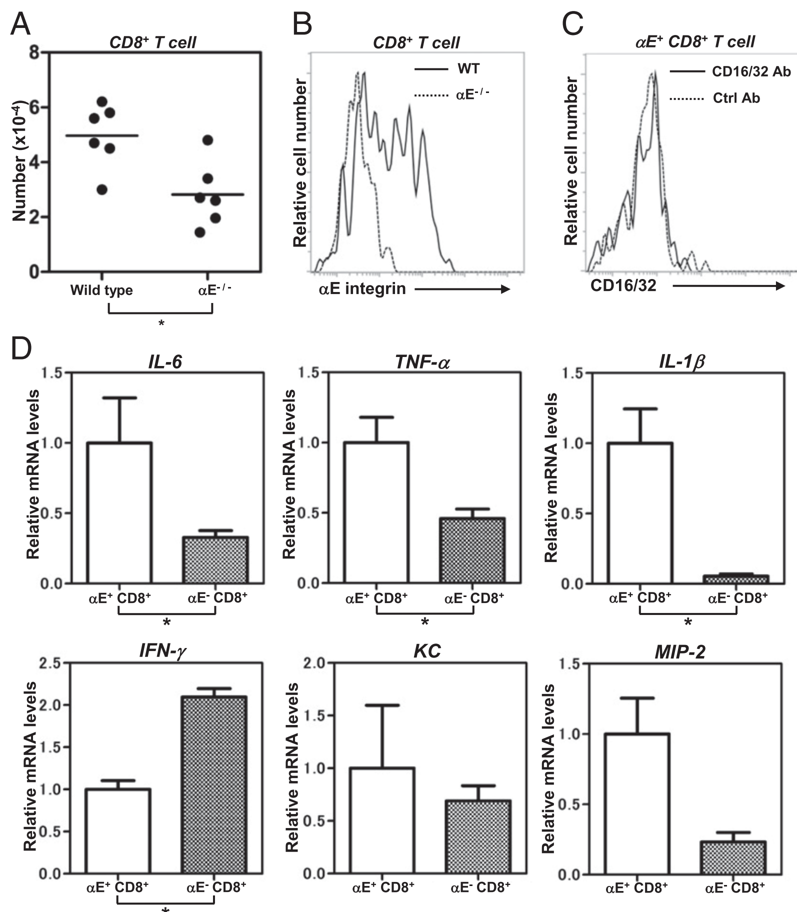

FIGURE 6. (**A**) Arthus reaction–induced recruitment of $CD8^+$ T cells in the peritoneum of $\alpha E^{-/-}$ and WT mice at 2 h after IC challenge. Horizontal bars indicate mean values for each group of mice. (**B**) Expression of αE integrin on peritoneal $CD8^+$ T cells. The dotted lines represent control staining obtained from $\alpha E^{-/-}$ mice. (**C**) Expression of FcγR (CD16/32) on αE^+ $CD8^+$ T cells. The dotted lines represent control staining using an unreactive isotype-matched mAb. (**D**) Cytokine expression levels of αE^+CD8^+ and αE^-CD8^+ T cells after IC challenge in vivo. Two hours after IC challenge, peritoneal lavage was collected. Collected cells were separated using MACS system, and then mRNA expression was evaluated using quantitative real-time PCR. All values represent the mean + SEM of results obtained from five to six mice in each group. $*p < 0.05$.

αE^+CD8^+ T cells to the peritoneal cavity and the infiltration of neutrophils (Fig. 8B, C). Thus, α4β7 integrin is straightly involved in the recruitment of mast cells, whereas αEβ7 integrin is directly required for αE^+CD8^+ T cell recruitment to facilitate neutrophil infiltration in the peritoneal reverse-passive Arthus reaction.

Discussion

Our current results demonstrate that the loss of β7 integrin significantly attenuated neutrophil infiltration in the peritoneal and cutaneous reverse-passive Arthus reaction (Figs. 1A, 3A). To our surprise, these Arthus reactions were also significantly reduced in $\alpha E^{-/-}$ mice. In the peritoneal reverse-passive Arthus reaction, the number of mast cells within the peritoneal cavity was significantly reduced only in $\beta 7^{-/-}$ mice (Fig. 1A, 1B), which is consistent with the result that mast cells expressed only β7 integrin, not αE integrin (Fig. 5A), and α4β7 integrin is known to facilitate mast cell migration to the inflammatory site (29, 36). As for $\alpha E^{-/-}$ mice, we found that $CD8^+$ T cell infiltration was significantly reduced (Fig. 6A), and nearly half of $CD8^+$ T cells in the peritoneal cavity expressed αE integrin in WT mice (Fig. 6B). Furthermore, these αE^+ $CD8^+$ T cells expressed more proinflammatory cytokines such as IL-6, TNF-α, and IL-1β (Fig. 6D). Finally, adoptive transfer of αE^+CD8^+ T cells restored the peritoneal Arthus reaction, whereas αE^-CD8^+ T cells did not (Fig. 7A), suggesting that αE integrin regulates the Arthus reaction both by recruiting $CD8^+$ T cells to the inflammatory sites and augmenting cytokine production from $CD8^+$ T cells. Thus, this is the first report, to our knowledge, to reveal that αE and β7 integrin highly contribute to IC-induced inflammation.

β7 integrin is a β-chain component in integrin heterodimer, which can either pair with α4 or αE integrin. $\beta 7^{-/-}$ mice therefore lack both α4β7 and αEβ7 integrin. The attenuated Arthus reaction in $\beta 7^{-/-}$ mice is presumably caused by the reduced number of mast cells because mast cells are critical for Arthus reaction (3). Indeed, when we adoptively transferred BMMCs to $\beta 7^{-/-}$ mice, the number of infiltrated neutrophils significantly increased to the level comparable to that of WT mice (Fig. 5B). We also confirmed this by using α4β7-neutralizing Ab in the peritoneal reverse-passive Arthus reaction (Fig. 8A, 8C). Mast cells are a strong producer of TNF-α and IL-6, which are involved in the Arthus reaction (5, 6, 37, 38). We also found that the levels of proinflammatory cytokines such as IL-6 and TNF-α were significantly reduced in $\beta 7^{-/-}$ mice. The lower cytokine levels presumably caused by reduced mast cell recruitment in the absence of β7 integrin expression might impair neutrophil recruitment to the sites of inflammation, leading to the inhibited Arthus reaction.

αE integrin deficiency also reduced cutaneous and peritoneal Arthus reaction. As αE integrin can pair with β7 integrin alone, only αEβ7 integrin is deficient in $\alpha E^{-/-}$ mice. As αEβ7 integrin is not expressed on neutrophils or mast cells (Fig. 5A), and the number of mast cells was not reduced in the peritoneal reverse-passive Arthus reaction, the attenuated neutrophil infiltration was supposed to be caused by cells other than mast cells. αE integrin expression is limited to hematopoietic cells such as subpopulations of $CD8^+$ T cells (17), γδ T cells (18), and DCs (19). The numbers of DCs and γδ T cells in the peritoneal cavity were not reduced in $\alpha E^{-/-}$ mice (data not shown); however, the number of $CD8^+$ T cells was significantly lower in $\alpha E^{-/-}$ mice after 2 h of IC challenge (Fig. 6A). αE integrin mediates local retention of T lymphocytes via its ligands such as E-cadherin (39). αE integrin also triggers CCR5-dependent $CD8^+$ T cell retention to the tumor site (40), and loss of αE integrin prevents GvHD with reduced infiltration of αE^+CD8^+ T cells to the epithelium (41). Considering that nearly half of $CD8^+$

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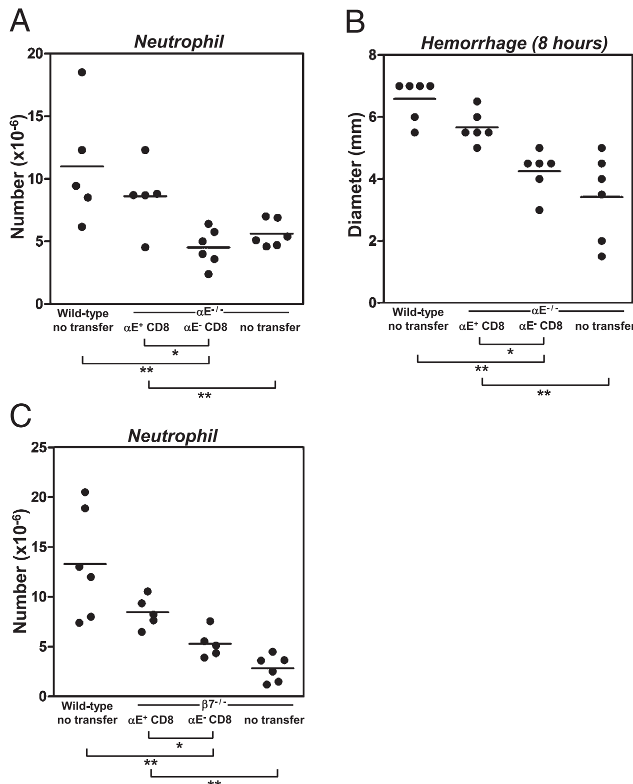

FIGURE 7. (**A**) Adoptive transfer of αE$^+$CD8$^+$ T cells into the peritoneal cavity of αE$^{-/-}$ mice. Mice were adoptively transferred i.p. either with αE$^+$CD8$^+$ T cells or αE$^-$CD8$^+$ T cells from WT mice and then challenged with IC. (**B**) Adoptive transfer of αE$^+$CD8$^+$ T cells into the skin of αE$^{-/-}$ mice. Mice were adoptively transferred intradermally either with αE$^+$CD8$^+$ or αE$^-$CD8$^+$ T cells from WT mice and then challenged with IC. (**C**) Adoptive transfer of αE$^+$CD8$^+$ T cells into the peritoneal cavity of β7$^{-/-}$ mice. Mice were adoptively transferred i.p. either with αE$^+$CD8$^+$ or αE$^-$CD8$^+$ T cells from WT mice and then challenged with IC. Horizontal bars indicate mean values for each group of mice. *$p < 0.05$, **$p < 0.01$.

T cells in the peritoneal cavity expressed αE integrin in WT mice (Fig. 6B), it is possible that αE integrin has some role in the recruitment or retention of CD8$^+$ T cells in the peritoneal cavity. We confirmed the role of αE integrin by using its neutralizing Ab. The numbers of αE$^+$CD8$^+$ T cells and neutrophils infiltrating to the peritoneal cavity were reduced by the blockade of αEβ7 integrin (Fig. 8B, 8C). Thus, αEβ7 integrin is directly involved in recruiting αE$^+$CD8$^+$ T cells to the peritoneal cavity and plays important roles in Arthus reaction.

We next evaluated the functional differences between αE$^+$CD8$^+$ and αE$^-$CD8$^+$ T cells. The mRNA expression levels of proinflammatory cytokines including IL-6, TNF-α, and IL-1β were significantly greater in αE$^+$CD8$^+$ T cells, whereas the expression level of IFN-γ was significantly lower in αE$^+$CD8$^+$ T cells. Because IFN-γ is known to reduce Arthus reaction (34), these results were consistent with the notion that αE$^+$CD8$^+$ T cells make a contribution in initiating Arthus reaction. Indeed, the adoptive transfer of αE$^+$CD8$^+$ T cells into αE$^{-/-}$ mice restored neutrophil infiltration, whereas αE$^-$CD8$^+$ T cells did not show such effect in the peritoneal reverse-passive Arthus reaction (Fig. 7A). We examined reconstitution studies of αE$^+$CD8$^+$ T cells in cutaneous Arthus reaction as well, and cutaneous Arthus reaction was also restored by the reconstitution of αE$^+$CD8$^+$ T cells (Fig. 7B). Furthermore, we adoptively transferred αE$^+$CD8$^+$ T cells into β7$^{-/-}$ mice and found that αE$^+$CD8$^+$ T cells augmented the reaction in β7$^{-/-}$ mice, whereas αE$^-$CD8$^+$ T cells did not (Fig 7C), indicating that αE$^+$CD8$^+$ T cells are able to increase neutrophil infiltration even when mast cell number is low. Collectively, these results indicate that αE integrin regulates the peritoneal and cutaneous Arthus reaction by augmenting proinflammatory cytokine production from CD8$^+$ T cells as well as by recruiting CD8$^+$ T cells to the inflammatory sites.

As αE$^+$CD8$^+$ T cells did not express FcRs, which are essential for IC recognition (3), it is unlikely that these αE$^+$CD8$^+$ T cells are directly stimulated by IC. Rather, cytokine production by αE$^+$CD8$^+$ T cells may indirectly augment IC-induced inflammation. Several cytokines are known to modulate cytokine production by CD8$^+$ T cells. IL-6 accelerates the proliferation of CD8$^+$ T cells, whereas the blockade of IL-6 reduces the production of TNF-α and IL-1β by CD8$^+$ T cells (42). Deficiency of p55 TNF-α receptor in CD8$^+$ T cells also reduces their proliferation (43). Moreover, IL-1β enhances proliferation and effector function of CD8$^+$ T cells (44), especially when combined with IL-6 (45). As mast cells and macrophages produce various cytokines including IL-6, TNF-α, and IL-1β (37, 38, 46–48), and αE$^+$CD8$^+$ T cells in our study produced greater amount of proinflammatory cytokines, these αE$^+$CD8$^+$ T cells activated by mast cells or macrophages may amplify local inflammation by producing various cytokines, which instigated neutrophil infiltration and IC-mediated tissue injury.

Although the neutrophil recruitment was significantly different at 4 h after peritoneal Arthus reaction, neutrophil numbers were

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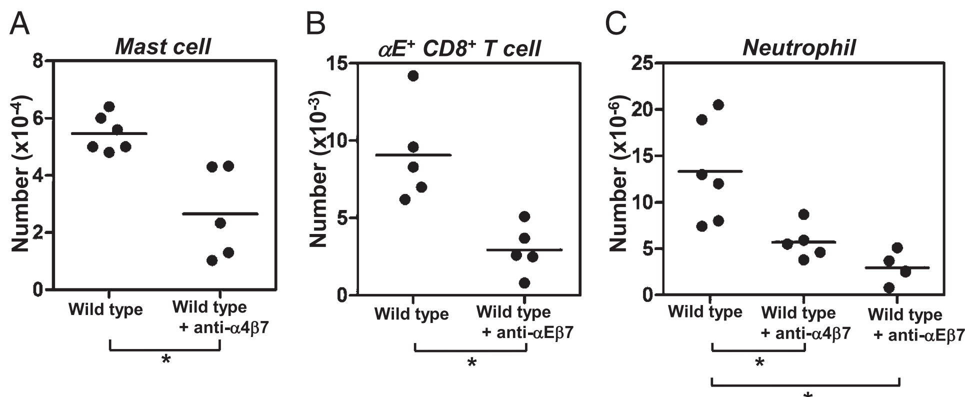

FIGURE 8. (**A**) Arthus reaction–induced recruitment of mast cells in the peritoneum of WT mice with the blockade of α4β7 integrin using its neutralizing Ab. (**B**) Arthus reaction–induced recruitment of αE$^+$CD8$^+$ T cells in the peritoneum of WT mice with the blockade of αEβ7 integrin using its neutralizing Ab. (**C**) Arthus reaction–induced recruitment of neutrophils in the peritoneum of WT mice with the blockade of α4β7 or αEβ7 integrin using neutralizing Abs. Horizontal bars indicate mean values for each group of mice. *$p < 0.05$.

not significantly different among $\beta7^{-/-}$, $\alpha E^{-/-}$, and WT mice 8 h after IC challenge (Fig. 1B). In addition to mast cells, macrophages are known to highly contribute to Arthus reaction (49). In that article, Heller et al. (49) showed that peritoneal neutrophil recruitment after 8 h of Arthus reaction was not significantly reduced in FcγRIII-deficient mice. In contrast, mice deficient in FcRγ-chain, which is the common component of both FcγRI and FcγRIII, showed significant reduction of neutrophil accumulation after 8 h. As mast cells express only FcγRIII, and macrophages express both FcγRI and FcγRIII, macrophages were thought to be important in peritoneal Arthus reaction after 8 h. Thus, we assume that mast cells are critical after 4 h, whereas other cells such as macrophages compensate after 8 h. Moreover, $CD8^+$ T cell may also have an important role. $\alpha E^{-/-}$ mice showed reduced $CD8^+$ T cell numbers 2 h after inducing peritoneal Arthus reaction (Fig. 6A). However, the numbers of $CD8^+$ T cells did not differ between $\alpha E^{-/-}$ and WT mice 4 h after the IC formation (data not shown). These results indicate that the number of $CD8^+$ T cells affect the subsequent neutrophil infiltration, causing normalization of neutrophil infiltration after 8 h of IC formation.

As for cutaneous Arthus reaction, the loss of either β7 or αE integrin reduced the later response of hemorrhage, but not the earlier response of edema. FcR γ-chain–deficient mice reveal significantly reduced edema and hemorrhage (33), whereas FcγRIII-deficient mice do not show reduced edema uniformly (50). Moreover, W/W^v mice that lack mast cells show reduced edema and hemorrhage, although reduction in hemorrhage is more prominent (51). Thus, mast cells that express FcγRIII seem to have more prominent roles on later response of hemorrhage than on earlier response of edema. As for edema, we assume that cells other than mast cells or αE^+CD8^+ T cells are important, and macrophage is the likely candidate because the numbers of macrophages in the skin tissue before and after IC challenge did not differ among WT, $\alpha E^{-/-}$, and $\beta7^{-/-}$ mice (Fig. 3D). Thus, the earlier responses of edema in $\alpha E^{-/-}$ or $\beta7^{-/-}$ mice are supposably maintained by macrophages for which the numbers in the skin tissue are not influenced by αE or β7 integrin deficiency.

Taken together, our findings suggest that in the peritoneal and cutaneous reverse-passive Arthus reaction, α4β7 integrin plays a role in mast cell migration for initial IC recognition, whereas αEβ7 integrin contributes by amplifying $CD8^+$ T cell function independent of IC recognition as well as recruiting αE^+CD8^+ T cells. As IC-mediated tissue injury is deeply involved in various diseases, especially in vasculitis, β7 or αE integrin could be a potential and selective therapeutic target for these human IC-mediated diseases.

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Disclosures

The authors have no financial conflicts of interest.

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