

Quinacrine inhibits the epidermal dendritic cell migration initiating T cell-mediated skin inflammation

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Quinacrine (QC) is an anti-inflammatory drug that has been used for the treatment of malaria and rheumatoid diseases. The mechanism(s) underlying the anti-inflammatory activity of QC remains poorly understood. We recently reported the QC-mediated inhibition of the NF- κ B pathway using an *in vitro* model. To test this potential mechanism *in vivo*, we used the contact hypersensitivity response (CHS) to chemical allergen sensitization and challenge in mice as a model of skin inflammation. The results indicated that QC treatment inhibited NF- κ B activation in the skin during allergen sensitization. This inhibition was reflected by decreased mRNA expression and protein production of the NF- κ B-dependent cytokines TNF- α and IL-1 β and the chemokine CCL21 in the skin. The decreases in these cytokines resulted in reduced migration of allergen-presenting dendritic cells from the skin into skin-draining lymph nodes and markedly decreased activation of effector CD8⁺ T cells for the CHS response to allergen challenge (inhibitory concentration 50% or IC₅₀ was 55 mg/kg). These findings reveal a previously unrecognized mechanism of QC-mediated inhibition of inflammation.

Received 12/9/06

Revised 10/4/07

Accepted 13/6/07

[DOI 10.1002/eji.200636708]

Key words:
Contact hyper-
sensitivity · Cytokines
· Dendritic cells
· NF- κ B

Introduction

The molecular mechanisms mediating the therapeutic effects of many drugs used to treat inflammation remain poorly defined, and that is a major factor limiting clinical application of such anti-inflammatory drugs. Quinacrine (QC) has been widely used for the treatment of malaria and chronic inflammatory conditions, including

lupus erythematosus and rheumatoid arthritis [1]. Our recent studies [2] indicated that QC inhibits the activation of NF- κ B, a transcription factor regulating the expression of many genes that initiate and regulate immune responses, proliferation and tumorigenesis [3–5]. These *in vitro* studies suggested blockade of the NF- κ B pathway as a potential anti-inflammatory mechanism of QC. Whether this mechanism is operative *in vivo*, as well as the identification of specific targets of the drug, are untested.

Allergic contact hypersensitivity (CHS) is a T cell-mediated cutaneous immune response to sensitization and subsequent challenge with a hapten. Contact skin allergies to various chemicals, including cosmetics and urushiol, the reactive compound of poison ivy, affect about 3% of the population in industrialized countries. Initial contact of skin with a hapten (*i.e.*, sensitization) mediates rapid production of NF- κ B-dependent pro-inflammatory cytokines including TNF- α and IL-1 β [6]. These cytokines induce maturation of epidermal den-

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Abbreviations: CHS: contact hypersensitivity · DNFB: 2,4-dinitrofluorobenzene · LC: Langerhans cells · LNC: LN cell · QC: quinacrine

dritic cells (DC), Langerhans cells (LC), and emigration of these hapten-presenting LC from the skin through the draining lymphatics into lymph nodes (LN) where hapten-specific T cells are primed [7, 8]. Subsequent contact of the skin with the sensitizing hapten (e.g., challenge) directs the hapten-primed T cells into the challenge site to mediate local inflammation, resulting in the characteristic tissue edema or swelling that peaks 24–48 h after challenge and then quickly resolves [9]. Studies from this and other laboratories have indicated that hapten-specific CD8⁺ T cells producing IFN- γ are the primary effector cells of CHS responses to many haptens including 2,4-dinitrofluorobenzene (DNFB), oxazolone and urushiol [10–12].

The maturation and migration of hapten-presenting LC from the sensitized skin into draining LN is a critical step in the initiation of the CHS response. The production of TNF- α and IL-1 β in response to hapten application promotes maturation of LC in the skin, in part through down-regulation of E-cadherin and up-regulation of CCR7 expression [13, 14]. The ligand for CCR7, CCL21/SLC, is expressed in the afferent lymphatics and is required for LC trafficking from the skin into the draining LN [15, 16]. Production of CCL21 is also induced through the NF- κ B pathway [17–19]. These NF- κ B pathway-dependent cytokines that regulate the initiation of CHS responses provide targets to test the ability of QC to inhibit an inflammatory response. The results suggest blockade of NF- κ B as a mechanism mediating the anti-inflammatory functions of QC during immune responses in the skin. Overall, these studies reveal novel insights into the mechanism of QC anti-inflammatory functions *in vivo* and suggest the use of this drug for preventive therapy of contact skin allergies in target populations.

Results

QC inhibits CHS responses to DNFB

The effect of QC treatment on CD8⁺ T cell-mediated immune responses to sensitization and challenge of the skin with hapten was tested. Groups of C57BL/6 mice were given varying doses of QC in PBS or PBS alone on 2 consecutive days before sensitization with DNFB. The sensitized, as well as unsensitized, mice were challenged on the ear with DNFB on day +5 and the increase in ear swelling was measured 24 h later. QC decreased the magnitude of the CHS response in a dose-dependent manner. Greater than 70% inhibition of ear swelling was observed in mice given 80 mg/kg QC when compared to mice treated with PBS during sensitization (Fig. 1A). Whereas the ear swelling response in the control group remained at peak levels by 48 h post-challenge, the CHS

response in QC-treated mice declined to baseline levels by that time (not shown). QC treatment of mice after hapten challenge either by i.p. injection or by topical application of QC on challenged ears did not inhibit the ear swelling response to hapten challenge (not shown). Overall, these results indicate that QC inhibits the induction, but not elicitation, of the CHS response.

The magnitude and duration of the CHS response directly correlates with the numbers of hapten-specific IFN- γ -producing CD8⁺ T cells in skin-draining LN during sensitization for CHS [20]. The development of these effector cells in QC-treated vs. the control mice was tested by ELISPOT assay. As typically observed [21], the numbers of hapten-specific CD8⁺ T cells producing IFN- γ were high in the LN of control sensitized mice on day +5 post sensitization and were not detected in non-sensitized, naïve mice. The number of hapten-specific CD8⁺ T cells producing IFN- γ decreased by half in the group treated with QC during sensitization (Fig. 1B). QC given at later time points (days 0, +1, +2 and +3 post sensitization) did not inhibit hapten-specific CD8⁺ T cell development or CHS responses (not shown).

To compare the effector function of hapten-specific CD8⁺ T cells from control and QC-treated mice, the ability of these T cells to mediate CHS following passive transfer to naïve recipients was tested. CD8⁺ T cells from hapten-sensitized control donors transferred ear swelling responses to hapten challenge in the naïve recipients. In contrast, substantially lower responses were induced in recipients by delivery of the same number of CD8⁺ T cells from donors treated with QC before sensitization (Fig. 1C). Since the numbers of hapten-specific effector CD8⁺ T cells in the LN of QC-treated mice were reduced by half, we also compared responses induced by transfer of twice the number of cells from QC-treated donors. This twofold increase in transferred cells resulted in ear swelling responses comparable to the responses observed following transfer of half the number of CD8⁺ T cells from control donors (Fig. 1D). Thus, the development and function of effector CD8⁺ T cells was not completely ablated by QC treatment. Together, these results indicate that QC treatment before hapten sensitization decreases the development of hapten-specific CD8⁺ T cells producing IFN- γ , and this inhibition accounts for the reduced skin inflammation in the treated animals.

QC decreases hapten-presenting LC numbers in the LN

Recent studies from this laboratory demonstrated that the presence of hapten-presenting DC in the LN of sensitized mice directly correlates with the magnitude of IFN- γ -producing effector CD8⁺ T cell development as well as the CHS responses elicited to hapten challenge

[20, 22]. To test the effect of QC on the presence of hapten-bearing DC during T cell priming for CHS, the numbers of these DC in the skin-draining LN of control and QC-treated mice sensitized with FITC were compared. Application of FITC as a hapten allows detection of hapten-presenting DC as FITC⁺/CD11c⁺ cells in the skin-draining LN [22, 23]. To distinguish between LC and other DC (residential and dermal DC) in the LN, cells were also stained with anti-CD207 mAb. Mice were sensitized with FITC and 48 h later LN cell (LNC) suspensions were prepared and stained with PE-labeled anti-CD11c and AlexaFluor 647-labeled anti-CD207 mAb. CD11c⁺CD207⁺ cells were gated as LC (Fig. 2A, gate R2) and CD11c⁺CD207[−] cells were gated as residential and dermal DC (Fig. 2A, gate R3) and analyzed for FITC expression. All CD11c⁺ residential DC in the LN of naïve mice were FITC negative (not shown). The majority of CD11c⁺CD207⁺ cells in the LN of FITC-

sensitized animals were FITC positive, indicating FITC-bearing LC that had emigrated from the sensitized epidermis to the LN. The frequencies and total numbers of these cells in draining LN were decreased by 70% in the LN of QC-treated mice vs. the control group (Fig. 2A and B). The majority of CD11c⁺CD207[−] cells did not express FITC (Fig. 2A, gate R3). The small numbers of these cells that were FITC positive indicated either dermal DC emigrating from sensitized skin or residential DC labeled with the hapten due to lymphatic drainage into the LN and were similar in the LN of QC-treated and control mice (Fig. 2A and B). The results indicated decreased migration of hapten-presenting LC to the lymphoid T cell-priming site following QC treatment and hapten sensitization.

To test the potential effects of QC treatment on the stimulatory function of DC, including LC that migrated into LN after hapten sensitization, total CD11c⁺ cells

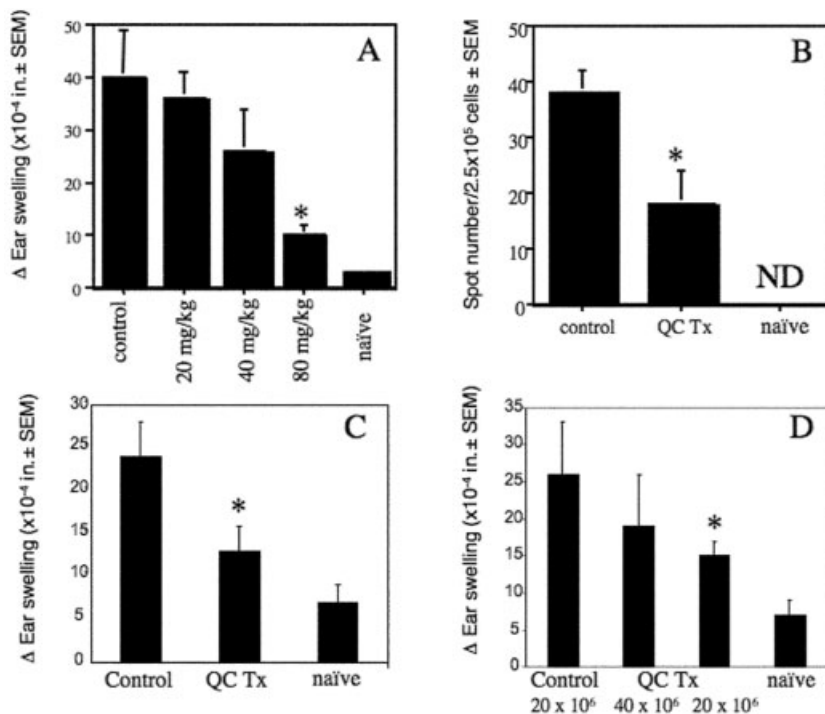


Figure 1. QC treatment inhibits CHS responses to DNFB. (A) Mice were treated with PBS (control) or with QC in PBS (QC Tx) on days −2 and −1 and sensitized on days 0 and +1 with 0.25% DNFB. On day +5 sensitized and naïve mice were ear challenged with 0.2% DNFB and the change in ear thickness was measured at 24-h intervals. The mean increase in ear thickness is shown in 10^{-4} inches $\pm$ SEM for groups of four mice. * $p < 0.05$ when compared to control. (B) CD8⁺ T cell-enriched cell suspensions were prepared from the LN of sensitized control and QC-treated mice (80 mg/kg) on day +5 post sensitization and cultured with hapten-labeled syngeneic splenocytes on anti-IFN- γ mAb-coated plates. ELISPOT assay was developed to detect hapten-specific CD8⁺ T cells producing IFN- γ . Mean number $\pm$ SEM of cytokine-producing hapten-specific T cells in triplicate culture is shown. * $p < 0.05$ when compared to control. (C) Mice were treated with PBS or with QC (80 mg/kg) and sensitized with 0.25% DNFB. CD8⁺ T cell-enriched LNC suspensions were obtained on day +5 post sensitization. Groups of naïve mice were injected i.v. with 50×10^6 of CD8⁺ T cell-enriched suspensions from PBS-treated donors (control) or from QC-treated donors (QC Tx) and were immediately challenged with 0.2% DNFB. Mean increase in ear thickness following hapten challenge is shown in 10^{-4} inches $\pm$ SEM for groups of four mice. * $p < 0.05$ when compared to control. (D) Donor mice were treated with PBS or with QC (80 mg/kg) and sensitized with 0.25% DNFB. Recipient naïve mice were injected i.v. with 20×10^6 CD8⁺ T cell-enriched LNC from PBS-treated donors (control) or with either 20×10^6 or 40×10^6 CD8⁺ T cell-enriched LNC from QC-treated donors (QC Tx) before the challenge. The mean increase in ear thickness following hapten challenge is shown in 10^{-4} inches $\pm$ SEM for groups of three mice. * $p < 0.05$ when compared to control.

were isolated from skin-draining LN of control and QC-treated mice at 48 h after hapten-sensitization and used as stimulatory cells to induce proliferation of CFSE-labeled hapten-immune CD8⁺ T cells from hapten-sensitized mice. The total percentage of CD8⁺ T cells proliferating during 96 h of culture with DC from control mice was slightly increased in comparison to CD8⁺ T cell proliferation during the culture with DC from QC-treated mice (Fig. 3A, gate M1, 45.6% vs. 37.5%, respectively and Fig. 3B). These results indicated that DC in QC-treated mice retained their stimulatory functions.

QC inhibits NF- κ B-dependent production of TNF- α , IL-1 β and CCL21 in the skin

The decreased presence of hapten-presenting LC in the draining LN after QC treatment suggested impaired migration of these DC from the sensitized skin. This migration is dependent on the cytokines TNF- α and IL-1 β and the chemokine CCL21 [14–16, 23–25]. Since our previous *in vitro* studies indicated that QC inhibits NF- κ B activation [2], we tested the ability of QC to inhibit the induction of these NF- κ B-regulated cytokines

in response to skin sensitization. The mRNA expression of these cytokines in the skin of sensitized mice treated with PBS (control) vs. QC (QC Tx) was tested at 3 h post sensitization when this expression reaches peak levels [6]. The expression of I κ B α , an NF- κ B-activated protein [26], was used as a marker of NF- κ B inhibition by QC. The expression of I κ B α mRNA was detectable in the skin of naïve mice and was up-regulated 3 h after sensitization, indicating NF- κ B activation in the skin by hapten sensitization (Fig. 4A and B, upper panel). This expression was inhibited by QC in both naïve and sensitized mice when compared to respective controls. Thus, QC inhibits both basal and hapten-induced activation of NF- κ B in the skin. Consistent with this, basal and hapten-induced mRNA expression of NF- κ B-dependent pro-inflammatory cytokines, TNF- α and IL-1 β was inhibited by QC (Fig. 4A and B, lower panels). These results were confirmed when homogenates of sensitized skin were prepared and tested for TNF- α and IL-1 β protein levels by ELISA. QC inhibited the hapten-induced production of these cytokines in the skin at 8 and 20 h post sensitization (Fig. 5). In contrast, hapten-induced production of CXCL1/KC was similar in control and QC-treated mice at 8 h post sensitization,

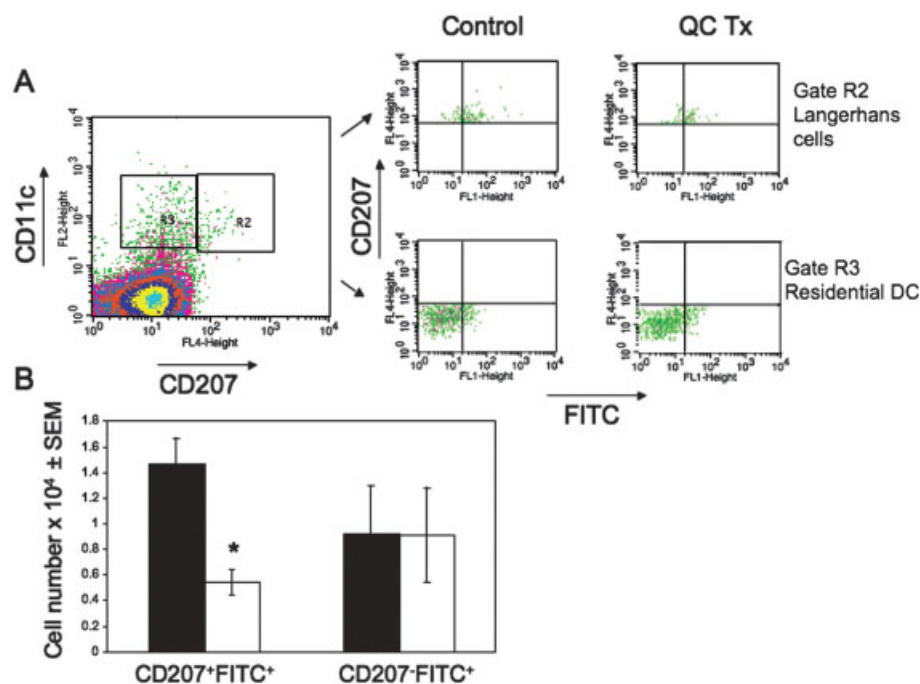


Figure 2. QC treatment decreases the number of hapten-bearing DC in the LN of hapten-sensitized mice. (A) Mice were sensitized with FITC and treated with PBS (control) or 80 mg/kg QC (QC Tx). LNC were prepared 48 h later and stained with PE-labeled anti-CD11c mAb and with AlexaFluor647-labeled anti-CD27 mAb. CD11c⁺ cells were gated based on CD27 expression (gates R2 and R3). Gated cells were analyzed for the percentages of CD27⁺/CD11c⁺ cells bearing FITC defined as hapten-presenting LC (gate R2) or for the percentages of CD27⁻/CD11c⁺ cells defined as residential DC (gate R3). (B) Total numbers of indicated DC populations in the skin-draining LN of each individual mouse (three per group) were calculated based on percentages in each 5 × 10⁴ LNC aliquot analyzed and the total number of cells in the LNC suspension. The mean number ± SEM of FITC⁺ DC per mouse is shown for each group of three individual mice. **p* < 0.05 when compared to control.

but was significantly inhibited by QC treatment at the later time point (Fig. 5). These results indicated differential effects of QC on the production of different cytokines and chemokines during initiation of the CHS response.

CCL21 is a chemokine critical for directing hapten-presenting DC from the skin through the afferent lymphatics into the LN [15, 16, 23]. The expression of CCL21 mRNA in the skin of naïve and hapten-sensitized mice was also inhibited by QC (Fig. 6A). Previous studies

from this and other laboratories have demonstrated that neutralization of either TNF- α or CCL21 during hapten sensitization inhibited hapten-presenting DC migration and subsequent activation of hapten-specific T cells to mediate effector functions in CHS [23, 27]. Based on these studies and on the results indicating inhibition of TNF- α and CCL21 expression by QC, we reasoned that QC would inhibit DC migration and T cell priming for CHS to the same level as neutralization of these cytokines. To address this, activated hapten-presenting

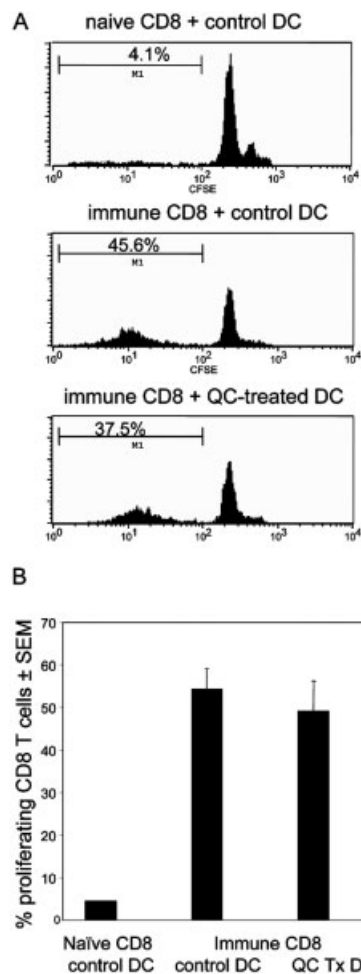


Figure 3. DC from QC-treated mice retain the ability to stimulate proliferation of hapten-specific CD8⁺ T cells. (A) Mice were treated with PBS (control DC) or 80 mg/kg QC (QC-treated DC) on days -2, -1 and then sensitized with 0.25% DNFB on days 0 and +1. DC were purified from LNC of sensitized mice at 48 h post sensitization and cultured in triplicate with CFSE-labeled LNC from naïve or sensitized mice obtained at day +5 post sensitization (0.6×10^5 DC: 3×10^5 LNC). After 96 h of culture the cells were collected and stained with PE-labeled anti-CD8 mAb. CD8⁺ cells were gated and analyzed for proliferation by evaluation of CFSE dilution. The numbers in the histograms indicate the percentages of dividing CD8⁺ T cells within the gated population (M1). (B) Mean percentages of total dividing CD8⁺ T cells in triplicate cultures is shown. * $p < 0.05$ when compared to control.

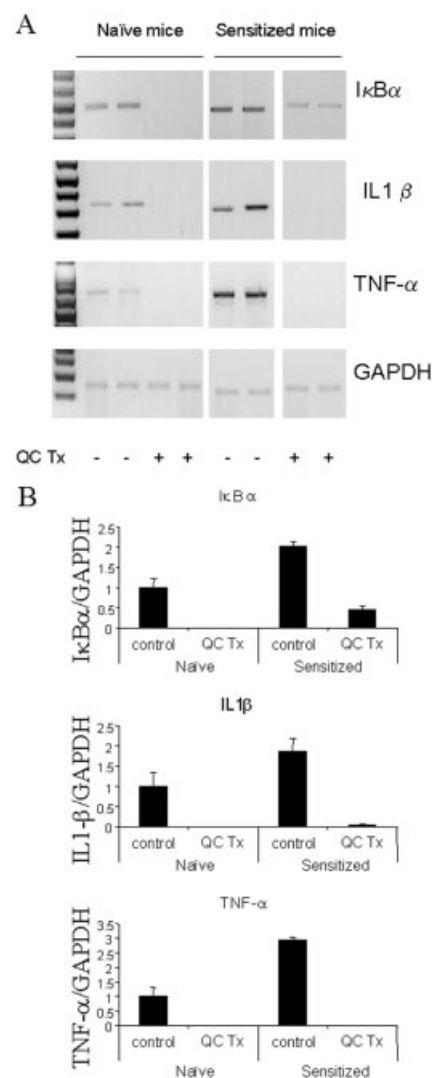


Figure 4. QC treatment inhibits IL-1 β and TNF- α expression in the skin of naïve and hapten-sensitized mice via NF- κ B blockade. (A) Mice were treated with PBS (control) or 80 mg/kg QC (QC Tx) on days -2, -1 and then left unsensitized (naïve) or sensitized with 0.25% DNFB on day 0, and 3 h later the skin was extracted and total RNA isolated. The expression of I κ B α , IL-1 β and TNF- α was evaluated using semi-quantitative RT-PCR. (B) Densitometric analysis of the gels was performed to quantify amplification of I κ B α and cytokine mRNA signals when compared to GAPDH signal.

DC were isolated from the LN of sensitized mice and adoptively transferred by intradermal injection into naïve mice treated with or without QC. The levels of hapten-specific T cell priming in the recipient LN induced by the transfer was compared in the control group and in the groups treated with QC or with anti-TNF- α mAb. DC transfer induced high numbers of hapten-specific CD8⁺ T cells producing IFN- γ . Treatment of recipients before DC transfer inhibited hapten-specific CD8⁺ T cell priming with either QC or anti-TNF- α mAb (Fig. 6B). These results provided direct evidence that the migration of transferred DC into the T cell priming site was TNF- α dependent and was inhibited by QC treatment.

Since these studies suggested the blockade of NF- κ B as a primary mechanism of QC-mediated inhibition of CHS responses, we then tested if treatment of mice with a different NF- κ B inhibitor had the similar effects on CHS responses. Mice were treated with a single injection of the NF- κ B inhibitor BAY 11–085 [28] (50 mg/kg, i.p.) 1 day before hapten sensitization. This treatment inhibited ear swelling response to hapten challenge almost to the levels observed following challenge of unsensitized (naïve) mice, indicating that NF- κ B function is critical for the development of CHS responses (Fig. 6C).

Discussion

The transcription factor NF- κ B regulates the expression of many genes involved in immune responses and inflammation. For example, NF- κ B activates genes encoding pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, chemokines including CXCL8, CCL2, CCL3, CCL21, and the adhesion molecules ICAM-1, VCAM-1 and E-selectin [3–5]. These proteins are important components of innate and adaptive immunity that mediate activation and trafficking of various immune

cells in response to infection or injury. NF- κ B also induces the expression of anti-apoptotic proteins such as Bcl-2 that promote the survival of T and B lymphocytes [29, 30]. These findings implicate NF- κ B as a therapeutic target for the development of reagents to attenuate many inflammatory responses.

The role of NF- κ B in the regulation of immune responses in the skin has been insufficiently investigated. Recent *in vitro* studies used pyrrolidine dithiocarbamate as an inhibitor of NF- κ B to demonstrate that NF- κ B blockade did not prevent the activation of human monocytes and monocyte-derived DC to various contact sensitizers [31]. We extended our recent *in vitro* studies identifying QC as a potent inhibitor of NF- κ B activation [2] by testing the *in vivo* effects of NF- κ B blockade on the induction of CHS responses. QC-mediated inhibition of skin inflammation in response to hapten sensitization and subsequent challenge correlated with the decreased development of hapten-specific effector CD8⁺ T cells in the skin-draining LN of sensitized mice. It is noteworthy that the inhibitory effect of QC on hapten-specific T cell development and CHS responses was observed when QC was given before hapten sensitization, but not during hapten sensitization or afterwards. Also, administration after hapten challenge did not inhibit the magnitude of CHS responses elicited (A. Gorbachev, unpublished observations). This suggested that QC inhibited early events required for the sensitization phase of the response. Consistent with this hypothesis, the numbers of hapten-specific CD8⁺ T cells that expand in draining LN in response to hapten sensitization were markedly reduced by QC treatment. However, CD8⁺ T cells from the LN of mice treated with QC before hapten sensitization were capable of mediating CHS responses to hapten challenge in recipient naïve mice when the numbers of cells transferred were increased twofold in comparison to CD8⁺ T cells from control PBS-treated donors. These results indicated that the activation and/or expansion of hapten-specific effector CD8⁺ T cells was

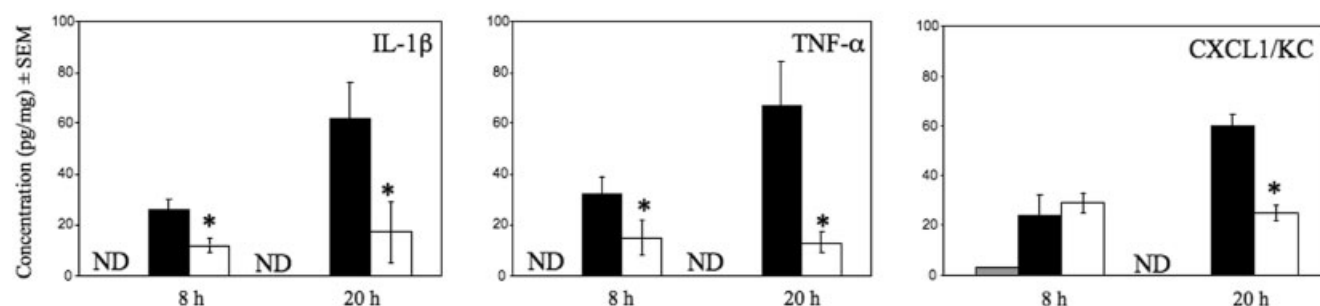


Figure 5. QC treatment inhibits IL-1 β , TNF- α and KC production in the skin of hapten-sensitized mice. Total protein extracts were prepared from the skin of naïve (gray bar) or sensitized control (black bars) and QC-treated (white bars) mice at 8 and 20 h post sensitization and tested for IL-1 β , TNF- α and KC production by ELISA. The results are expressed as a mean concentration of cytokine per mg of total protein $\pm$ SEM. * p < 0.05 when compared to control. ND – not detected.

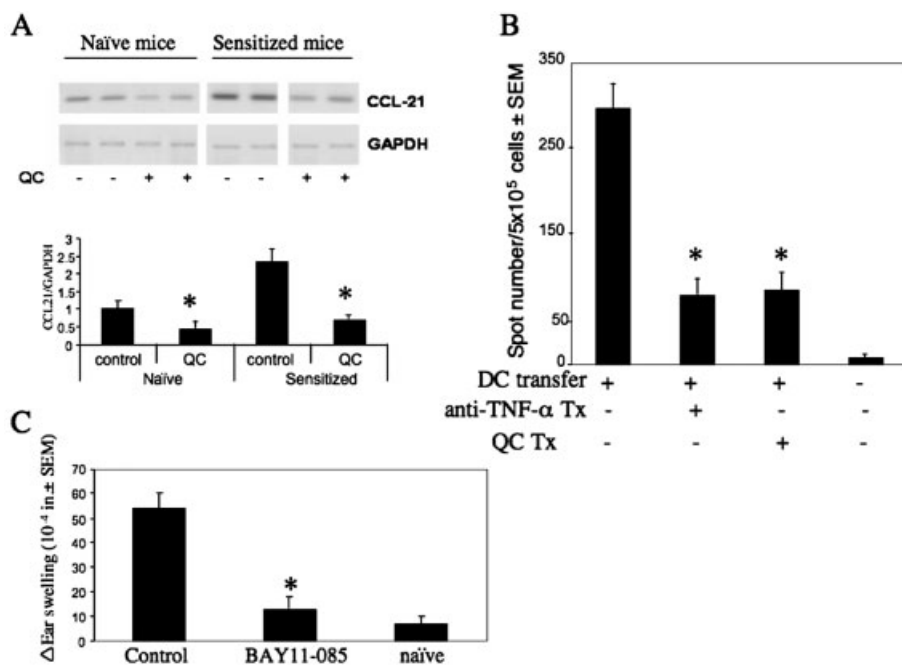


Figure 6. QC treatment inhibits CCL21 expression in the skin and migration of hapten-presenting DC. (A) Mice were treated with PBS (control) or 80 mg/kg QC (QC Tx) on days -2, and -1 and sensitized with 0.25% DNFB. Total RNA was extracted from skin of naïve or hapten-sensitized mice excised 3 h after sensitization and tested for CCL21 mRNA expression by semi-quantitative RT-PCR. (B) DC were purified from LNC of sensitized mice at 24 h post sensitization and transferred to naïve mice by intradermal injection. The recipient mice were pre-treated with PBS or with QC (QC Tx) on days -2 and -1 before DC transfer. To neutralize TNF- α , recipients were given 1 mg anti-TNF- α mAb i.v. At 5 days after DC transfer, CD8⁺ T cell-enriched LNC suspensions were prepared from DC recipients and hapten-specific CD8⁺ T cells producing IFN- γ were enumerated by ELISPOT. * $p < 0.05$ when compared to control. (C) Mice were injected with 200 μ L 10% DMSO in PBS (control) or 250 μ g/mL solution of BAY11-085 in 10% DMSO/PBS (BAY11-085) i.p. on day -1. Then mice were sensitized and ear challenged with DNFB. The mean increase in ear thickness following hapten challenge is shown in 10⁻⁴ inches $\pm$ SEM for each group of four individual mice. * $p < 0.05$ when compared to control.

reduced by QC treatment, but the effector function of CD8⁺ T cells that were activated in the presence of QC was not. One potential mechanism of the inhibition of CHS by QC could be a direct effect on hapten-specific T cell activation and expansion in LN. However, this activation and expansion typically occurs within 4–5 days following hapten sensitization [20]. The results showing that QC treatment during this time period did not inhibit hapten-specific T cell development and CHS responses argue against this mechanism of inhibition. Another potential mechanism that was considered and tested was the inhibition of hapten-presenting DC migration from the sensitized skin to LN, which precedes T cell activation. In support of this mechanism, the numbers of hapten-presenting DC, specifically LC, that migrate from the sensitized skin to the LN to activate hapten-specific T cells were decreased by QC treatment.

The migration of hapten-presenting LC from sensitized skin into draining LN during initiation of CHS is dependent on TNF- α and IL-1 β produced by epidermal keratinocytes in response to hapten application [6–8]. These cytokines down-regulate the expression of E-

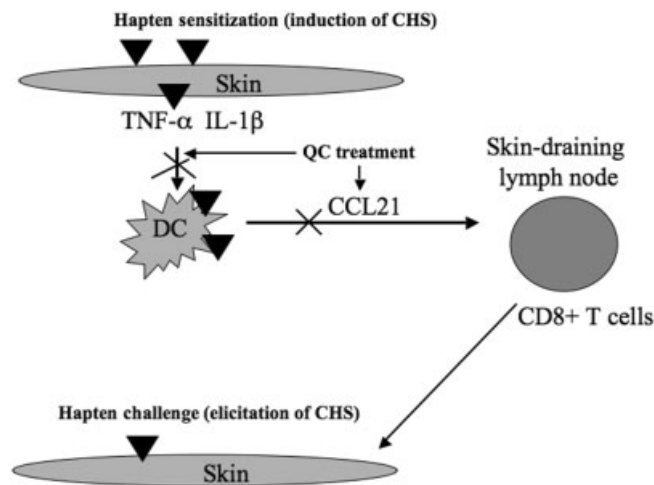


Figure 7. QC inhibits induction of CHS responses through blockade of pro-inflammatory cytokines critical for DC migration from the skin. Pre-treatment of mice with QC before hapten sensitization inhibits the NF- κ B-dependent cytokines TNF- α , IL-1 β and CCL21 required for the migration of hapten-presenting DC from the skin to LN. This inhibition reduces the number of hapten-presenting LC in the LN and priming of hapten-specific CD8⁺ T cells that mediate the inflammatory response to hapten challenge in the skin.

cadherin that tethers the DC in the epidermis and up-regulate expression of CCR7 that binds to CCL21 and directs the DC into the afferent lymphatics and into the draining LN [13]. Studies using neutralizing antibodies or knockout mice have demonstrated that disruption of either TNF- α - and IL-1 β -mediated activation or CCL21-dependent migration of DC resulted in impaired hapten-specific T cell priming and CHS responses [16, 23, 25, 27]. TNF- α , IL-1 β and CCL21 expression is induced through the NF- κ B pathway [17–19]. In the current studies QC treatment inhibited I κ B α expression in the skin, an indication of QC-mediated inhibition of NF- κ B activation. In line with the inhibition of NF- κ B activation, QC treatment resulted in marked decreases in TNF- α , IL-1 β and CCL21 mRNA expression and protein production in the skin. It seems unlikely that inhibition of these cytokines was mediated by non-specific toxic effects of QC on cells in the skin, as production of another chemokine, CXCL1/KC, by these cells was not affected at early time points of CHS initiation by QC treatment. Moreover, the migration of adoptively transferred hapten-presenting DC from a skin injection site to the draining LN was dependent on TNF- α and was inhibited by QC as evidenced by decreases in hapten-specific T cell priming. Collectively, these results provide evidence that QC inhibits hapten-presenting DC migration mediated by cytokines that are dependent on NF- κ B activation and suggest that the NF- κ B pathway is important for providing factors initiating the sensitization phase of CHS responses.

Overall, the results of these studies highlight the role of NF- κ B in the induction of inflammatory immune responses in the skin. Blockade of NF- κ B activation disrupts the induction of molecular events at several steps that are critical for the induction of CHS. First, blockade of NF- κ B inhibits the production of pro-inflammatory cytokines required for activation and mobilization of DC in the skin. Second, this blockade results in inhibition of CCL21 expression, which is necessary for directing hapten-activated DC trafficking into the lymphoid tissue where hapten-specific T cells are primed. Inhibition of these factors is likely to account for the decreased priming of the hapten-specific CD8⁺ T cells that mediate the inflammatory response to hapten challenge (Fig. 7). Previous studies have suggested inhibition of phospholipase A2 as an inhibitory mechanism of QC in inflammation [32]. It is worth noting that the inhibition of phospholipase A2 actually increases rather than decreases CHS responses [33], which in light of the current results argues against inhibition of this enzyme as a mechanism of QC-mediated suppression of CHS responses. The current results are consistent with recent studies from our group indicating that QC inhibits NF- κ B activation [2]. Keeping in mind that QC is a drug that is relatively safe and well tolerated in humans, these

studies suggest new clinical applications of QC for the preventive treatment of inflammatory diseases dependent on NF- κ B functions.

Materials and methods

Mice

C57BL/6 mice were purchased through Dr. Clarence Reeder (National Cancer Institute, Frederick, MD). Adult 8–10-week-old female mice were used throughout these studies. All animal experiments were performed according to the National Institutes of Health Guides for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care Use Committee (IACUC) of The Cleveland Clinic.

Reagents and antibodies

Quinacrine (QC) and FITC were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). mAb for the capture and detection of IFN- γ in ELISPOT assays and PE-labeled mAb specific for mouse CD11c were purchased from BD Bioscience (San Diego, CA). AlexaFluor 647-labeled mAb specific for mouse CD207 (Langerin) was purchased from eBioscience (San Diego, CA). NF- κ B inhibitor BAY11–085 was purchased from Calbiochem (EMD Biosciences Inc., La Jolla, CA).

Hapten sensitization and elicitation of CHS

For sensitization to DNFB, mice were painted on days 0 and +1 with 25 μ L 0.25% DNFB (Sigma) on the shaved abdomen and 5 μ L on each footpad. On day +5 sensitized and, as a negative control, unsensitized mice were challenged with 10 μ L 0.2% DNFB on both sides of each ear. Ear thickness was measured in a blinded manner at 24-h intervals after challenge using an engineer's micrometer (Mitutoyo, Elk Grove Village, IL) and expressed in units of 10^{–4} inches as previously described [12]. The magnitude of ear swelling responses is presented as the mean increase of each group of three sensitized or non-sensitized mice (i.e., 6 ears) $\pm$ SEM over the thickness measured just prior to hapten challenge on day +5 post sensitization. To test the effect of QC on CHS responses, groups of mice were injected i.p. with the indicated amounts of QC diluted in sterile PBS on days –2 and –1 before hapten sensitization. Control mice received injections with an equal volume of PBS.

To elicit CHS responses by adoptive transfer of hapten-presenting DC, skin-draining LN harvested from eight to ten mice on day +2 post sensitization were used to prepare cell suspensions and DC were purified from the LNC using anti-CD11c mAb-coated microbeads (Miltenyi Biotec Inc., Auburn, CA). The purity of DC was >90% as assessed by flow cytometry and 4 $\times$ 10⁵ DC were injected intradermally into the lower abdominal area of each animal.

To transfer CHS to naïve mice, hapten-immune T cells were obtained on day +5 post sensitization from sensitized mice treated with PBS or with QC. CD8⁺ T cells were enriched by negative selection using Dynabeads (Dynal A. S., Oslo,

Norway), resuspended at 12.5×10^7 cells/mL in PBS and 400- μ L aliquots (50×10^6 cells) were injected i.v. to naïve mice. Immediately following cell transfer, ears were challenged with hapten and the increase in ear swelling was measured 24 h later.

ELISPOT assays

ELISPOT assays to enumerate hapten-specific T cells producing IFN- γ were performed as previously described [21]. Briefly, ELISPOT plates (Millipore, Bedford, MA) were coated with 100 μ L 4 μ g/mL anti-IFN- γ mAb R26A2 and incubated overnight at 4°C. The plates were blocked with 1% BSA in PBS for 90 min at 37°C and washed four times with PBS. LNC suspensions from DNFB-sensitized mice were prepared on day +5 after sensitization and CD8⁺ T cells were enriched by negative selection using Dynabeads (Dynal A. S., Oslo, Norway). The efficiency of CD4⁺ T cell depletion was >95% as assessed by flow cytometry. Syngeneic spleen cells from naïve mice were treated with 50 μ g/mL mitomycin C, hapten-labeled by incubation with DNBS (100 μ g/mL), and used as stimulator cells. Responder LNC were resuspended in serum-free HL-1 medium (BioWhittaker, Walkersville, MD) and cultured at 3×10^5 cells/well with 5×10^5 stimulator cells/well for 24 h at 37°C in 5% CO₂. In all experiments responder CD8⁺ T cells cultured with unlabeled splenocytes and LNC from naïve mice cultured with DNBS-labeled stimulator cells were used as negative controls. After 24 h, cells were removed from the culture wells by extensive washing with PBS and then PBS/0.2% Tween 20 (PBS-T). Biotinylated anti-IFN- γ mAb XMG1.2 (2 μ g/mL) was added and the plate was incubated overnight at 4°C. The following day the wells were washed three times with PBS-T and incubated with anti-biotin mAb conjugated with alkaline phosphatase for IFN- γ . After 2 h at room temperature, the wells were washed with PBS and nitroblue tetrazolium 5-bromo-4-chloro-3-indolyl substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added for the detection of IFN- γ . The resulting spots were counted on an ImmunoSpot Series 1 Analyzer (Cellular Technologies Ltd., Cleveland, OH) that was designed to detect spots with predetermined criteria for size, shape and colorimetric density. To determine the number of hapten-specific cytokine-producing T cells, the number of spots resulting from the culture of T cells with unlabeled splenocytes (typically less than five spots per well) was subtracted from the number of spots resulting from the culture of T cells with hapten-labeled cells.

Flow cytometry

Skin-draining LNC suspensions were prepared from hapten-sensitized mice on day +2 post sensitization. Two-color flow cytometry analyses were performed as previously described [34]. To prevent nonspecific antibody binding cells were incubated with rat serum (Rockland, Gilbertsville, PA) diluted 1:1000 in staining buffer (Dulbecco's PBS with 2% FCS/0.02% NaN₃) for 20 min on ice. The cells were then washed and stained with PE-labeled anti-CD11c mAb to detect DC. To specifically detect hapten-bearing LC, LNC were obtained at 48 h post sensitization with FITC and were stained with PE-

labeled anti-CD11c mAb first and then were fixed, permeabilized and stained with AlexaFluor 647-labeled anti-CD207 mAb. Stained cells were washed five times, resuspended in staining buffer and analyzed by three-color flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). CD11c⁺CD207⁺FITC⁺ cells were gated and their numbers were defined for each analyzed sample. Total numbers of LC in the skin-draining LN of each mouse were calculated based on the percentage of LC in analyzed cell aliquot.

Proliferation assay

DC were purified from pooled LNC of sensitized mice at 48 h post sensitization using anti-CD11c mAb-coated microbeads and cultured in triplicate with CFSE-labeled LNC from naïve or sensitized mice obtained at day +5 post sensitization (0.6×10^5 DC: 3×10^5 LNC per well) in serum-free HL-1 media at 37°C, 5% CO₂. After 96 h of culture, cell suspensions were washed and stained with PE-labeled anti-CD8 mAb. CD8⁺ cells were gated and analyzed for proliferation by evaluation of CFSE dilution.

Analysis of IL-1 β , TNF- α , CCL21 and I κ B α expression by RT-PCR

Naïve and sensitized mouse skin (~200 mg) were snap frozen in liquid nitrogen and then ground using a mortar and pestle in liquid nitrogen. Total RNA was extracted using the RNeasy Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. For synthesis of cDNA, 2 μ g total RNA from each sample was resuspended in 10 μ L of the reaction buffer. cDNA synthesis was performed using M-MLV reverse transcriptase (Promega Corporation, Madison, WI) with random primers according to the manufacturer's protocol. The reaction was stopped by denaturing the enzyme at 99°C for 5 min, and the mixture was diluted with distilled water to a final volume of 50 μ L. Aliquots (3 μ L) of the synthesized cDNA were added to 47 μ L PCR mixture, containing 1 $\times$ PCR buffer, MgCl₂ (1.5 mM), dNTP (0.2 mM each), primer mix (0.5 μ M each) and 2.0 U Taq DNA polymerase (all from Invitrogen Corp., Carlsbad, CA). PCR primers for mouse GAPDH, I κ B α , TNF- α , IL-1 β and CCL21 were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) with the following sequences: mGAPDH sense: 5'-tcgtggatctgacgtgccgcctg-3', antisense: 5'-caccacccgtgtgctgtagccgtat-3'; mI κ B α sense: 5'-ctgggagctggctgtgatcc-3', antisense: 5'-tctgtgcatagctctctca-3'; mIL-1 β sense: 5'-aaatgcctcgtgctgtgacc-3', antisense: 5'-ctgcttgagaggtgctgtatgacc-3'; mTNF- α sense: 5'-atgagcagaagcatgatccgc-3', antisense: 5'-ccaaagtagacctgccggactc-3'; mCCL21 sense: 5'-caggactgctgccttaagta-3', antisense: 5'-gcatagctcaggcttaga-3'.

Amplification was initiated by 1 min of denaturation at 95°C for 1 cycle. This was followed by 25–35 cycles at 94°C for 45 s, 56°C for 30 s, and 72°C for 30 s. After the last amplification cycle, the samples were incubated for 7 min at 72°C. For each set of primers, dilutions of cDNA were amplified for 20–40 cycles to define optimal conditions for linearity and to permit semi-quantitative analysis of signal strength. Abundant mRNA (i.e., GAPDH and I κ B α) were amplified 25 cycles, whereas cytokine mRNA were amplified 30–35 cycles.

The amplified PCR products were separated on a 2% agarose gel with 1× Tris-acetate buffer at 75 V for 40–60 min and visualized by UV light after staining with 0.5 µg/mL ethidium bromide. Gels were photographed using the Bio Doc-It Imaging System (UVP, Inc., Upland, CA). Densitometric analysis of the captured images was performed using NIH 1.54 image analysis software and normalized to the intensity of the GAPDH band.

Measurement of TNF- α , IL-1 β and CXCL1/KC proteins in the skin

To test cytokine and chemokine production in sensitized skin, skin was excised at indicated times after DNFB sensitization and homogenized in the presence of proteinase inhibitors (10 µg/mL PMSF, 2 µg/mL aprotinin, 2 µg/mL leupeptin and 100 µg/mL chymostatin). Following standardization of protein levels to 5 mg total protein/mL, aliquots were tested by ELISA to quantitate TNF- α , IL-1 β and CXCL1/KC levels using Quantikine Mouse Cytokine Immunoassay Kits (R&D Systems Inc., Minneapolis, MN).

Statistical analysis

Statistical analysis to assess differences between experimental groups was performed using Student's *t*-test. Differences were considered significant when $p < 0.05$. Three or four mice per each group were used in all experiments. All experiments were repeated at least two times with similar results.

Acknowledgements: The authors thank the staff of the Cleveland Clinic Biological Resources Unit for excellent animal care. This work was supported by National Institutes of Health Grant RO1AI45888 (PI is Dr. Robert Fairchild).

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