Latent HIV-1 reactivation in transgenic mice requires cell cycle -dependent demethylation of CREB/ATF sites in the LTR

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Objective: We previously produced a line of transgenic mice that carried the HIV-1 genome deficient in the *pol* gene. Although the HIV-1 genome in the lymphocytes was dormant under normal physiological conditions, it could be reactivated *in vivo* by lipopolysaccharide (LPS) administration via induction of interleukin-1α/β and tumour necrosis factor-α. In this report, we analysed further the reactivation mechanism of the latent HIV-1 using this transgenic mouse model.

Design and methods: Possible involvement of CpG methylation in HIV-1 latency was examined by treating transgenic lymphocytes with a demethylating agent, 5'-azacytidine. CpG methylation in the HIV-1 long terminal repeat (LTR) was analysed using the bisulfite genomic sequencing method. As previous studies suggested that CpG demethylation depended on the cell cycle progression, we analysed the relation between cell cycle progression and LPS-induced reactivation of HIV-1 by labelling lymphocytes with an intracellular fluorescein, carboxyfluorescein diacetate succinimidyl ester.

Results: We found that 5'-azacytidine enhanced HIV-1 expression ninefold compared to treatment with LPS alone. Furthermore, HIV-1 p24 induction by LPS was observed only in cells that had undergone cell division, while induction was prevented in cells in which cell cycle progression was blocked either by mimosine, aphidicolin, or nocodazole. LPS-induced HIV-1 reactivation was associated with demethylation of two CpG sites located in the CREB/ATF binding sites in the HIV-1 LTR in a cell cycle-dependent manner.

Conclusions: These observations indicate that cell cycle progression-dependent demethylation of the CREB/ATF sites in the LTR is crucial for the reactivation of latent HIV-1 genome in transgenic mice.

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Keywords: HIV-1, transgenic mouse, DNA methylation, cell cycle, viral latency

Introduction

The HIV-1 long-terminal-repeat (LTR) contains a set of *cis*-acting transcriptional control elements located both upstream and downstream of the mRNA start site [1]. The upstream control elements in the U3 region of the LTR are composed of the negative regulatory element, the enhancer, the modulator, and the basal promoter elements. Several transcriptional enhancer elements such as CREB, NF-κB, C/EBP1, Ets and AP-1 binding sites are known to be located in the enhancer/modulatory region [2]. It has been shown that various agents including chemicals, ultraviolet radiation, bacterial and viral products, and cytokines acting through these enhancer elements, activate HIV-1 expression, and that this enhancer/modulatory region

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is essential for any significant level of viral replication in the course of normal infection *in vivo* [1,3].

The molecular mechanisms of latency and reactivation of HIV-1 have not been completely elucidated. However, several models that might explain how HIV-1 exists in the host chromosome as a latent virus have been proposed. These models include transcriptional repression of the proviral DNA by binding of specific inhibitory DNA binding proteins, modifying the chromatin conformation, and methylating the DNA at the enhancer/modulatory region [4–7]. Methylation of the cytosine residues in the enhancer/modulatory region is of special interest, because CpG methylation has been implicated in the silencing of integrated provirus genomes, such as Moloney murine leukaemia virus (M-MuLV) [8,9] and mouse mammary tumour virus (MMTV) [10], as well as in the regulation of many imprinted genes [11]. These observations suggest that CpG methylation may also be involved in HIV-1 latency *in vivo*. However, the elucidation of the mechanism of latency and reactivation of HIV-1 has proven difficult, because in an HIV-infected person most T cells are infected with active HIV-1 [12,13] and therefore it is difficult to isolate and purify cells infected with HIV in the latent stage.

In this report, we have taken advantage of an animal carrier model in order to analyse activation mechanisms of latent HIV-1 *in vivo*. Previously, we produced a transgenic mouse (HIV-Tg mouse) carrying the HIV-1 proviral genome in which the *pol* gene is deleted [14]. Although the transgene expression in lymphoid tissues is barely detectable under normal physiological conditions, its expression was significantly enhanced when bacterial lipopolysaccharide (LPS) was injected into mice [15]. We also found that tumour necrosis factor (TNF)-α and interleukin (IL)-1α/β, but not IL-6 or interferon-γ, were involved in the activation of HIV-1 genes upon induction with LPS. After induction with LPS, three HIV-1 transcripts, encoding a 9.0-, 4.0-, and 1.8-kb mRNA, and properly processed protein products were detected in lymphatic organs. High levels of p24 (up to 400 pg/ml) together with the viral RNA were detected in serum at the same density as in the normal virion, indicating that viral particles had been produced. Thus, HIV-Tg mice are considered to be a good model for persistent infection of HIV-1.

We have investigated molecular mechanisms of HIV-1 gene activation after LPS treatment using this HIV-1-carrier model. We found that CpG sites in the U3 enhancer region were heavily methylated under physiological conditions, while demethylation was observed upon induction with LPS at specific CpG sites located in the putative binding sites for the CREB/ATF family transcription factors. Moreover, we found that HIV-1 p24 induction by LPS occurred only in cells that had undergone cell division while it was abolished in cells in which cell cycle progression was blocked by specific inhibitors, indicating that cell cycle progression is crucial for the reactivation of HIV-1. Our observations provide clues with which to delineate the mechanisms related to signal-mediated reactivation of HIV.

Materials and methods

HIV-Tg mice

HIV-Tg mice of the T1607 line, which carry the reverse transcriptase gene-deficient HIV-1 genome (NL-4-3-2) on the C3H/HeN background, were used throughout this study [14]. The mice were housed under specific pathogen-free conditions in the environmentally controlled clean rooms of the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo. The experiments were conducted according to the institutional ethical and safety guidelines for animal experiments and safety guidelines for gene manipulation experiments.

LPS stimulation *in vivo* and *in vitro*

LPS derived from *Escherichia coli* (055:B5) (Difco Laboratories, Inc., Detroit, Michigan, USA) was injected intraperitoneally to HIV-Tg mice (2.5 μg/g body weight) and total RNA was extracted 48 h after injection. For *in vitro* experiments, spleen cells and lymph node cells were combined and lymphocytes were purified using Ficol-Hypaque (Pharmacia, Uppsala, Sweden), and 1×10^7 cells were cultured in RPMI 1640 medium (Gibco BRL, Ibaraki, Japan) supplemented with 50 mM 2-mercaptoethanol and 10% foetal calf serum (FCS; JRH Bioscience, Tokyo, Japan), with or without 4 μg/ml LPS. Cells and the culture supernatant were harvested after 48 h. 5′-azacytidine (5-AzaC) was added to the culture at the indicated concentrations simultaneously with LPS.

Measurement of the HIV-1 p24 Gag protein and viral RNA

To measure the HIV-1 p24 in the culture supernatant, an ELISA kit (RETRO-TEK HIV-1 p24 Antigen ELISA, ZeproMetrix Corporation, Buffalo, NY, USA) was used. Northern blotting analysis was performed by the standard method using total RNA extracted from tissues of HIV-Tg mice and cultured splenocytes. Ten μg total RNA were used for each lane. A 7.5-kb *Sph*I–*Xho*I fragment of pNL4-3-2 was used as the probe. Levels of radioactivity of hybridized bands were quantified using a BAS 2000 Bio-Image-Analyzer (Fujifilm, Tokyo, Japan).

Analysis of cell division and intracellular HIV-1 p24 antigen

In a modification of the previously described technique [16], the single-cell suspension of Ficol-Hypaque-purified Tg lymphocytes from the spleen and lymph nodes was labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE: Molecular Probes Inc., Eugene, OR, USA) by incubating 1×10^7 cells/ml in phosphate buffered saline (PBS) with 10 mM CFSE for 8 min at room temperature. Staining for intracellular p24 was performed by using a standard technique and a rabbit anti-p24 polyclonal antibody (Ab). Briefly, cells were stimulated with LPS 48 h and 6 h before harvest and monensine (Sigma, St. Louis, Missouri, USA) was added to the culture. After the harvest, cells were fixed in 4% paraformaldehyde/PBS for 10 min at room temperature and stained with anti-p24 Ab (Shigematu-boueki, Ibaraki, Japan) for 60 min, followed by staining with phycoerythrin (PE)-conjugated anti-rabbit IgG in the permeabilization buffer (Hanks' solution with 0.1% saponin and 2% FCS) for 30 min. Then cells were washed twice and analysed by flow cytometry, using Becton-Dickinson Lysis II Software. To inhibit cell cycle progression, either L-mimosine (300 μM), aphidicolin (2 μg/ml), or nocodazole (1 μg/ml) (Sigma) was added together with LPS.

Bisulfite genomic sequencing

Analysis of CpG methylation was carried out according to the method of Clark *et al.* with some modifications [17]. Briefly, 5 μg genomic DNA was denatured in 0.3 N NaOH for 20 min. Then, 4.8 M $Na_2S_2O_5$ and 10 mM hydroquinone were added to make the final concentration of 4.2 M and 0.5 M, respectively, and the solution was incubated at 55°C for 5 h. After purification of the DNA, 10 N NaOH was added to a final concentration of 0.3 N and the sample was incubated at 37°C for 20 min. The modified DNA was purified and dissolved in 20 μl distilled H_2O, and an aliquot (1–2 μl) was subjected to PCR amplification. Primers used for amplification of the modified sense sequence were as follows: NL4-3 LTR forward primer, 5′-TTTGTTATATTTTATGAGTTAGTAT-3′ (−240 to −217); reverse primer, 5′-CAAAAAACTCCCAAA CTCAAATCTA-3′ (+20 to +42). Amplified products were cloned by the TA method followed by sequence determination using an automatic sequencer (Hitachi DNA sequencer 5500; Hitachi, Tokyo, Japan).

Results

Demethylation of provirus by an inhibitor of DNA methyltransferase, 5-AzaC, activates HIV-1 gene expression in a dose-dependent manner

We previously reported that *in vivo* LPS stimulation of HIV-Tg mice induced viral gene expression in spleen cells [15]. To examine the effects of CpG demethylation in LPS-mediated reactivation of latent HIV-1, we treated transgenic lymphocytes with 5-AzaC in the presence of LPS and studied the HIV-1 mRNA expression in the lymphocytes using Northern blot hybridization analysis, and p24 levels in the culture supernatant using ELISA. As shown in Fig. 1, treatment with LPS enhanced HIV-1 mRNA expression about 10-fold compared with non-treated lymphocytes. On the other hand, treatment with 5-AzaC in the absence of LPS did not enhance HIV-1 expression at all (Fig. 1a and b). Under these conditions, 5-AzaC was not incorporated into DNA, as no cell proliferation was observed (see Fig. 3a, left panel). However, increasing concentrations of 5-AzaC in combination with LPS enhanced the levels of HIV mRNA two- to ninefold compared with those obtained for LPS alone (Fig. 1a). The maximum enhancement was observed at 5 μM 5-AzaC. The activation was associated with cell proliferation and no cytotoxic effect from 5-AzaC was observed for 48 h even at 10 μM, as evaluated by Trypan blue dye exclusion test (data not shown). The levels of HIV-1 p24 Gag protein released into the culture supernatant increased in parallel with the levels of mRNA (Fig. 1b).

We previously showed that LPS activates HIV-1 gene expression in transgenic mice by inducing TNF-α and IL-1. Then, we examined the effect of 5-AzaC treatment on the expression of TNF-α, IL-1α/β, TNF receptors (TNF-RI and RII) and the IL-1 receptor (IL-1 RI). We found that the expression of these molecules was not affected by the treatment, indicating that the induction of HIV-1 mRNA by 5-AzaC is not caused by the over-expression of these cytokines or their receptors (data not shown). These findings suggest that demethylation of HIV-1 proviral DNA is directly involved in the reactivation of the latent virus.

LPS-induced reactivation of HIV-1 is associated with demethylation of specific CpG sites in the enhancer region

As HIV-1 gene expression was enhanced by 5-AzaC treatment *in vitro*, we next examined whether or not demethylation of CpG sites was involved in the LPS-induced HIV-1 gene expression in animals. As reported previously, the administration of LPS to HIV-Tg mice resulted in activation of HIV-1 genes in lymphocytes after 48 h (Fig. 2a). The CpG methylation in the U3 enhancer/modulatory region of HIV-1 LTR in lymphocytes, with or without LPS treatment, was analysed using the bisulfite genomic sequencing method. PCR amplified fragments of the U3 enhancer region of HIV-1 LTR were cloned into plasmids and the methylation status of each CpG site in a single allele was analysed. The results showed that all eight CpG sites were almost completely methylated in most clones from lymphocytes without LPS treatment, with a few

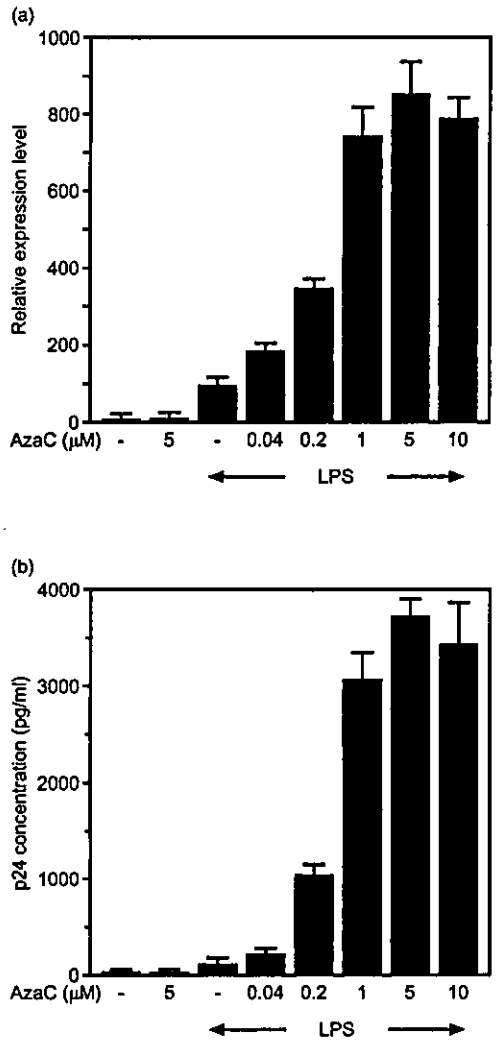

Fig. 1. 5-AzaC dose-dependent induction of HIV-1 mRNA expression and p24 Gag protein in HIV-Tg lymphocytes stimulated with LPS. Cultured HIV-Tg lymphocytes were stimulated with or without LPS (4 μg/ml) for 48 h. Then HIV-1-specific mRNA was measured by Northern blot hybridization (a), and p24 Gag protein concentration was measured by ELISA (b). (a) Total RNA was extracted from the lymphocytes and the relative levels of the 4.0-kb HIV-1 mRNA are shown. The same blot was reprobed with a β-actin probe to examine the relative amount of the RNA in the lane. Data are expressed as relative values to the activation level in splenocytes stimulated with LPS in the absence of 5-AzaC. (b) The levels of p24 Gag protein in the culture supernatant were measured by ELISA. Results were reproducible in three independent experiments, and the average and standard deviation (SD) of a representative experiment are shown.

clones having single or double demethylated CpG sites [Fig. 2b, LPS (−) panel]. However, 10 out of 25 clones from lymphocytes after LPS stimulation presented demethylation at the fifth CpG site from the 5′ end, whereas other sites remained methylated [Fig. 2b, LPS (+) panel]. Thus, it was shown that LPS stimulation induced demethylation of a specific CpG site(s) in the enhancer region of HIV-1 LTR, suggesting that HIV-1 gene was transcribed from the allele having these demethylated CpG sites.

Cell cycle progression is required for HIV-1 p24 expression upon stimulation with LPS

As 5-AzaC treatment alone could not activate HIV-1 expression and cell proliferation induced by LPS was required for the activation, we next examined potential roles of cell proliferation in the activation of HIV-1. For this, we labelled lymphocytes with an intracellular fluorescein, CFSE, allowing us to successfully distinguish each generation of dividing cells based on its dilution [16]. CFSE did not interfere with the proliferative capacity of the stimulated cells, as evaluated by flow cytometer and [^{3}H]thymidine incorporation (data not shown). We found that high levels of p24 expression after treatment with LPS were detected only in cells with lower concentrations of CFSE, whereas low levels of p24 expression were observed without LPS stimulation (Fig. 3a). These results indicate that p24 expression is induced only in cells that undergo cell divisions. In the absence of LPS, most lymphocytes did not replicate and p24 expression was not significantly induced after 48 h of culture (Fig. 3a, left panel).

Because these results suggested that p24 induction depended on cell cycle progression, we next analysed the specific stages of the cell cycle which are required to induce HIV-1 p24 expression. For this, we blocked cell cycle progression with various inhibitors and examined p24 induction along with cell division analysis using CFSE. As shown in Fig. 3b, HIV-1 p24 induction was almost completely prevented in cells in which cell cycle progression was blocked either at G1 phase with mimosine, at S phase with aphidicolin, or at G2/M phase with nocodazole. These treatments did not affect the expression of TNF-α, IL-1α/β, TNF receptors and IL-1 receptor, excluding the possibility that these cell cycle blockers inhibit HIV-1 induction by suppressing the expression of these molecules (data not shown). These observations are in clear contrast with the HIV-1 p24 induction in cells with normal cell cycle progression. These findings indicate that the induction of HIV-1 p24 expression in lymphocytes depends on cell cycle progression, suggesting that DNA replication is required for LPS-induced HIV-1 gene activation.

The CpG at the binding sites for the CREB/ATF family transcription factors is specifically demethylated after activation with LPS

As it was indicated that HIV-1 reactivation depended on cell proliferation, we next examined cell cycle dependency of the CpG demethylation in the U3

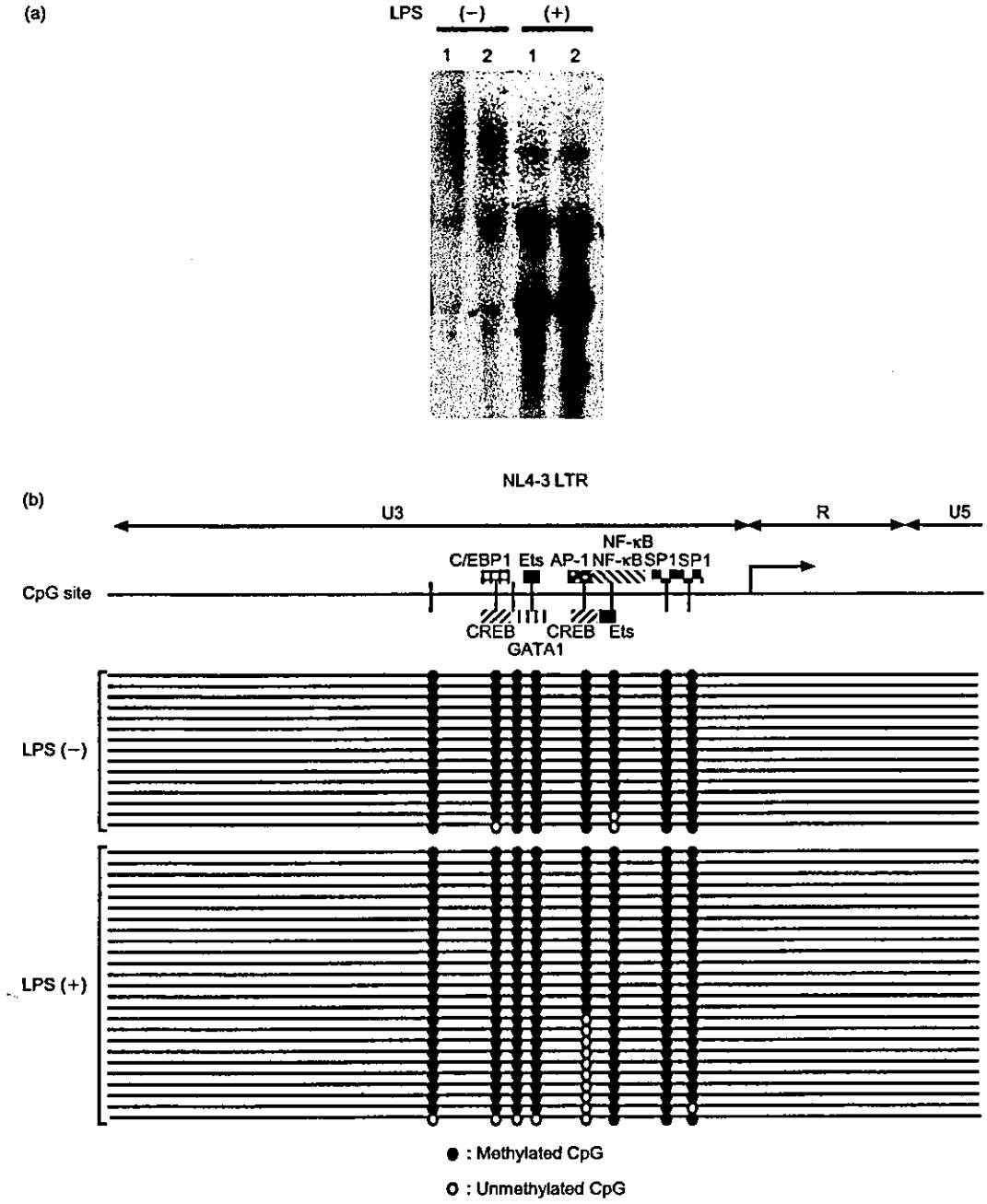

Fig. 2. Demethylation of specific CpG sites in the LTR U3 region of HIV-1 after stimulation with LPS. (a) Induction of the HIV-1 mRNA in the lymphocytes of HIV-Tg mice by LPS. HIV-Tg mice received intraperitoneal injections of PBS (−) or LPS (+). Total RNA was extracted from the lymphocytes 48 h after injection and analysed by Northern blot hybridization for HIV-1-specific mRNA. Two samples from different mice were analysed in parallel. (b) CpG demethylation after stimulation with LPS for 48 h. PCR amplified fragments of the HIV-1 LTR from lymphocytes of HIV-Tg mice with PBS (−) or LPS (+) treatment were cloned into plasmids and the CpG methylation pattern in each plasmid clone was analysed using bisulfite genomic sequencing. Upper panel: schematic map of the CpG sites in the U3 region of NL4-3 LTR is shown in relation to the binding motifs for transcriptional factors which were identified by TSEARCH program [18]. Lower panel: results of bisulfite genomic sequencing of the lymphocytes from HIV-Tg mice with PBS (−) or LPS (+) treatment are shown, where the single line indicates the methylation status of an allele. The CpG sites in the U3 region reside at −216, −169, −157, −144, −118, −95, −62 and −46 relative to the transcription start site (nucleotide +1).

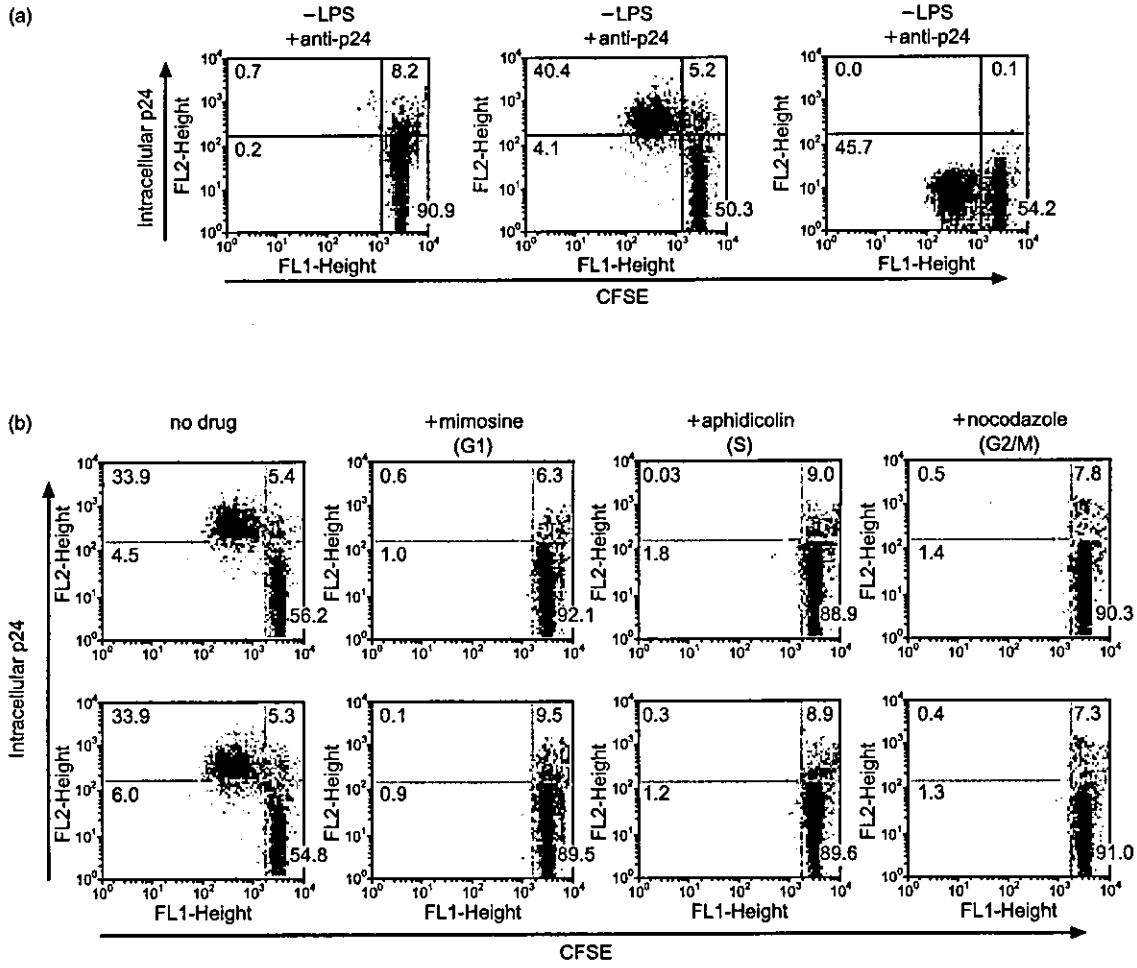

Fig. 3. Cell cycle-dependent induction of HIV-1 p24 expression. (a) Induction of HIV-1 p24 expression only in cells that had undergone cell division. CFSE-labelled HIV-Tg lymphocytes were cultivated with LPS or without stimulant for 48 h. After fixation and permeabilization of the cells, intracellular p24 was stained with rabbit anti-p24 polyclonal Ab, and then with PE-conjugated anti-rabbit IgG. The staining pattern was analysed by flow cytometry. The x-axis represents the intensity of the green fluorescence, CFSE. The intensity decreased with the progression of cell division due to dilution of the intracellular fluorescein. The y-axis represents the intensity of the red fluorescence of PE-conjugated anti-p24 antibody. Left panel: cultivated without LPS, detected with anti-p24 Ab. Middle panel: stimulated with LPS, detected with anti-p24 Ab. Right panel: stimulated with LPS, detected without anti-p24 Ab. Results are representative in four independent experiments. (b) Inhibition of HIV-1 p24 induction in cells in which cell cycle progression was blocked. CFSE-labelled HIV-Tg lymphocytes were stimulated with LPS in the presence of the drugs indicated above the panels. The specific phase at which the cell cycle was arrested is shown in parenthesis. Each panel shows result obtained from a mouse. Results are representative of three independent experiments using two mice for each experiment.

region of HIV-1 LTR. After LPS stimulation, we sorted lymphocytes according to the intensity of CFSE fluorescence. Thus, cells presenting high-intensity fluorescence represent non-dividing cells, and cells presenting low-intensity fluorescence represent those that had undergone cell division. Next, we examined CpG methylation of the integrated HIV-1 LTR using bisulfite genomic sequencing method. The results showed that CpG sites in most alleles from cells with high-intensity CFSE fluorescence were heavily methylated, with only a few alleles having a single demethylated CpG site (Fig. 4, CSFE high). On the other hand, eight of the 16 clones from cells with low-intensity CSFE fluorescence presented demethylation at a single or double CpG site(s). Demethylation was limited to the second and fifth CpG sites in the U3 region in most alleles (Fig. 4, CSFE low). Sequence analysis around these demethylated CpG sites per-

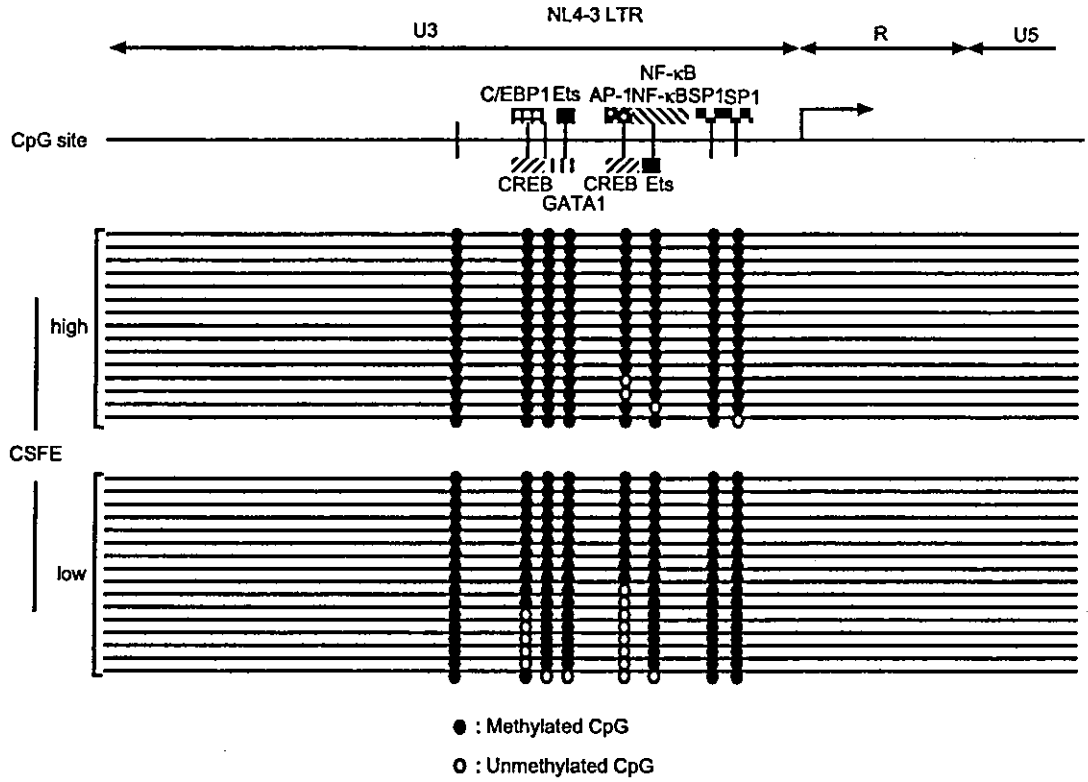

Fig. 4. Demethylation of specific CpG sites after cell cycle progression. CFSE-labelled HIV-Tg lymphocytes were stimulated with LPS for 48 h, and sorted according to the intensity of CFSE fluorescence, high and low. PCR amplified fragments were cloned into plasmids and the methylation status of each CpG site in a single allele was analysed. CFSE 'high' shows the result from cell populations that had not divided yet, and CFSE 'low' shows that from cell populations that had undergone cell division. Upper panel: locations of CpG sites in NL4-3 LTR U3 region are shown in relation to the binding motifs for transcriptional factors which were analysed by TSEARCH program. Lower panel: methylation status of the CpG sites analysed by bisulfite genomic sequencing methods.

formed with a TSEARCH program [18] revealed that both CpG sites were located in the putative CREB/ATF binding motif [19]. Taken together, these results indicate that LPS stimulation of HIV-Tg mouse lymphocytes induces viral gene expression, accompanied by the specific demethylation of CpG in the CREB/ATF sites, in a cell cycle dependent manner.

Discussion

Involvement of proviral DNA methylation in viral latency has been known for a long time in other retroviruses such as M-MuLV or MMTV [8–10]. However, the mechanism of latency has not been elucidated completely for HIV-1 [20]. With regard to this, it was reported that methylation of sequences in the upstream region of HIV-1 promoter rendered genes transcriptionally inactive [4,21], and that treatment of latently infected cultured cells with 5-AzaC reactivated the latent provirus [4,22,23], although reactivation was not observed in some cases [24]. These observations suggest that methylation of the sequences in the promoter region might be involved in the latency of HIV-1. However, as cells infected with the latent form of HIV-1 are difficult to obtain directly from patients, no direct evidence of the involvement of this mechanism in HIV-1 latency *in vivo* has been presented to date. Here, we have first shown that methylation of proviral DNA is crucial for the latency of HIV-1 *in vivo* using HIV-Tg mice.

We showed that only two CpG sites in the U3 enhancer/modulatory region are preferentially demethylated upon reactivation with LPS, in contrast with the notion that the promoter activity is regulated by the density of CpG methylation [25]. Sequence analysis around the demethylated CpG sites indicated that both sites were located in the putative CREB binding sites. Indeed, binding of CREB1 to the CREB/ATF binding sites in the U3 enhancer/modulatory region was previously demonstrated [19,26]. Also, as binding of CREB/ATF proteins to DNA is blocked

by CpG methylation [27], demethylation of CREB binding sites should make them available to the CREB family transcription factors. It has been reported that demethylation of a specific CpG site is involved in the activation of other viruses [28]. Thus, methylation of a specific site(s) important for transcriptional regulatory factor binding is thought to be one of the major regulatory mechanisms for the viruses to control their gene expression, as was suggested for various cellular genes [29].

The mechanism by which LPS activates CREB transcription factors is not completely understood. However, it was reported that p38 mitogen-activated protein kinase (MAPK), which is activated by TNF-α and IL-1, is involved in the activation of HIV-1 genes [30–32], and that it indirectly activates CREB by activating downstream kinase MSK1 [33]. We also observed that one of the p38 MAPK specific inhibitors, SB203580 [34], suppressed LPS-induced HIV-1 gene activation in HIV-Tg mouse splenocytes (unpublished data). Thus, it is possible that a link between CREB transcription factors and p38 MAPK is involved in the reactivation of silenced HIV-1 genes, although the identification of the interacting factor(s) for the two CREB/ATF sites in the U3 region remains to be elucidated.

Formally, demethylation of CpG sites can be achieved by either active or passive mechanisms. With the active mechanism, methylated residues are believed to be removed by demethylases, whereas with the passive mechanism, demethylation of cytosine residues is caused by prevention or failure of CpG site methylation of newly synthesized DNA strands [35]. Using the Epstein–Barr virus latent replication origin, oriP, Hsieh reported that protein binding specifies the site of DNA demethylation and that the first strand demethylation is a passive process that depends on DNA replication [36]. In this study, we have shown that the inhibition of cell cycle progression suppresses the reactivation of the latent HIV-1 genes. This suggests that DNA replication is required for the demethylation of the HIV-1 gene enhancer, as indicated for the passive mechanism. We showed that HIV-1 reactivation is inhibited not only by G1 phase or S phase inhibitors, but also by G2/M phase inhibitors. This is not because hemizygous demethylation is insufficient to activate genes, because many of the HIV-1 genomes that actively produced p24 were still hemizygotes (Fig. 3). These observations indicate that single round of cell cycle progression is not sufficient for demethylation.

So far, few reports have investigated cell cycle dependency of HIV-1 gene activation [37–40]. Kashanchi *et al.* demonstrated by *in vitro* transcription analysis using cell lines transfected with an HIV-1 LTR chloramphenicol acetyltransferase construct that Tat activated gene expression in the G1 and G2 phases of the cell cycle [39]. On the other hand, Park *et al.* showed in an *in vivo* study using HIV-based vectors that lentiviral transduction to hepatocytes was enhanced when hepatocellular regeneration was stimulated under the conditions that stimulate cell cycle progression [40]. Furthermore, Roberts *et al.* have reported that the influence of cell cycle on constitutive and induced HIV-1 expression varied in each of the cellular models of latent HIV-1 infection: OM-10.1 promyelocytes, ACH-2 T-lymphocytes, and U1 promonocytes [41]. Although these reports suggested cell cycle dependency of the viral replication, no information has been presented to date about the participation of the cell cycle progression in the reactivation of viruses from the latent state.

In summary, our study demonstrated that extracellular signal-induced reactivation of heavily methylated latent HIV-1 requires cell cycle-dependent demethylation of specific CpG sites. Although there is no perfect animal model for the HIV-1 carrier at this time, these findings may provide a rationale for the assumption that methylation of the proviral DNA might also be involved in the latency of HIV-1 in humans. It is obvious, however, that other factors such as cellular and viral regulatory factors or integration sites of the proviral DNA are also involved in the reactivation of the latent virus. Therefore, integration of these different mechanisms may provide a complete understanding of the HIV-1 reactivation mechanism.

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IL-1-induced tumor necrosis factor-α elicits inflammatory cell infiltration in the skin by inducing IFN-γ-inducible protein 10 in the elicitation phase of the contact hypersensitivity response

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Abstract

Contact hypersensitivity (CHS) is a typical inflammatory response against contact allergens. Inflammatory cytokines, including IL-1 and tumor necrosis factor (TNF)-α, are implicated in the reaction, although the precise roles of each cytokine have not been completely elucidated. In this report, we dissected the functional roles of IL-1 and TNF-α during CHS. CHS induced by 2,4,6-trinitorochlorobenzene as well as oxazolone was suppressed in both IL-1α/β$^{-/-}$ and TNF-α$^{-/-}$ mice. Hapten-specific T cell activation, as examined by T cell proliferation, OX40 expression and IL-17 production, was reduced in IL-1α/β$^{-/-}$ mice, but not in TNF-α$^{-/-}$ mice, suggesting that IL-1 but not TNF-α is required for hapten-specific T cell priming in the sensitization phase. On the other hand, TNF-α, induced by IL-1, was necessary for the induction of local inflammation during the elicitation phase. We also found that the expression of IFN-γ-inducible protein 10 (IP-10) was augmented at the inflammatory site. Although IP-10 mRNA expression was abrogated in TNF-α$^{-/-}$ mice, both CHS development and TNF-α mRNA expression occurred normally in IFN-γ$^{-/-}$ mice, indicating that the induction of IP-10 during CHS was primarily controlled by TNF-α. Interestingly, CHS was suppressed by treatment with anti-IP-10 mAb, suggesting a critical role for IP-10 in CHS. Reduced CHS in TNF-α$^{-/-}$ mice was reversed by IP-10 injection during the elicitation phase. Thus, it was shown that the roles for IL-1 and TNF-α are different, although both cytokines are crucial for the development of CHS.

Introduction

The development of contact hypersensitivity (CHS) requires several distinctive events including the capture of contact allergens by Langerhans cells (LC), LC migration into lymph nodes (LN) and maturation, hapten-specific T cell activation within the LN, and leukocyte infiltration into the skin requiring a series of adhesion events: rolling, firm adhesion and eventual

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transmigration into inflamed regions (1,2). Various cytokines are produced by keratinocytes (KC) in the skin and LC in LN (2), and they are involved in both the sensitization and elicitation phases of CHS (1,2). The pro-inflammatory cytokines IL-1 and tumor necrosis factor (TNF)-α may play crucial roles in this reaction, as CHS is greatly suppressed in mice deficient for these molecules (3,4). These cytokines promote LC migration (4–8) by regulating E-cadherin expression on KC and LC (9,10), CCR7 expression on immature dendritic cells (11,12), and the ligands for CCR7, SLC and ELC, expression in LN (13). Adhesion molecules, ICAM-1, VCAM-1, and E- and P-selectin on vascular endothelium, are up-regulated by IL-1 and TNF-α during the elicitation phase to facilitate leukocyte rolling and firm adhesion in inflammatory sites (14,15). Although these cytokines are critical, various additional chemokines produced by KC, LC and lymphatic endothelial cells are also involved in leukocyte migration during CHS (2,16).

In earlier studies, it was reported that CHS was suppressed by the treatment with anti-IL-1β antibody, but not by anti-IL-1α antibody (17). CHS induced by low-dose 2,4,6-trinitorochlorobenzene (TNCB) sensitization, but not by high-dose sensitization, was reduced in IL-1β$^{-/-}$ mice on a 129 × B6 background (18), suggesting the involvement of IL-1β in CHS. On the other hand, Zheng *et al.* reported that oxazolone-induced CHS was normal in IL-1β$^{-/-}$ mice (19), and we also showed that TNCB-induced CHS is suppressed only in IL-1α$^{-/-}$ and IL-1α/β$^{-/-}$ mice, but not in IL-1β$^{-/-}$ mice, on a C57BL/6 background using both low- and high-dose TNCB, indicating that IL-1α is responsible for the development of this reaction (4). Reduced CHS in IL-1α/β$^{-/-}$ mice is caused by a defect in antigen-specific T cell activation rather than impairment of LC migration into LN (4). IL-1 potentiates T cell priming through the CD40 ligand (CD40L) and OX40 induction on CD4$^+$ T cells (20), and specifically IL-1α, which is produced by LC, plays a crucial role in the hapten-specific T cell activation during CHS (4). The possible role for IL-1 in the elicitation phase, however, remains to be elucidated.

CHS is suppressed by the injection with anti-TNF-α antibody (21). Likewise, oxazolone-induced CHS is reduced in TNF-α$^{-/-}$ and TNF receptor II$^{-/-}$ mice (3,6), suggesting the involvement of TNF-α in the development of CHS—although, again, the mechanisms remain unclear. Furthermore, CHS suppression in these single cytokine-deficient mice was incomplete, suggesting that additional cytokines or these cytokines mutually compensate for the deficiency. Therefore, although IL-1 and TNF-α are clearly involved in CHS, their individual functional roles in CHS pathogenesis, including a functional discrimination of each and their relationship, remain to be elucidated. The current study sought to address these points using IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. Our results demonstrate that IL-1 and TNF-α play important roles in the sensitization phase and elicitation phase respectively. During the sensitization phase, both cytokines are implicated in the migration of LC from the skin to LN, although IL-1 alone is required for hapten-specific T cell activation. In the elicitation phase, however, IL-1 induces TNF-α expression that then activates the expression of IFN-γ-inducible protein 10 (IP-10), a chemokine crucial for the recruitment of inflammatory cells into inflammatory sites.

Methods

Mice

IL-1α$^{-/-}$, IL-1β$^{-/-}$, IL-1α/β$^{-/-}$, IL-1Ra$^{-/-}$ and IFN-γ$^{-/-}$ mice were generated by homologous recombination as described. Mice from each group were backcrossed to C57BL/6J and BALB/cA mice respectively for eight generations (22,23). TNF-α$^{-/-}$ mice were backcrossed to C57BL/6J and BALB/cA mice for 10 and eight generations respectively (24). IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice and IL-1Ra$^{-/-}$ × TNF-α$^{-/-}$ mice were obtained by intercrossing IL-1α/β$^{-/-}$ mice and TNF-α$^{-/-}$ mice, and IL-1Ra$^{-/-}$ mice and TNF-α$^{-/-}$ mice respectively. These mice were housed under specific pathogen-free conditions in an environmentally controlled clean room at the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo. The experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments. Sex- and age-matched mice of 8–12 week in age were used for experimentation.

CHS

TNCB (Tokyo Kasei, Tokyo, Japan)-induced CHS was assayed as described previously (4,19). Briefly, the abdomens of mice were shaved and sensitized epicutaneously with 25 μl of 3.0% TNCB dissolved in acetone mixed with olive oil (4:1). Five days after sensitization, the outside of one ear (auricle) was challenged with 25 μl of 1.0% TNCB while the other ear was given 25 μl of vehicle alone. Mice were euthanized 24 h after TNCB challenge. A disk of ear tissue from both ears of each mouse was removed using a 6-mm biopsy punch. Each disk was weighed and ear swelling was calculated as follows: increase of ear swelling = [(weight of TNCB challenged ear) – (weight of vehicle-treated ear)]. For reconstitution or neutralization of CHS, either 25 ng of mouse rTNF-α (Peprotech, Rocky Hill, NJ), 50 ng of mouse rIL-1α/β mixture (Peprotech), 1 μg of mouse rIP-10 (Peprotech) or 100 μg of anti-mouse IP-10 mAb (25) was injected intradermally into the ear skin immediately after TNCB challenge. At 24 h after TNCB challenge, ear swelling was then measured as described above.

Migration and maturation of LC

Measurement of LC migration and maturation was carried out as described (4). The backs and abdomens of mice were shaved and painted with 50 μl of 0.5% FITC isomer I (Sigma, St Louis, MO) dissolved in an equal volume mixture of acetone and dibutylphthalate. After 24 h, inguinal, axillary and brachial LN were harvested. Single-cell suspensions were prepared from collagenase-treated LN, and stained with biotinylated anti-mouse CD11c mAb (HL3; PharMingen, San Diego, CA) and PerCP–streptavidin (PharMingen). The content of FITC$^+$ cells within the CD11c$^+$ population was analyzed to estimate migration of LC (5000 cells) from the skin to the LN using flow cytometry on a FACScan cytometer (Becton Dickinson, Mountain View, CA). To examine maturation state of the LC, CD11c$^+$FITC$^+$ cells in LN were analyzed for staining with either phycoerythrin (PE)–anti-mouse CD40 mAb (3.23; Immunotech, Marseille, France) or PE–anti-mouse CD86 mAb (RMMP-1; Immunotech).

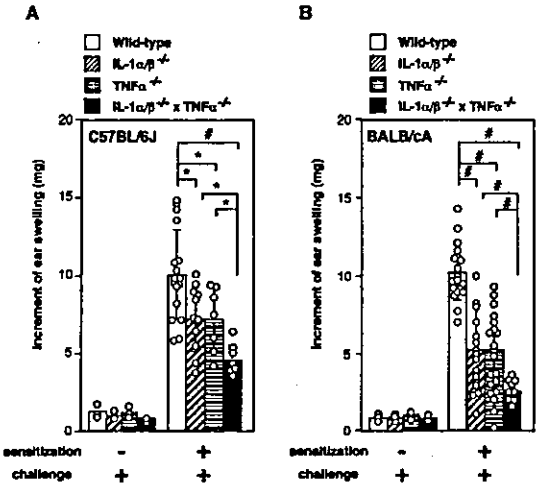

Fig. 1. Effect of IL-1 and TNF-α deficiency on TNCB-induced CHS. Ear swelling following TNCB-induced CHS was measured in IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. (A) TNCB-induced CHS using C57BL/6J wild-type, IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. (B) TNCB-induced CHS using BALB/cA wild-type, IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. Each circle represents an individual mouse. Averages ± SD for groups of mice are shown. Student's *t*-test was used for statistical evaluation of the results. $^{*}P < 0.05$ and $^{\#}P < 0.001$.

T cell response

Five days after 3.0% TNCB sensitization, single-cell suspensions were prepared from inguinal, axillary and brachial LN. T cells were purified by the depletion of B220^{+} cells and Mac-1^{+} cells using a MACS system (Miltenyi Biotec, Bergish Gladbach, Germany). To prepare TNP-conjugated spleen cells, Thy-1.2^{+} cells were also depleted by MACS columns. Cells were incubated with 100 mM trinitrobenzene sulfonate (Wako, Osaka, Japan) in PBS for 5 min at 37°C and irradiated at 35 Gy with ^{137}Cs. T cells derived from TNCB-sensitized mice (5×10^5 cells/well) were cultured with TNP-conjugated spleen cells (2×10^5 cells/well) in 200 μl RPMI 1640 (Sigma) containing 50 μM 2-mercaptoethanol (Gibco/BRL, Gaithersburg, MD), 50 μg/ml streptomycin (Meiji, Tokyo, Japan), 50 U/ml penicillin (Meiji) and 10% heat-inactivated FCS (Sigma) in a 96-well flat-bottom plate for 72 h. Following a 72-h incubation, cultures were pulsed with [^{3}H]thymidine (0.25 μCi/ml) (Amersham, Little Chalfont, UK) for 6 h. Cells were harvested with a Micro 96 cell harvester (Skatron, Lier, Norway); incorporated [^{3}H]thymidine was measured with a Micro Beta counter (Pharmacia Biotech, Piscataway, NJ). To measure OX40 expression, dinitrobenzene sulfonate (DNBS)-sensitized LN cells were cultured for 72 h in the absence or presence of 40 μg/ml DNBS as described elsewhere (26). Cells were then incubated with FITC-anti-mouse CD4 mAb (BD PharMingen) and PE-anti-mouse OX40 (OX86) (Immunotech), and analyzed by flow cytometry. IL-17 levels in culture supernatants were measured by ELISA using monoclonal rat anti-mouse IL-17 and polyclonal biotinylated goat anti-mouse IL-17 antibody (Dako, Carpinteria, CA) as capture and detection antibody respectively. Horseradish peroxidase–avidin was obtained from BD PharMingen and TMB substrate was purchased from Dako.

CHS of mice transferred with T cells

T cells (2×10^7 cells/mouse) derived from TNCB-sensitized mice were suspended in PBS and injected i.v. into an unchallenged mouse. After 12 h, the outside of one ear was challenged with 25 μl of 1.0% TNCB, while the other was given vehicle alone. Then, 24 h after challenge with TNCB, ear swelling was measured as described above.

Induction of IP-10 mRNA expression by injection with rIL-1 and rTNF-α

rIL-1α/β (100 ng) was injected into the ear with or without anti-TNF-α mAb (XT22; Endogen). rTNF-α (100 ng) was also injected into the ear. Total RNA was prepared from ear tissue 3 h after the injection. IP-10 mRNA expression was then detected by Northern blot hybridization.

Northern blot hybridization analysis

Total RNA was prepared from the ear 3 and 24 h after either TNCB challenge or cytokine injection. Northern blot hybridization analysis was carried out as described (23).

Statistics

Student's *t*-test was used for statistical evaluation of results.

Results

Effects of IL-1α/β and TNF-α deficiency on the development of CHS

To elucidate the role of IL-1 and TNF-α in CHS development, the ear swelling response against TNCB was assessed in IL-1α/β$^{-/-}$ and TNF-α$^{-/-}$ mice of the C57BL/6J background. The CHS response was significantly suppressed in both TNCB-sensitized IL-1α/β$^{-/-}$ mice and TNF-α$^{-/-}$ mice, indicating that both IL-1 and TNF-α are involved in the reaction (Fig. 1A). Without sensitization, no ear swelling was observed. The suppression, however, was not complete, indicating that additional cytokines may compensate for the deficiency. We then examined the possible mutual compensation between IL-1 and TNF-α using IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. The suppression of ear swelling was significantly greater in double cytokine knockouts than in IL-1α/β$^{-/-}$ or TNF-α$^{-/-}$ mice, clearly indicating that IL-1 and TNF-α have distinct roles in the CHS response.

As the genetic backgrounds of mice can affect the CHS response (27), we also examined the effects of cytokine deficiency on mice of the BALB/cA background. We obtained similar results to those seen for the C57BL/6J background. Suppression was significantly greater in IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice than in either IL-1α/β$^{-/-}$ or TNF-α$^{-/-}$ mice (Fig. 1B).

We also examined effects of these deficiencies on the development of CHS using oxazolone treatment. We obtained similar results (data not shown), demonstrating that both IL-1 and TNF-α play important roles in CHS pathogenicity irrespective of allergens.

Effect of IL-1α/β and TNF-α deficiency on LC migration and maturation

LC of the skin are the major antigen-presenting cells (APC) involved in CHS. Following painting of the skin with FITC, we examined the effect of cytokine deficiency on LC migration by measuring the migration of FITC-labeled LC to regional LN by flow cytometry. Migration of FITC$^+$ LC from the skin to the draining LN was impaired in IL-1α/β$^{-/-}$ mice, consistent with previous observations (4); while 44% of CD11c$^+$ LN cells from wild-type C57BL/6J mice were labeled with FITC, only 27% of CD11c$^+$ LN cells from IL-1α/β$^{-/-}$ mice were labeled with FITC (Fig. 2A). LC migration was also suppressed in TNF-α$^{-/-}$ mice, indicating that TNF-α also plays a role in the migration of LC. Interestingly, the phenotype in triple cytokine knockout mice was more severe; <3% of CD11c$^+$ cells in draining LN of IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice were labeled with FITC. Similar results were obtained in BALB/cA mice (data not shown). These observations suggest that these cytokines have partially redundant functions in the induction of LC migration.

CD40 and CD86 expression on FITC$^+$ CD11c$^+$ cells was similar among IL-1α/β$^{-/-}$, TNF-α$^{-/-}$, IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ and wild-type mice on either the C57BL/6J (Fig. 2B) or BALB/cA background (data not shown). The expression of CD80 and ICAM-1 was also similar among these mice (data not shown), indicating that LC in mutant mice mature normally, measured by cell surface molecule expression.

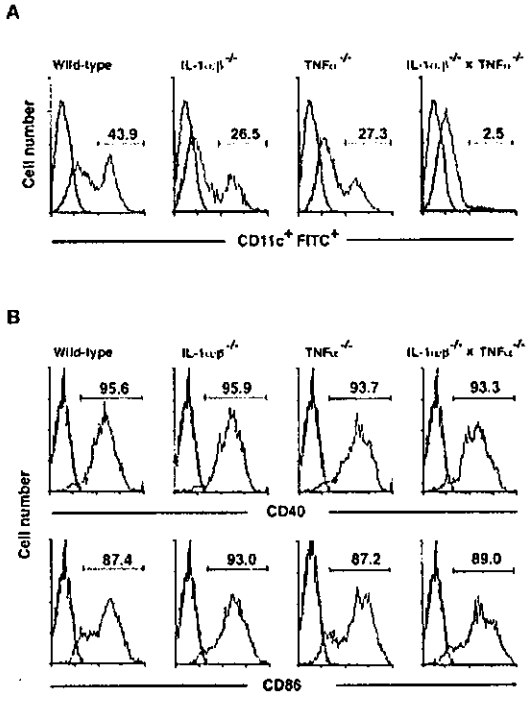

Fig. 2. Effect of IL-1 and TNF-α deficiency on LC migration and maturation. Mice were sensitized epicutaneously with 0.5% FITC. After 24 h, draining LN were harvested and analyzed for FITC staining (A) and CD40/CD86 expression (B) by flow cytometry. (A) Content of FITC$^+$ cells within the CD11c$^+$ population derived from wild-type, IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. Shaded area shows non-treated mice. (B) The expression of CD40 and CD86 among CD11c$^+$ FITC$^+$ cells from wild-type, IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. Solid lines show isotype-matched control Ig staining. One representative result from four independent experiments is shown.

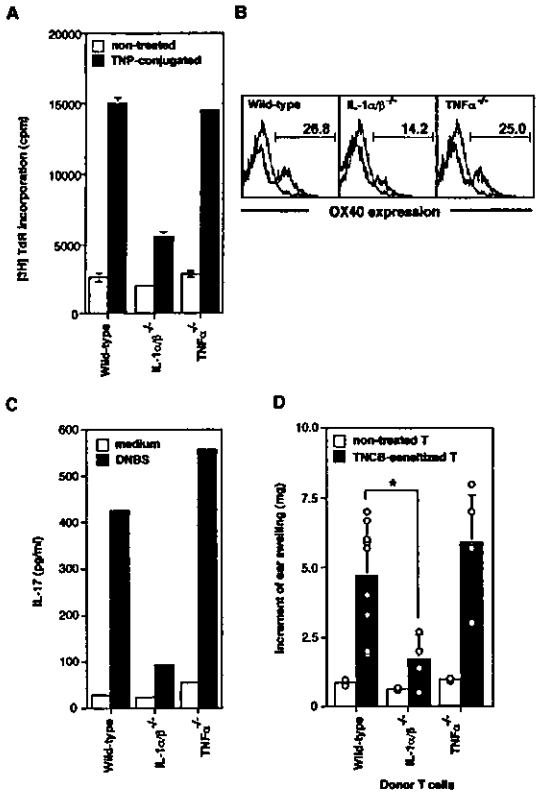

Fig. 3. Effect of IL-1 and TNF-α deficiency on hapten-specific T cell induction. (A) LN were harvested 5 days after sensitization with 3.0% TNCB. T cells were purified using MACS separation columns. LN T cells (5×10^5 cells) from TNCB-sensitized mice were cultured for 72 h with either irradiated TNP-conjugated APC (2×10^5 cells) or non-treated APC from C57BL/6J splenocytes depleted of T cells. The proliferative response was assessed by [^{3}H]thymidine incorporation. The result was reproduced in three independent experiments. (B) LN cells (4×10^5 cells) from 0.2% DNFB-sensitized mice were cultured in the absence or presence of 40 μg/ml DNBS for 72 h. After 72 h of culture, cells were harvested and stained with FITC–anti-mouse CD4 mAb and PE–anti-mouse OX40 mAb. The expression of OX40 on CD4$^+$ T cells was analyzed by FACS. Thin lines indicate isotype-matched control Ig staining and solid lines demonstrate staining with anti-OX40 mAb. The result was reproducible in three independent experiments. (C) IL-17 levels in supernatants from (B) were measured by ELISA. The result was reproducible in three independent experiments. (D) LN T cells (2×10^7 cells) from non-treated or TNCB-sensitized wild-type, IL-1α/β$^{-/-}$ and TNF-α$^{-/-}$ mice were injected i.v. into non-sensitized wild-type mice. Ear swelling following TNCB-induced CHS was measured 5 days later, as performed in Fig. 1. Each circle represents an individual mouse. An average ± SD for each group of mice is shown. Student's *t*-test was used for statistical evaluation of the results. $^*P < 0.05$.

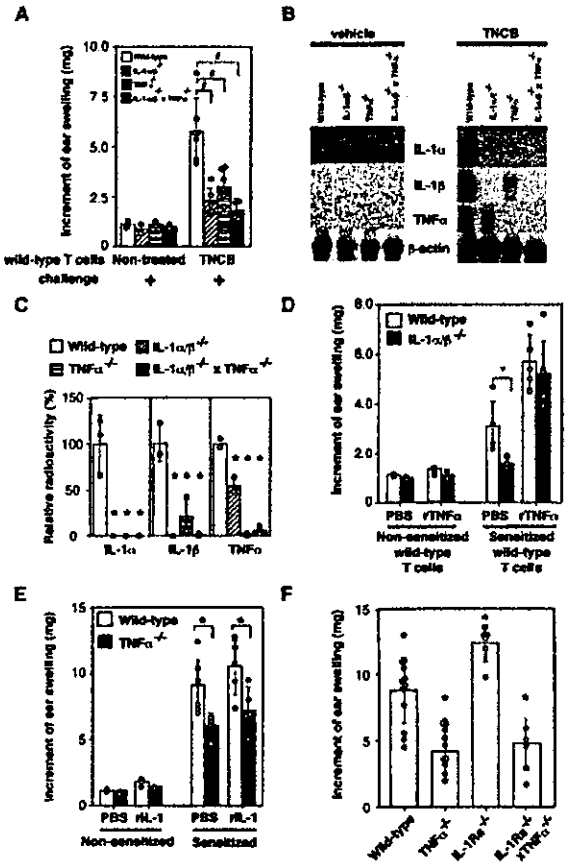

Fig. 4. Roles of IL-1 and TNF-α in the elicitation phase of CHS. (A) Non-treated or TNCB-sensitized wild-type LN T cells were injected i.v. into non-sensitized wild-type, IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. One day later, mice were challenged with 1.0% TNCB and ear swelling was measured after 24 h. (B) Northern blot hybridization analysis of cytokine expression in the vehicle- or TNCB-challenged ear skin from TNCB-sensitized mice measured in (A). (C) Cytokine mRNA levels relative to those of wild-type control mice. Ratios of examined expression to TNCB-sensitized and -challenged wild-type control expression were determined by the radioactive intensity of the bands shown in (B). The radioactive intensity of each band was normalized to the β-actin band signal. The relative values of deficient mice against wild-type mice are shown. Each circle represents an individual mouse. An average ± SD for each group of mice is shown. Wild-type (n = 3), IL-1α/β$^{-/-}$ (n = 3), TNF-α$^{-/-}$ (n = 3) and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ (n = 3) mice. (D) Non-treated or TNCB-sensitized wild-type LN T cells were injected i.v. into non-sensitized wild-type and IL-1α/β$^{-/-}$ mice. One day after transplantation, mice were challenged with 1.0% TNCB and injected with 50 ng of rTNF-α intradermally. Ear swelling was measured after 24 h. (E) Five days after sensitization with 3.0% TNCB, wild-type and TNF-α$^{-/-}$ mice were challenged with 1.0% TNCB and injected 50 ng of rIL-1α/β intradermally. Ear swelling was measured after 24 h. Each circle represents an individual mouse. An average and SD for each group of mice is shown. (F) Ear swelling following TNCB-induced CHS was measured in wild-type, IL-1Ra$^{-/-}$, TNF-α$^{-/-}$ and IL-1Ra$^{-/-}$ × TNF-α$^{-/-}$ mice as shown in Fig. 1. Each circle represents an individual mouse. Averages ± SD for groups of mice are shown. Student's t-test was used for statistical evaluation of the results. $^{*}P < 0.05$ and $^{\#}P < 0.001$.

Effects of IL-1α/β and TNF-α deficiency on hapten-specific T cell activation

To address the defects in CHS responses in TNF-α$^{-/-}$ mice, we analyzed the hapten-specific T cell response. Purified T cells from TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ mice, sensitized with TNCB, were co-cultured with TNP-conjugated splenocytes to examine their proliferative responses. The IL-1α/β$^{-/-}$ T cell response was lower than the wild-type response, in agreement with previous findings (4). The response of T cells from TNCB-sensitized TNF-α$^{-/-}$ mice, however, was comparable to that of wild-type T cells (Fig. 3A), demonstrating normal T cell priming in TNF-α$^{-/-}$ mice. We also examined OX40 expression on CD4^{+} T cells, as OX40 induction by IL-1 is crucial for T cell activation (20). OX40–OX40 ligand co-signaling is required for hapten-specific T cell priming and the development of CHS (26). Although OX40 expression on IL-1α/β$^{-/-}$ T cells was lower than that on wild-type T cells, expression on TNF-α$^{-/-}$ T cells was comparable to wild-type, consistently with normal hapten-specific T cell proliferative responses (Fig. 3B). Moreover, we found that IL-17 production, which reflects T cell activation in the sensitization phase of the CHS response (28), was also reduced in IL-1α/β$^{-/-}$ mice, whereas that in TNF-α$^{-/-}$ mice was comparable with that in wild-type mice (Fig. 3C). These results suggest that TNF-α is not involved in antigen-specific T cell priming in CHS.

These results were further confirmed by T cell transfer experiments. Non-sensitized wild-type mice were given LN T cells from TNCB-sensitized wild-type, IL-1α/β$^{-/-}$ or TNF-α$^{-/-}$ mice, and then challenged with TNCB. Ear swelling was impaired in mice given transferred TNCB-sensitized IL-1α/β$^{-/-}$ T cells, but not TNF-α$^{-/-}$ T cells (Fig. 3D). The impairment was observed in mice receiving sensitized IL-1α/β$^{-/-}$ T cells from mice on either the C57BL/6J or BALB/cA backgrounds (Fig. 3D; data not shown for the BALB/cA background mice). These results indicate that IL-1, but not TNF-α, is required for T cell activation during the sensitization phase of CHS.

Roles of IL-1 and TNF-α in the elicitation phase of CHS

Next, we examined the role of IL-1 and TNF-α in the elicitation phase. We transferred TNCB-sensitized wild-type T cells into IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ or IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice to avoid effects on the sensitization phase, as IL-1 deficiency affects T cell priming. Ear swelling responses upon challenge with TNCB were significantly impaired in mutant mice receiving wild-type T cells sensitized with TNCB compared with wild-type mice (Fig. 4A). Under these experimental conditions, mice that received non-sensitized T cells did not develop the CHS response. The suppression was observed on both the C57BL/6J (Fig. 4A) and BALB/cA (data not shown) backgrounds. Thus, both IL-1 and TNF-α play important roles in the elicitation phase of CHS. These findings also suggest that both TNF-α and IL-1 mediate local inflammation during CHS through common mechanisms as the suppression in IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice was similar. Consistent with this possibility, mRNAs encoding TNF-α and IL-1α/β were reduced in IL-1α/β$^{-/-}$ and TNF-α$^{-/-}$ mice respectively in comparison with wild-type mouse tissue (Fig. 4B and C). Thus, the results indicate that the expression of IL-1α and β mRNA is induced by TNF-α, and TNF-α

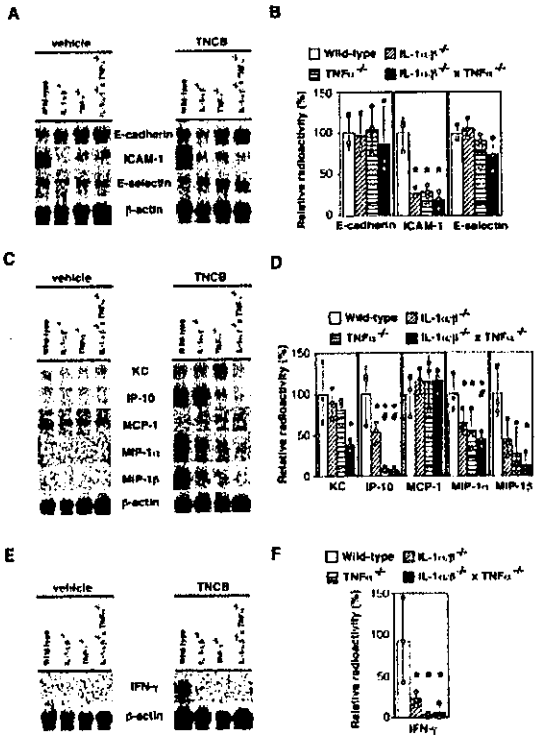

Fig. 5. Effect of IL-1 and TNF-α deficiency on the mRNA expression of adhesion molecules and chemokines. mRNA levels for adhesion molecules (A and B), chemokines (C and D) and IFN-γ (E and F) were measured in vehicle- or TNCB-challenged ear skin of wild-type, IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice using Northern blot hybridization. (A, C and E) Northern blot analysis. (B, D and F) Quantitated relative radioactivity of the mRNA bands on the autoradiograms. *P < 0.05 versus wild-type mice and #P < 0.05 versus IL-1α/β$^{-/-}$ mice.

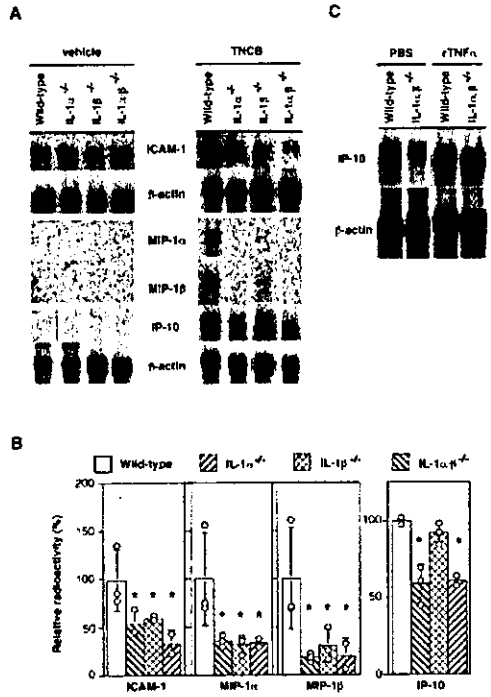

Fig. 6. mRNA expression of adhesion molecules and chemokines in IL-1α$^{-/-}$, IL-1β$^{-/-}$ and IL-1α/β$^{-/-}$ mice. mRNA levels relative to wild-type control levels for the adhesion molecules and chemokines in vehicle- or TNCB-challenged ear skin of wild-type, IL-1α$^{-/-}$, IL-1β$^{-/-}$ and IL-1α/β$^{-/-}$ mice were measured as described in Fig. 5. (A) Northern blot analysis. (B) Quantitated relative radioactivity of the mRNA bands on the autoradiograms. *P < 0.05 versus wild-type mice. (C) The reduced IP-10 mRNA expression in the skin of IL-1α/β$^{-/-}$ mice after treatment with TNCB recovered following an injection with rTNF-α. Five days after TNCB-sensitized T cell injection, mice were challenged with 1.0% TNCB and given 50 ng of TNF-α by intradermal injection in the ear. mRNA was collected from the ear 3 h after TNF-α injection and analyzed by Northern blot hybridization.

expression is induced by IL-1α/β respectively in the elicitation phase of CHS, suggesting that these cytokines amplify the expression of each other.

We next examined the possibility that TNF-α and IL-1 act within a cascade. After transplantation with TNCB-sensitized wild-type T cells, we injected rTNF-α or rIL-1α/β into IL-1α/β$^{-/-}$ mice or TNF-α$^{-/-}$ mice respectively. IL-1α/β$^{-/-}$ mice receiving rTNF-α demonstrated strong responses comparable to those seen in wild-type mice injected with rTNF-α, although the wild-type response was also enhanced by rTNF-α (Fig. 4D). The response in TNF-α$^{-/-}$ mice, however, was not affected by rIL-1α/β administration (Fig. 4E). Similarly, rTNF-α administration rescued the defects observed in IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice, while rIL-1α/β could not compensate (data not shown). We also examined CHS using IL-1 receptor antagonist (IL-1Ra)$^{-/-}$ × TNF-α$^{-/-}$ mice on the BALB/cA background. IL-1Ra$^{-/-}$ mice exhibited exacerbated CHS compared with wild-type mice, while the CHS response in IL-1Ra$^{-/-}$ × TNF-α$^{-/-}$ mice was suppressed to levels seen in TNF-α$^{-/-}$ mice (Fig. 4F). Although H-2 loci are b/b in the TNF-α$^{-/-}$ mice that we used, the CHS response in wild-type BALB/cA (H-2$^{d/d}$) was comparable with that in wild-type BALB.B (H-2$^{b/b}$), indicating that the difference in H-2 loci did not affect the results (data not shown). Taken together, these results suggest that IL-1-induced TNF-α plays an important role in induction of local inflammation during the elicitation phase of CHS, although TNF-α also can induce IL-1α/β.

Effects of IL-1α/β and TNF-α deficiency on the expression of genes for adhesion molecules and chemokines in the inflammatory sites of CHS

We next investigated the expression of various adhesion molecule and chemokine genes suspected to play roles in the development of inflammation. Ear tissues from IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice were examined following the induction of CHS with TNCB. The expression of ICAM-1 mRNA, but not E-cadherin and E-selectin mRNA, was severely reduced in IL-1α/β$^{-/-}$, TNF-α$^{-/-}$ and IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice (Fig. 5A and B). Of the examined chemokine mRNAs, MCP-1 mRNA expression was not impaired in any of the deficient mice. KC mRNA was reduced in only IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice, while the mRNA expression of MIP-1α, MIP-1β and IP-10

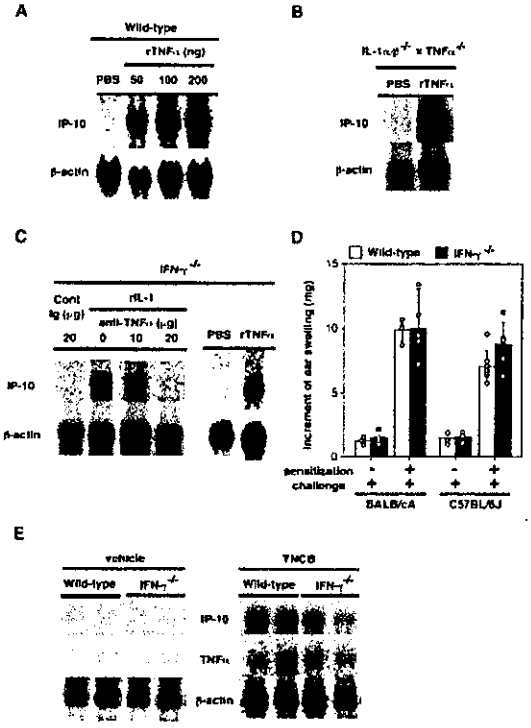

Fig. 7. IFN-γ-independent IP-10 production by TNF-α. rIL-1 or rTNF-α was injected intradermally into the ear skin of wild-type, IFN-γ$^{-/-}$, IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. At 3 h after the injection, total mRNA from the ear was prepared. IP-10 mRNA expression was analyzed by (A) Northern blot hybridization. rTNF-α was injected intradermally into the ears of wild-type mice. Dose-dependent expression of IP-10 was measured. (B) rTNF-α (100 ng) was injected intradermally into the ears of IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice. (C) The effect of anti-TNF-α on rIL-1-induced IP-10 expression was analyzed by intradermal injection of rIL-1 (100 ng) concurrently with either anti-TNF-α or control Ig (20 μg) into the ears of IFN-γ$^{-/-}$ mice. The effect of rTNF-α (100 ng) injection was also analyzed. (D) TNCB-induced CHS using wild-type and IFN-γ$^{-/-}$ mice on the BALB/cA background, and wild-type and IFN-γ$^{-/-}$ mice on the C57BL/6J background. Ear swelling was measured after 24 h. Each circle represents an individual mouse. An average ± SD for each group of mice is shown. (E) The effect of IFN-γ deficiency on IP-10 and TNF-α expression in vehicle- or TNCB-challenged ear skin was analyzed. Each lane represents an individual mouse.

was impaired in all mice deficient in either IL-1α/β or TNF-α (Fig. 5C and D). The suppression of IP-10 mRNA expression was more severe in TNF-α$^{-/-}$ mice than in IL-1α/β$^{-/-}$ mice. As IFN-γ is a potent inducer of IP-10 (29), we also examined IFN-γ mRNA expression in mutant mice; although IP-10 mRNA levels in IL-1α/β$^{-/-}$ mice were ~50% of the wild-type level, the expression of IFN-γ was more severely affected in the mutant mice (Fig. 5E and F). Similar results were obtained using both the C57BL/6J and BALB/cA backgrounds (data not shown for BALB/cA mice). Although CHS was impaired only in IL-1α$^{-/-}$ mice (4), the expression of ICAM-1, MIP-1α and MIP-1β mRNA at the inflammatory site was reduced in both IL-1α$^{-/-}$ and IL-1β$^{-/-}$ mice (Fig. 6A and B). IP-10 mRNA expression, however, was reduced in only IL-1α$^{-/-}$ mice, demonstrating a correlation between IP-10 expression and CHS sensitivity (Fig. 6A and B). Furthermore, IP-10 mRNA expression in IL-1α/β$^{-/-}$ mice receiving TNCB-sensitized wild-type T cells was lower than that seen in wild-type mice (Fig. 6C). This expression, however, recovered to wild-type levels following treatment with rTNF-α after transplantation. These results suggest that, during the elicitation phase, TNF-α induces IP-10 expression, resulting in ear inflammation.

Critical role of IP-10 in the elicitation of CHS

We examined the possibility that TNF-α induces IP-10 mRNA expression by injecting rTNF-α intradermally into the ears of wild-type mice. rTNF-α injection increased IP-10 mRNA expression in a dose-dependent manner (Fig. 7A). IP-10 mRNA expression was also strongly induced following the injection of rTNF-α into IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice, consistent with the possibility that TNF-α is induced by IL-1 (Fig. 7B).

IP-10 mRNA expression was analyzed in IFN-γ$^{-/-}$ mice to evaluate the possible involvement of IFN-γ in IP-10 induction and CHS (Fig. 7C). IP-10 mRNA was induced by either TNF-α or IL-1 even in the absence of IFN-γ. Furthermore, IP-10 mRNA expression, induced by rIL-1, was suppressed by anti-TNF-α administration (Fig. 7C), suggesting that IL-1 induces IP-10 through TNF-α. To exclude the possible involvement of IFN-γ in CHS, we examined the sensitivity of IFN-γ$^{-/-}$ mice against TNCB. IFN-γ$^{-/-}$ mice on either the C57BL/6J or BALB/cA background demonstrated a similar sensitivity to CHS as wild-type mice (Fig. 7D). Although the expression levels were slightly reduced, IP-10 mRNA expression was clearly shown in the mutant mice and TNF-α mRNA expression was normal in their ear tissues (Fig. 7E). These results suggest that TNF-α directly induces IP-10 mRNA expression in the ear through an IFN-γ-independent mechanism.

To address the role of IP-10 in the development of CHS, we examined the effect of a neutralizing anti-IP-10 mAb. TNCB-induced CHS in mice treated with antibody was significantly reduced in comparison with mice treated with control Ig (Fig. 8A), indicating IP-10 plays an important role in CHS. Moreover, the ear swelling of TNF-α$^{-/-}$ mice after challenge with TNCB was restored to the levels of wild-type mice following the injection of rIP-10 into the ear. This is not due to the inflammatory activity of IP-10, as ear swelling was not observed without TNCB challenge (Fig. 8B). Similar observations was obtained using IL-1α/β$^{-/-}$ × TNF-α$^{-/-}$ mice injected with rIP-10 (data not shown). Collectively, these observations indicate that TNF-α induces IL-1α first, then the IL-1α induces TNF-α, resulting in the induction of IP-10 that plays a critical role in CHS elicitation in an IFN-γ-independent manner.

Discussion

IL-1α/β and TNF-α are believed to exert similar activities in CHS responses to contact allergens, such as LC migration, LC maturation and the expression of adhesion molecules. The precise relationship between these cytokines and their distinctive roles in CHS, however, has not been well elucidated. In this report, we demonstrated that IL-1 is required for hapten-specific T cell priming during the sensitization phase of CHS, consistent with our previous report indicating that IL-1 plays a crucial role in antigen-specific T cell activation through

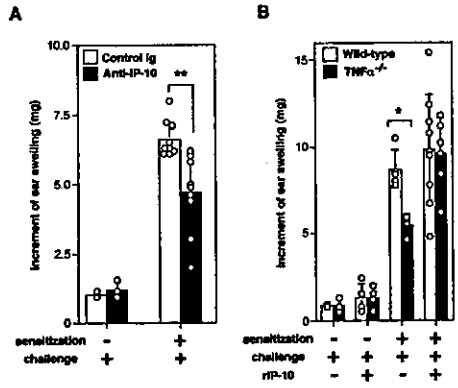

Fig. 8. The role of IP-10 in CHS. Five days after sensitization with 3.0% TNCB, mice were challenged with 1.0% TNCB and injected with either 100 μg of anti-mouse IP-10 mAb or 1 μg of rmIP-10 intradermally into the ear. Ear swelling was then measured. (A) Wild-type mice were treated with either control Ig or anti-IP-10 mAb. (B) Wild-type mice or TNF-$\alpha^{-/-}$ mice were treated with PBS or rIP-10 before or after sensitization with TNCB and then ear swelling was measured. Each circle represents an individual mouse. An average ± SD for each group of mice is shown. Student's *t*-test was used for statistical evaluation of the results. $^{*}P < 0.05$ and $^{**}P < 0.001$.

the induction of CD40L and OX40 on T cells (4,20). In contrast, TNF-α deficiency did not affect antigen-specific T cell priming. Furthermore, TNF-α-deficient T cells sensitized with TNCB were capable of efficiently transferring CHS to wild-type recipient mice, indicating that TNF-α is not involved in the sensitization phase of CHS. We showed that TNF-α plays an important role in the elicitation phase, because TNCB-sensitized wild-type T cells cannot evoke CHS in TNF-$\alpha^{-/-}$ mice upon transplantation. IL-1 is also involved in the elicitation phase. This is because TNF-α is induced by the action of IL-1 and the deficiency of CHS in IL-$1^{-/-}$ mice at the elicitation phase is recovered by the addition of TNF-α. Thus, it was clearly shown that IL-1 and TNF-α play distinctive roles during CHS.

Although the involvement of other cytokines such as IL-4 in CHS development is only obvious under conditions using specific allergens or specific genetic backgrounds (27), the effects of IL-1 or TNF-α deficiency were clearly observed using both TNCB and oxazolone as contact allergens on both the C57BL/6J and BALB/cA genetic backgrounds. Thus, the requirement for IL-1 and TNF-α may be critical for CHS development irrespective of the nature of the allergen and the genetic backgrounds.

LC are the major APC in hapten-specific T cell activation during the sensitization phase (1). Although both IL-1 and TNF-α are involved in the migration of LC from the skin to LN, we demonstrated in a recent report that the suppression of LC migration in IL-1α/$\beta^{-/-}$ mice was only transient; migration recovered gradually to levels similar to wild-type mice by 36 h after stimulation (4). We obtained similar results in TNF-$\alpha^{-/-}$ mice (data not shown). Thus, the reduced CHS in IL-1α/$\beta^{-/-}$ and TNF-$\alpha^{-/-}$ mice cannot be completely explained by a deficiency in LC migration.

Recently, it has been reported that the CD40–CD40L interaction is important for the induction of CHS (30). It was suggested that TNF-α, elicited by the CD40–CD40L interaction, plays a critical role in LC migration and maturation. Our results, however, demonstrated that LC maturation, as examined by the expression of activation surface marker and T cell priming activity, was normal in TNF-$\alpha^{-/-}$ and IL-1α/$\beta^{-/-}$ × TNF-$\alpha^{-/-}$ mice, indicating TNF-α is not essential for these processes. It is likely that similar cytokines, such as granulocyte macrophage colony stimulating factor, may compensate the activity.

TNF-α is produced by various cell types including KC and LC in the skin (2). The specific cells producing this cytokine during the elicitation phase of CHS, however, have not been identified. Although TNF-α is known to be produced by $CD4^{+}$ T cells (31), we demonstrated that T cell-derived TNF-α is not directly involved in the development of CHS (Fig. 3D). In regard to this, it is reported that mast cell-derived TNF-α plays an important role in CHS induction (32). CHS is induced normally in mast cell-deficient *Kit^{w}/Kit^{w-v}* mice receiving wild-type mast cells, while the response could not be restored in *Kit^{w}/Kit^{w-v}* mice receiving TNF-$\alpha^{-/-}$ mast cells (32). Thus, mast cells, in addition to KC and LC, may contribute to the production of TNF-α.

ICAM-1 (14,33), MCP-1 (1,16), MIP-1α and MIP-1β (34) are involved in ear swelling during the elicitation phase. We showed that the expression of these genes was reduced in both IL-1α/$\beta^{-/-}$ and TNF-$\alpha^{-/-}$ mice. The expression of these cytokines was also reduced in IL-$1\beta^{-/-}$ mice, in which ear swelling develops normally (4,19). In addition, the CHS response was exacerbated in CCR5 (a receptor of MIP-1β)-deficient mice (35) and that in MCP-$1^{-/-}$ mice developed normally (36). Therefore, these molecules do not appear to be involved in the suppression of ear swelling in IL-1α/$\beta^{-/-}$ and TNF-$\alpha^{-/-}$ mice.

IP-10 mRNA is expressed by KC in the skin (2,37) and IP-10 plays an important role in the trafficking of effector T_h1 cells to inflammatory sites (16,38–41). We found that the levels of IP-10 mRNA correlated well with the severity of ear swelling in IL-$1\alpha^{-/-}$, IL-$1\beta^{-/-}$, IL-1α/$\beta^{-/-}$, TNF-$\alpha^{-/-}$ and IL-1α/$\beta^{-/-}$ × TNF-$\alpha^{-/-}$ mice. Furthermore, anti-IP-10 mAb significantly suppressed the development of ear swelling and the reduced CHS in TNF-$\alpha^{-/-}$ mice recovered to wild-type levels following injection with rIP-10. Thus, we conclude that IP-10 plays a critical role in the development of CHS at the elicitation phase. In agreement with our notion, recently it was shown that CHS is reduced in IP-$10^{-/-}$ mice (42). It should be noted, however, that IP-10 mRNA expression was much more severely impaired in TNF-$\alpha^{-/-}$ mice than in IL-1α/$\beta^{-/-}$ mice (Fig. 5C and D), although the suppression of ear swelling in IL-1α/$\beta^{-/-}$ mice was similar to that in TNF-$\alpha^{-/-}$ mice (Fig. 1). Moreover, the CHS response was suppressed only partially by anti-IP-10 mAb treatment (Fig. 8). Thus, in addition to the IP-10 induction by inducing TNF-α, IL-1 may also contribute to the local inflammation through IP-10-independent pathways during CHS response.

It is well known that IFN-γ is a potent inducer of IP-10 (29) and actually it was shown that IP-10 is induced by IFN-γ, but not by TNF-α, upon infection with *Trypanosoma cruzi* (43). Recently, however, it was shown that TNF-α could potentiate IP-10 mRNA expression in hepatocytes more effectively than IL-1β and IFN-γ, while the induction of IP-10 mRNA in Kupffer and endothelial cells by these cytokines was equivalent (44).

We demonstrate here that IP-10 in the skin is mainly induced by TNF-α, not by IFN-γ. Consistent with this observation, we showed that TNCB-induced CHS was normal in IFN-$\gamma^{-/-}$ mice, in agreement with the normal development of CHS in IFN-γRI$^{-/-}$ mice (45). On the other hand, FITC-induced CHS was reduced in IFN-γRII$^{-/-}$ mice (46). This apparent discrepancy may be caused by the difference of the inducers (47). Normal expression of TNF-α is observed in IFN-$\gamma^{-/-}$ mice at inflammatory sites, supporting our claim that TNF-α-induced IP-10 is responsible for the induction of inflammation.

In summary, we have demonstrated specific functions for IL-1 and TNF-α in the development of CHS. These findings may provide a critical cue allowing for the development of novel therapeutics to treat inflammatory diseases of the skin.

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Abbreviations

APC	antigen-presenting cell
CD40L	CD40 Ligand
CHS	contact hypersensitivity
DNBS	dinitrobenzene sulfonate
IP-10	IFN-γ-inducible protein 10
IL-1Ra	IL-1 receptor antagonist
KC	keratinocyte
LC	Langerhans cell
LN	lymph node
PE	phycoerythrin
TNCB	2,4,6-trinitorochlorobenzene
TNF	tumor necrosis factor

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IL-1 is required for tumor invasiveness and angiogenesis

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Here, we describe that microenvironmental IL-1β and, to a lesser extent, IL-1α are required for *in vivo* angiogenesis and invasiveness of different tumor cells. In IL-1β knockout (KO) mice, local tumor or lung metastases of B16 melanoma cells were not observed compared with WT mice. Angiogenesis was assessed by the recruitment of blood vessel networks into Matrigel plugs containing B16 melanoma cells; vascularization of the plugs was present in WT mice, but was absent in IL-1β KO mice. The addition of exogenous IL-1 into B16-containing Matrigel plugs in IL-1β KO mice partially restored the angiogenic response. Moreover, the incorporation of IL-1 receptor antagonist to B16-containing plugs in WT mice inhibited the ingrowth of blood vessel networks into Matrigel plugs. In IL-1α KO mice, local tumor development and induction of an angiogenic response in Matrigel plugs was less pronounced than in WT mice, but significantly higher than in IL-1β KO mice. These effects of host-derived IL-1α and IL-1β were not restricted to the melanoma model, but were also observed in DA/3 mammary and prostate cancer cell models. In addition to the *in vivo* findings, IL-1 contributed to the production of vascular endothelial cell growth factor and tumor necrosis factor in cocultures of peritoneal macrophages and tumor cells. Host-derived IL-1 seems to control tumor angiogenesis and invasiveness. Furthermore, the anti-angiogenic effects of IL-1 receptor antagonist, shown here, suggest a possible therapeutic role in cancer, in addition to its current use in rheumatoid arthritis.

The cytokine IL-1 mainly affects inflammatory processes but also possesses various immune, degradative, and growth-promoting properties. There are two IL-1 agonistic proteins, IL-1α and IL-1β, and one antagonistic protein, the IL-1 receptor antagonist (IL-1Ra, reviewed in ref. 1). IL-1Ra binds to the IL-1 receptor type I without transmitting an activation signal and, thus, represents a physiological inhibitor of IL-1 activity. IL-1α and IL-1β bind to the same receptors, and there are no significant differences in the spectrum of activities induced by recombinant IL-1α or IL-1β by using *in vitro* and *in vivo* assays, including injection into humans (1). However, processing of IL-1α and IL-1β differ: IL-1β is active only as secreted mature product, whereas IL-1α is active as a precursor or membrane-associated molecule. IL-1α remains cytosolic but IL-1β is abundantly secreted by activated macrophages and blood monocytes. Also, IL-1β is present in the circulation of patients undergoing infectious or inflammatory responses, whereas IL-1α is rarely found in the circulation (1).

Tumor enlargement requires blood vessel growth that originates with the sprouting of new capillaries from preexisting blood vessels; in the absence of access to an adequate vasculature, tumor cells become necrotic and/or apoptotic (reviewed in refs. 2–8). Tumors may exist as microscopic *in situ* lesions for years in the absence of their own microcirculation. For tumor progression and metastasis, an angiogenic switch permits rapid growth and invasion (2–8). The angiogenic switch is a discrete genetic event that results in a change in the local equilibrium between pro- and anti-angiogenic regulators, which are produced by tumor cells as well as by the surrounding stromal cells and infiltrating leukocytes.

In various experimental models, IL-1 increases tumor invasiveness and metastasis (1, 9–17). For example, IL-1 expression at the site of tumor development enhances the expression of adhesion molecules on endothelial and malignant cells and facilitates the invasion of malignant cells into the circulation and their dissemination to remote tissues (11, 13). Although recombinant IL-1 stimulates the proliferation of endothelial cells, adhesion molecule expression and production of cytokines and small/inflammatory mediator molecules (1, 18, 19), endogenous IL-1 has not been characterized as major cytokine in mediating *in vivo* tumor angiogenesis. In the present studies, we have assessed the role of IL-1 as a regulator of tumor angiogenesis by using transplantable tumors in IL-1β and IL-1α knockout (KO) mice. We also assessed whether inhibition of IL-1 activity by IL-1Ra can function as an anti-tumor agent by inhibiting angiogenesis.

Materials and Methods

Reagents. Recombinant human IL-1Ra was obtained from Amgen Biologicals; recombinant mouse IL-1α was obtained from R & D Systems. Matrigel was purchased from Biological Industries, Beit HaEmek, Israel.

Tumor Cells. B16 melanoma cells (20) and the DA/3 mammary adenocarcinoma (21) were maintained in DMEM (GIBCO/Invitrogen) supplemented with 10% (vol/vol) FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine in a humidified 5% CO_2 atmosphere at 37°C. All medium ingredients were purchased from Biological Industries.

Mice. IL-1β and IL-1α KO mice were described (22–24). WT C57BL/6 and BALB/c strains were obtained from The Jackson Laboratory and Harlan, Jerusalem. Animal studies were performed according to the guidelines and approval of the Animal Care Committee of Ben-Gurion University. Female 8- to 10-week-old mice were used in all experiments.

Tumorigenicity Assays. WT or IL-1 KO mice were injected intrafootpad (i.f.p.) or intravenously (i.v.) with 2×10^5 B16 melanoma cells to assess local tumor growth or metastasis, respectively. Local tumor development was measured by using a caliper. The endpoint of such experiments was the time at which the tumor's size had reached a diameter of 12 mm. Mice injected i.v. were observed until the first signs of dyspnea and then killed. Lung metastases were evaluated histologically in formalin-fixed paraffin sections stained with hematoxylin/eosin. BALB/c or IL-1 KO mice from the same background were injected i.f.p. with

Abbreviations: IL-1Ra, IL-1 receptor antagonist; i.f.p., intrafootpad; MVD, mean vessel density; PEC, peritoneal exudate cells; VEGF, vascular endothelial growth factor; KO, knockout; TNFα, tumor necrosis factor α.

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