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An IL-12-Independent Role for CD40-CD154 in Mediating Effector Responses: Studies in Cell-Mediated Glomerulonephritis and Dermal Delayed-Type Hypersensitivity

Amanda-Jane Ruth, A. Richard Kitching, Ming Li, Timothy J. Semple, Jennifer R. Timoshanko, Peter G. Tipping, and Stephen R. Holdsworth

Crescentic glomerulonephritis (GN) results from IL-12-driven Th1-directed cell-mediated responses (akin to delayed-type hypersensitivity (DTH)) directed against glomerular Ags. CD40-CD154 interactions are critical for IL-12 production and Th1 polarization of immune responses. Crescentic anti-glomerular basement membrane GN was induced in C57BL/6 (wild-type (WT)) mice (sensitized to sheep globulin) by planting this Ag (as sheep anti-mouse glomerular basement membrane globulin) in their glomeruli. Crescentic GN did not develop in CD40−/− mice due to significantly reduced nephritogenic Th1 responses. IL-12 was administered to CD40−/− mice with GN to dissect interactions between IL-12 and CD40 in inducing nephritogenic immunity and injury. Administration of IL-12 to CD40−/− mice restored Th cell IFN-γ production, and up-regulated intrarenal chemokines and glomerular T cell and macrophage accumulation compared with WT control mice. Despite this, renal macrophages were not activated and renal injury and dermal DTH were not restored. Thus, CD40-directed IL-12 drives Th1 generation and effector cell recruitment but CD40 is required for activation. To test this hypothesis, activated OT-II OVA-specific CD4+ cells and OVA323-339-loaded nonresponsive APCs were transferred into footpads of WT, CD40−/−, and macrophage-depleted WT mice. WT mice developed significant DTH compared with CD40−/− and macrophage-depleted WT mice. This study demonstrated that CD40-induced IL-12 is required for generation of systemic Th1 immunity to nephritogenic Ags, and that IL-12 enhances Th1 effector cell recruitment to peripheral sites of Ag presentation via generation of local chemokines. Effector cell activation, renal DTH-like injury, and dermal DTH require direct Th1 CD154/macrophage CD40 interactions. The Journal of Immunology, 2004, 173: 136–144.

A n important costimulatory pair, CD40-CD154, is known to regulate adaptive immunity (1). These costimulatory molecules have been well defined as providing bidirectional “second signaling” to naive CD4+ T cells and APCs that confer their functional activity (2). The primary activation signal is delivered through CD4+ TCR recognition of the MHC/peptide complex expressed by APCs. Adhesion and costimulatory molecules are up-regulated on APCs through CD40 ligation with CD154 expressed on activated T cells (3). Up-regulation of these molecules enhances T cell activation (4).

It has been suggested that the CD40-CD154 interaction also regulates the nature of the immune response by preferentially driving the response toward the Th1 phenotype through the production of IL-12 (5). Ligation of CD40L (CD154) expressed by CD4+ T cell has been shown to trigger APC–IL-12 secretion through CD40 engagement (6, 7). However, ligation of CD40 has been shown to inhibit IL-12 production by human APCs when CD40 is engaged on the APCs before the cells are exposed to a primary stimulatory (IFN-γ/GM-CSF) signal (8). Thus, the timing of the costimulatory signal in concert with the presence of inflammatory mediators appears to dictate the regulatory outcome of the signal delivered by CD40-CD154. Evidence suggests that IL-12 is the prominent inducer of Th1 responses, as IL-12 alone or together with IFN-γ drives T cells to differentiate into the Th1 phenotype (9). Studies have shown that exogenous IL-12 replaces the requirement for CD40-CD154 in the generation of Th1 immune responses (5, 10).

Roles for CD40 and CD154 in mediating effector responses have been suggested. CD40 has been shown to be required for delivering optimal functional capacity to macrophages by increasing production of IL-12 and IL-1 (11, 12), and through up-regulation of ICAM-1, MHC class II, and CD86 (13). CD40-dependent activation of endothelial cells has been shown to up-regulate adhesion molecule expression that facilitates leukocyte migration to inflammatory sites (14, 15).

Crescentic glomerulonephritis (GN) is the most injurious form of GN that frequently progresses to renal failure. Renal injury in crescentic GN results from Th1-directed glomerular delayed-type hypersensitivity (DTH)-like responses (16). The glomerular influx of CD4+ T cells (16) and macrophages (17) results in local cell inactivation.
proliferation, leading to the formation of glomerular crescents and severe glomerular injury. Macrophages are the major effector cell responsible for the development of glomerular injury (18). Their recruitment and activation is dependent on nephrigenic Ag-specific Th1 cells (17).

Recruitment of nephrigenic Th1 cells to glomeruli also requires the expression of MHC class II by intrinsic nonimmune renal cells, as chimeric mice with MHC class II intact bone marrow but deficient renal MHC class II exhibited significantly diminished recruitment of Th1 effectors during the development of crescentic GN (19). Recent studies also demonstrate a requirement for CD40 expression by intrinsic renal cells in the development of Th1-directed nephrigenic responses. Chimeric wild-type (WT)→CD40−/− mice with CD40-intact bone marrow and CD40−/− renal cells failed to recruit glomerular Th1 effector cells or express Th1-recruiting chemokines following nephrigenic Ag challenge despite the development of normal systemic immunity (20). CD40-CD154 signals are important in effector responses leading to GN as WT (CD40-intact, C57BL/6) mice with established immune responses treated with a neutralizing Ab to CD154 also failed to recruit glomerular T cells and develop injury following glomerular antigenic challenge (20). Similar results have been observed in experimental autoimmune encephalomyelitis where the absence of CD40 expression in the CNS prevented the accumulation of injurious Th1 effectors and severe injury (21).

It was hypothesized that administration of exogenous IL-12 to CD40-deficient mice would restore the generation Th1 effectors in response to Ag priming and facilitate their recruitment to glomeruli following local challenge, leading to the development of crescentic GN. The current studies demonstrated that administration of IL-12 to CD40−/− mice leads to the restoration of Th1 cell Ag-specific responsiveness and the glomerular accumulation of effectors. However, in the absence of CD40, recruited glomerular effectors were not activated and failed to induce injury. Subsequent Th1 transfer studies in dermal DTH confirmed that T cell-mediated macrophage activation through CD40 ligation was required for the full in vivo expression of the DTH response.

Materials and Methods

Animals

Breeding pairs of mice with a targeted disruption of the CD40 gene (22) were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice have been backcrossed for nine generations to a C57BL/6 background. Breeding pairs of transgenic mice expressing MHC class II I-A* restricted CD4 cells that are TCR specific for OVA25-329 (OT-1) mice (C57BL/6 background) possessing OVA-specific transgenic CD4+ T cells (23) were obtained from Dr. W. R. Heath (Immunology Division, and Transplantation and Autoimmunity Division, Walter and Eliza Hall Institute, Melbourne, Victoria, Australia). Mice were housed and bred under specific pathogen-free conditions (Monash University, Victoria, Clayton, Australia).

Induction of accelerated anti-glomerular basement membrane (GBM) GN

Sheep anti-mouse GBM globulin was prepared as previously described (24). Accelerated experimental anti-GBM GN was induced by sensitizing 8- to 10-wk-old male mice with a s.c. injection in each flank consisting of a total of 1 mg of sheep globulin in 100 μl of CFA (Sigma-Aldrich, St. Louis, MO). GN was initiated 10 days later by i.v. challenge with 14 mg of sheep anti-mouse anti-GBM globulin. Immune responses and renal injury were assessed 10 days later in CD40+/- (n = 12) and WT (n = 6) mice. Urine was collected over the 24-h period before sacrifice with the aid of metabolic cages, and protein concentration was determined by a modified Bradford method (25).

Administration of recombinant murine rmIL-12

A total of 1 μg of rmIL-12 (a gift from Dr. M. Gately, Hoffmann-La Roche, Nutley, NJ; specific activity 3.5 × 10^6 U/mg) in 200 μl of 1% homologous mouse serum in PBS was administered (i.p.) to CD40−/− mice (n = 5) for 5 consecutive days each week for the duration of experiments (21 days). Treatment was initiated 2 h before sensitization of mice with normal sheep globulin. Control animals (C57BL/6 mice, n = 6; CD40−/− mice, n = 6) received 200 μl of homologous mouse serum in PBS without rmIL-12. The administered dose of rmIL-12 was based on a previous published dose having biological effects (Ref. 26, and M. Gately, unpublished observations).

Assessment of systemic immune responses

Humoral responses to sheep globulin. Mouse anti-sheep globulin Ab titers in sera were measured by ELISA at the end of each experiment as previously described (27).

Induction of active dermal DTH to sheep globulin. Mice with experimental anti-GBM GN were challenged by intradermal injection of 400 μg of sheep globulin in 40 μl of PBS into the right plantar footpad. The same dose of the irrelevant Ag (horse globulin) was injected into the contralateral footpad. Swelling was measured after 24 h using a micrometer (Mitutoyo, Kawasaki-shi, Japan). DTH was assessed as the difference measured in swelling between the two footpads and expressed in millimeters.

Assessment of splenocyte IFN-γ production

CD40+/− mice (n = 5) were treated with 1 μg of rmIL-12, and control mice (C57BL/6, n = 5) received vehicle alone as described above. Mice were immunized 2 h before IL-12 administration with s.c. injection of a total of 1 mg of sheep globulin in 100 μl of CFA, and spleens were removed aseptically 9 days later. Single cell suspensions were prepared in DMEM/5% FCS (27). A total of 4 × 10^5 splenocytes/ml in DMEM/10% FCS were incubated for 72 h at 37°C with protein G-purified normal sheep IgG (10 μg/ml). IFN-γ in supernatants was measured by ELISA as described previously (27). Rat anti-mouse IFN-γ (RA-6A2; BD Pharmingen, San Diego, CA) and biotinylated rat anti-mouse IFN-γ (XMG1.2; BD Pharmingen) mAbs were used.

Chemokine expression measured by RNase protection assay

RNA extraction. Total kidney RNA was extracted with TRizol reagent (Invitrogen Life Technologies, Victoria, Australia) according to the manufacturer’s protocol. The final product was air dried and dissolved in ultrapure Dnase/RNase-free water (Invitrogen Life Technologies) and then stored at −80°C. The concentration of RNA was determined by spectrophotometry at 260 nm.

RNase protection assay. Total Kidney RNA was assessed using the RiboQuant System (BD Pharmingen). Multiplexes incorporating [α-32P]UTP were transcribed from the template set mCK-5c using T7 RNA polymerase in vitro transcription. After Dnase I treatment, the riboprobes were isolated by phenol/chloroform extraction and precipitation with 4 M ammonium acetate and ethanol. The incorporation of [α-32P]UTP was determined by Cherenkov activity in a scintillation counter. The probe was diluted to 3.5 × 10^6 cpm/μl and then added to 20 μg of total kidney RNA. Hybridization and isolation was conducted according to the protocol in the RiboQuant manual. RNA hybrids were separated by electrophoresis on 5% polyacrylamide/8 M urea gel. The gel was dried at 80°C for 1 h before being exposed to the imaging plate of a FLA-2000 phosphoimager (Fuji Photo Film, Tokyo, Japan). Image Gauge software, version 3.46 (Fuji Photo Film), was used to evaluate the gel image. Chemokine expression was measured and normalized to the housekeeping gene L32 and the results expressed as arbitrary units. Levels of expression of mRNA for lymphoactin (LTN), RANTES, TCA-3, IFN-inducible protein-10 (IP-10), MIP-2, MCP-1, but not MIP-1α, were sufficient to allow quantitative analysis.

Measurement of urinary NO

Concentrations of urinary nitrite were determined by Griess assay. Urine samples (collected from mice for a 24-h period before sacrifice) were centrifuged at 2000 × g for 10 min. A total of 50 μl aliquots of urine were added to 50 μl of Griess reagent (1.5% sulfanilamide/0.15% naphthyl ethylene diamine) in a 96-well microtiter plate. Samples were incubated for 10 min at room temperature and absorbance read at 540 nm. Urinary nitrite concentration was determined from standards of sodium nitrite of known concentrations. Samples were tested in duplicate and measured as micro- moles per 24 h.

Passive transfer of dermal DTH

Recipient animals. Recipient mice for cell transfer strategies were as follows: CD40−/− mice (n = 6), WT mice (n = 6), and macrophage-depleted (clobrofate-treated) mice (n = 6).
Clodronate treatment of WT mice. Macrophage depletion was induced by i.v. administration of microencapsulated clodronate (obtained from Dr. M. D’Souza, Mercer University, Atlanta, GA). A total of 500 μg of microencapsulated clodronate in 250 μl of saline was administered 48 h before transfer. Macrophages were depleted by at least 80% as determined by immunoperoxidase staining of spleen sections.

Activation and purification of naive OT-II cells. OT-II TCR transgenic CD4+ T cells were derived from spleens of OT-II mice (n = 6). Single splenocyte suspensions were obtained by pushing spleens gently through stainless steel mesh sieves with the aid of a rubber policeman under aseptic conditions. Cells were suspended in HBSS and centrifuged at 4°C for 3 min at 4°C, and resuspended in RPMI 1640/10% FCS to obtain 1.5 × 10^6 cells/ml that were cultured (37°C, 5% CO_2) for 30 min and washed three times to remove unbound OVA323–339. Cells coated with OVA323–339 and uncoated splenocytes were resuspended in 10% HBSS containing 1 μM OVA\textsubscript{323–339} (obtained from Prof. F. R. Carbone, Department of Microbiology and Immunology, Melbourne University, Melbourne, Victoria, Australia) and incubated at 37°C for 30 min and washed three times to remove unbound OVA\textsubscript{323–339}. Cells coated with OVA\textsubscript{323–339} and uncoated cells were both counted and adjusted to 2 × 10^7 cells/30 μl for transfer into footpads of naive recipients.

OVA\textsubscript{323–339} coating. Spleens were removed aseptically from C57BL/6 mice (n = 10) and single cell suspensions were prepared in HBSS-10% FCS. Splenocytes were resuspended and fixed in 1% paraformaldehyde HBSS and incubated for 20 min at room temperature to render the cells unresponsive. Fixed cells were washed three times in 10% FCS-HBSS and resuspended into two equal volumes of 5 × 10^7 cells/ml. Half the total number of fixed splenocytes were resuspended in 10% HBSS containing 1 μM OVA\textsubscript{323–339} (obtained from Prof. F. R. Carbone, Department of Microbiology and Immunology, Melbourne University, Melbourne, Victoria, Australia) and incubated at 37°C for 30 min and washed three times to remove unbound OVA\textsubscript{323–339}. Cells coated with OVA\textsubscript{323–339} and uncoated cells were both counted and adjusted to 2 × 10^7 cells/30 μl for transfer into footpads of naive recipients.

Histology, immunohistochemistry, and immunofluorescence

Kidney tissue was fixed in Bouin’s fixative, embedded in paraffin, and 3-μm tissue sections were cut and stained with periodic acid-Schiff reagent. Glomerular crescent formation was assessed by a blinded protocol.
whereby a minimum of 25 glomeruli was assessed to determine the crescent score for each animal. Crescent formation was considered to be apparent when glomeruli exhibited two or more layers of cells in Bowman’s space.

Periodate lysine paraformaldehyde-fixed kidney, skin, and spleen sections (6 μm) were stained to demonstrate CD4+ T cells and/or macrophages using a three-layer immunoperoxidase technique (29). The primary mAbs used were GK1.5 (anti-mouse CD4; American Type Culture Collection (ATCC), Manassas, VA) and M1/70 (anti-mouse Mac-1; ATCC). A minimum of 25 glomeruli were assessed per animal and results were expressed as cells per glomerular cross-section (c/gcs).

Protein G-purified monoclonal rat anti-mouse MHC class II Ab (clone Y3P; a gift from Prof. K. Shortman, Walter and Eliza Hall Institute, Melbourne, Victoria, Australia) was conjugated with Alexa Fluor dye 488 and FA11 (rat anti-mouse CD68) conjugated to Alexa Fluor dye 594 (Molecular Probes). Snap frozen kidney and skin sections (6 μm) were blocked with 5% normal rat serum in 1% BSA/PBS and incubated with both Abs (1 in 50) for 60 min at room temperature. Confocal images were collected using a Bio-Rad (Hercules, CA) confocal inverted Nikon microscope equipped with an air-cooled 25-mW argon/krypton laser. MHC class II cells were quantitated in glomeruli as c/gcs and a total of 25 glomeruli were assessed per animal (30).

Statistical analysis

One way ANOVA was applied with Tukey’s posttest (Graph Pad, San Diego) to analyze differences for parameters examined. Statistical significance was defined as p < 0.05. Data were expressed as the mean ± SEM.

Results

Administration of IL-12 to CD40−/− mice restores IFN-γ production by CD40−/− splenocytes stimulated with the nephritogenic Ag

CD40-intact C57BL/6 (WT) mice immunized with sheep globulin develop humoral immunity (Fig. 1A) and dermal DTH (Fig. 1B) to sheep globulin. Ag-stimulated splenocytes from WT mice pro-

FIGURE 3. Representative samples from an RNase protection assay for the detection of chemokine mRNA in mice with GN. Lanes 1 and 2, WT mice with GN; lanes 3–7, mRNA from CD40−/− mice treated with exogenous IL-12 with up-regulated chemokine expression compared with mRNA from CD40−/− mice (lanes 8–10). The expression of LTN, RANTES, MIP-1β, IP-10, MCP-1, and TCA-3 were significantly up-regulated in CD40−/− mice by IL-12 administration, while the expression of MIP-1α and MIP-2 was not significantly changed.

FIGURE 4. Assessment of glomerular DTH effectors in experimental crescentic GN. Glomeruli of WT mice had significant CD4+ T cell (A) and macrophage (B) influx in glomeruli compared with CD40−/− mice. Administration of IL-12 to CD40−/− mice (CD40−/− + IL-12) restored glomerular accumulation of CD4+ T cells and macrophages to levels higher than that observed in the glomeruli of WT mice with GN. Dotted lines represent values for normal WT mice without GN. Results are measured as c/gcs.

FIGURE 5. Glomerular injury in mice with GN. Marked proteinuria (A) and crescent formation (B) was observed in WT mice. In contrast, glomerular injury was not observed in the absence of CD40 (CD40−/− mice) and was not restored by IL-12 (despite the influx of leukocytes into glomeruli). CD40−/− and CD40−/− given exogenous IL-12 (CD40−/− + IL-12) had normal urinary protein levels and minimal crescent formation. The dotted lines in A represent normal values for mice without GN.
administration to CD40−/− mice did not restore dermal DTH responses (Fig. 1B) or Ab responses (Fig. 1A). However, despite the absence of CD40 signaling, IL-12 restored Ag-specific IFN-γ production by CD40−/− splenocytes (p < 0.01 compared with CD40−/− mice; Fig. 1C).

Glomerular expression of chemokine mRNA was markedly up-regulated by IL-12 in CD40−/− mice

LTN, RANTES, TCA-3, IP-10, MIP-2, MIP-1β, and MCP-1 mRNA were significantly up-regulated in WT control mice with GN compared with normal WT mice (all p < 0.05; Figs. 2 and 3). CD40−/− mice had reduced expression of LTN, RANTES, IP-10, and MIP-1β mRNA compared with WT mice with GN that did not reach statistical significance. The treatment of CD40−/− mice (with GN) with exogenous IL-12 resulted in up-regulated expression of LTN, RANTES, TCA-3, IP-10, MCP-1, and MIP-1β mRNA. Levels of MIP-2 in CD40−/− mice were not significantly up-regulated by IL-12 administration.

IL-12 replaces the requirement for CD40 in the generation and accumulation of DTH effectors in glomeruli

WT mice with GN developed significant accumulation of DTH effectors, CD4+ T cells (0.94 ± 0.05 c/gcs; Fig. 4A), and macrophages (2.40 ± 0.36 c/gcs; Fig. 4B) at the site of glomerular antigenic challenge. Accumulation of effectors in glomeruli of CD40−/− mice was attenuated compared with WT mice (CD4+ T cell, 0.11 ± 0.04 c/gcs; macrophage accumulation, 0.41 ± 0.13 c/gcs; both p < 0.05). IL-12 administration restored cellular influx in CD40−/− mice and this cellular accumulation occurred to a higher degree than that observed in glomeruli from WT mice (CD4+ T cells, 3.0 ± 1.1 c/gcs, p < 0.01) and macrophages (4.05 ± 1.16 c/gcs, p < 0.01).

Despite the restoration of DTH effectors in IL-12-treated CD40−/− mice, glomerular injury did not occur in CD40−/− mice

Renal injury in WT mice was associated with proteinuria (31.4 ± 6.6 mg/24 h, p < 0.01 compared with normal, 3.0 ± 1.0 mg/24 h; Fig. 5A). In contrast, CD40−/− mice and CD40−/− mice given
IL-12 had urinary protein levels within the normal range (1.9 ± 0.4 mg/24 h and 1.5 ± 0.4 mg/24 h, both p < 0.001 compared with WT controls with GN). Glomerular crescent formation was prominent in WT mice with GN (21 ± 5%; Fig. 5B), however, CD40^−/− and CD40^+/− given IL-12 displayed minimal crescent formation (4 ± 2%, p < 0.01 and 8 ± 3%, p < 0.05, respectively, compared with WT controls with GN).

**IL-12 administration to CD40^−/− mice did not restore macrophage activation in the absence of CD40**

Cellular MHC class II expression in glomeruli was significantly attenuated in the absence of CD40 (CD40^−/−, 0.4 ± 0.05 c/gcs; CD40^−/− + IL-12, 0.5 ± 0.04 c/gcs) compared with cellular MHC class II up-regulation in the glomeruli of WT mice (3.40 ± 0.43 c/gcs, p < 0.01). Confocal microscopy revealed that glomerular MHC class II expression was associated with macrophages in WT mice (Fig. 6). However, glomerular macrophages in CD40^−/− mice treated with exogenous IL-12 failed to up-regulate MHC class II (as did CD40^+/+ mice; Fig. 7). The data suggest that macrophages in glomeruli require CD40 expression for activation as measured by MHC class II up-regulation. It has been shown previously that activated macrophages produce NO as an effector function. Urinary NO levels were reduced in CD40^−/− and IL-12-treated CD40^−/− mice compared with WT urinary NO levels (WT, 54.0 ± 14.3 μM; CD40^−/−, 2.5 ± 1.0 μM; CD40^−/− + IL-12, 3.2 ± 0.7 μM; p < 0.05) demonstrating that glomerular macrophages release less NO in the absence of CD40 expression.

**FIGURE 7.** Effector cell activation in glomeruli of mice with GN. Glomeruli from WT mice exhibited up-regulation of intraglomerular macrophage-associated MHC class II (measured as c/gcs) compared with glomeruli of CD40^−/− mice and CD40^+/− mice treated with exogenous IL-12 (CD40^−/− + IL-12) (A). B, Urinary NO levels were measured over 24 h and were significantly higher in WT mice than CD40-deficient animals (p < 0.05). IL-12 administration had no effect on NO production in CD40^−/− mice.

**Dermal DTH transfer studies reveal that host macrophages fail to induce DTH in the absence of CD40 expression**

To determine the ability of CD40-intact and CD40-deficient (CD40^+/−) host macrophages to affect dermal DTH, activated OT-II transgenic CD4^+ cells, together with unresponsive APCs coated with OVA\textsubscript{323-339} (control not coated with OVA\textsubscript{323-339}), were transferred to the footpads of naive recipients. WT recipients developed dermal DTH 24 h after cell transfer (0.39 ± 0.09 mm; Fig. 8). Footpad swelling was dependent on host CD40 expression and host macrophage accumulation as DTH was attenuated in CD40^−/− mice and macrophage-depleted (clodronate-treated) recipients (0.10 ± 0.04 mm, 0.05 ± 0.06 mm, respectively, p < 0.01 compared with WT; Fig. 8). Assessment of the activation status of the infiltrating host macrophages by MHC class II expression revealed that MHC class II was up-regulated by infiltrating macrophages when CD40 expression was intact (Fig. 9A) but not in the absence of CD40 (Fig. 9B).

**Discussion**

Crescentic GN in mice resembles crescentic GN in man (16, 31) and is similarly characterized by accumulation of Th1 effectors (CD4^+ T cells and macrophages), resulting in crescent formation and renal injury. Glomeruli are specialized filtering units that are highly vulnerable to immune injury. Many exogenous or endogenous Ags have the potential to directly deposit in glomeruli (inciting in situ immune complex formation in sensitized hosts) or deposit as immune complexes leading to crescentic GN (32–34). A large number of Ags (35) can induce GN in humans (including foreign serum proteins that can induce serum sickness associated GN (36)). Heterologous globulin (targeted to glomeruli as anti-mouse GBM globulin) acts as a planted Ag in sensitized mice (37). The current studies use a well-characterized model of crescentic GN in C57BL/6 mice that results from Th1 nephritogenic immune responses to sheep globulin, which induces glomerular DTH-like responses when the immunogen is planted in glomeruli (38).

Th1 (cell-mediated) responses are driven by the production of IL-12 that is primarily secreted by APCs in a CD40 contact-dependent fashion through ligation with CD4^+ T cell-expressed CD154 (7, 39). The development of GN in the model used in the current studies is IL-12 dependent (40). IL-12^−/− mice failed to develop nephritogenic polarized immune responses (30). Administration of exogenous IL-12 to C57BL/6 mice with GN greatly

**FIGURE 8.** Results of cell transfer studies in dermal DTH. Transfer of activated transgenic OT-II CD4^+ cells plus unresponsive APCs coated with OVA\textsubscript{323-339} into naive WT mice resulted in significantly greater DTH when compared with the contralateral control footpad from the same animal that received OT-II CD4^+ cells plus unresponsive APCs without Ag (OVA\textsubscript{323-339}). In contrast, transfer of activated OT-II CD4^+ cells to CD40^−/− mice and macrophage-depleted (clodronate-treated) WT mice resulted in significantly attenuated DTH.
enhanced disease severity by amplifying the Th1 response (40). We have recently demonstrated the requirement for CD40

CD40-CD154 interactions in the initiation of nephritogenic immune responses for the development of effector responses, and the requirement for nonimmune renal cell expression of CD40 in the development of glomerular DTH (20).

In the current studies we explored the capacity of IL-12 to restore Th1 cell-mediated immune responses to a nephritogenic Ag (sheep globulin) in CD40−/− mice developing GN. The results demonstrate that treatment of CD40−/− mice with IL-12 restored the ability of these animals to develop Ag-specific Th1 responses. Despite the restoration of Th1-polarized Ag-specific T cells in these animals, treatment with exogenous IL-12 did not restore Ag-specific humoral immune responses. This finding is consistent with previous studies confirming the Ag-specific requirement for T cell-CD154 B cell-CD40 cell-cell interactions for Ab production (41). Studies using agammaglobulinemic mice have shown that the development of severe crescentic injury in this model is Ab independent (42). Thus, the fact that IL-12 fails to restore humoral responses to the nephritogenic Ag would not be expected to effect the progression of crescentic GN in this model.

IL-12 treatment amplified CD4+ T cell and macrophage recruitment to glomeruli in CD40−/− mice. Inflammatory chemokines are important in the recruitment of leukocytes to the site of Ag challenge in GN (43, 44). IL-12 administration to CD40−/− mice amplified the expression LTN, RANTES, TCA-3, IP-10, and MCP-1, and enhanced the influx of Th1 effector cells. WT mice with GN developed significant crescentic injury and proteinuria, while CD40−/− mice did not develop crescentic GN due to their deficient adaptive immune responses and their inability to generate and recruit (and possibly activate) effector cells. Despite augmented effector cell infiltration in glomeruli, renal injury did not develop in CD40−/− mice given exogenous IL-12. It is likely that this lack of restoration of injury was due to inefficient effector cell activation. Macrophage activation was assessed by MHC class II expression, and functional activation of glomerular cells and infiltrating leukocytes was assessed by urinary NO production. MHC class II expression was significantly up-regulated on macrophages in glomeruli and urinary NO levels up-regulated in WT mice with GN compared with CD40−/− mice. Despite its capacity to augment CD4+ IFN-γ production, glomerular chemokine production, and Th1 effector cell recruitment, IL-12 treatment failed to induce macrophage MHC class II expression, restore glomerular NO production, or induce injury in CD40−/− mice. Therefore, exogenous IL-12 does not replace the requirement for CD40 in mediating macrophage activation.

Macrophage accumulation is dependent on Th1 cells in the development of DTH (17). To further explore the requirement for CD40 in macrophage activation in effector DTH responses in vivo, we developed a cell transfer system in the skin whereby DTH in the naive host was dependent on the recruitment and activation of host macrophages. Activated transgenic OT-II CD4+ T cells, together with unresponsive APCs pulsed with the OT-II TCR-binding peptide OVA123–339, were transferred into the dermis of three groups of naive C57BL/6 recipients: macrophage-depleted, CD40−/−, and WT mice. OT-II CD4+ cells were functionally active in this system, demonstrated by their capacity to recruit and activate macrophages and to induce DTH. OT-II cell activation required APCs presenting OVA123–339. To prevent transferred APCs from potentially contributing to DTH due to their possible effector cell functions, transferred APCs were fixed with paraformaldehyde, preserving Ag-presenting function while preventing effector function. DTH was present in WT mice receiving OT-II T cells and OVA123–339-coated cells but did not develop in clodronate-treated (macrophage-depleted) or CD40−/− recipients. The absence of DTH in macrophage-depleted mice, coupled with the absence of macrophages in the lesion in these mice, confirms that this lesion is macrophage dependent.

The failure to induce DTH in CD40−/− mice after transfer of OT-II cells and Ag-pulsed APCs confirms that macrophage CD40

**FIGURE 9.** Photomicrographs of dermal macrophage accumulation and activation status (MHC class II expression) in recipients of cell transfer studies. Immunohistochemical staining of the skin revealed a significant macrophage infiltrate (black reaction product, nuclear fast red counterstain) in both WT recipients (A) and CD40−/− mice (B), but minimal macrophage accumulation in clodronate-treated mice (C). Macrophages (red) in the WT recipients were colocalized with MHC class II expression (green), confirming macrophage activation (colocalization yellow, D). Macrophages in dermal lesions of CD40−/− recipients were apparent (E, red) but failed to express the activation marker MHC class II (green) in the absence of CD40. Clodronate-treated animals had little macrophage accumulation or MHC class II expression (F). Photomicrographs were taken at ×400 magnification.
expression is required for macrophage activation inducing DTH. Although OT-II cell transfer induced dermal macrophage recruitment, these macrophages had minimal MHC class II expression, consistent with the failure of Th1-directed macrophage activation in the absence of macrophage CD40 expression observed in GN.

The roles for CD40 and IL-12 have been explored previously in experimental leishmaniasis. Studies in CD154−/− mice demonstrated the requirement for CD40-CD154 in the generation of protective Th1 responses (45). Exogenous IL-12 treatment of infected CD154−/− mice led to protection (measured by attenuated footpad swelling) that seems to be at odds with the current studies in GN and dermal DTH. The Leishmania studies showed enhanced IFN-γ production by CD154−/− T cells in the presence of IL-12, but macrophage activation was not assessed (5, 45). Other studies have demonstrated that IFN-γ is insufficient to activate macrophages to a leishmanialistic state in the absence of CD154 (5, 46). Therefore, the effects of IL-12 in this system may involve other pathways distinct from macrophage activation.

In conclusion, these studies define the roles of CD40 and its interactions with IL-12 in Th1 responses, using two models of Th1 effector injury, crescentic GN and dermal DTH. They show that: 1) optimal production of Th1 cytokines is CD40-dependent but this can be overcome by IL-12; 2) IL-12 augments local chemokine production in CD40−/− mice, leading to recruitment of effector T cells and macrophages; and 3) injurious activation of macrophages requires cell-cell CD154-CD40 interactions, and this cannot be overcome by production of soluble factors such as chemokines, IL-12, or IFN-γ. Experiments in dermal DTH confirm that functional effector macrophage activation requires direct macrophage CD40 expression/signaling. Understanding the role of CD40-CD154 interactions in mediating Th1 effector responses and its interactions with IL-12 may have therapeutic relevance for Th1-mediated immune injury in man.

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References


