Gene Correction Reduces Cutaneous Inflammation and Granuloma Formation in Murine X-Linked Chronic Granulomatous Disease

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Our laboratory previously demonstrated that X-linked chronic granulomatous disease (X-CGD) mice develop exaggerated inflammatory responses and form granulomas following intradermal challenge with sterile *Aspergillus fumigatus* (AF) hyphae. In this study, we examined the efficacy of retroviral-mediated gene transfer (RMGT) into X-CGD bone marrow stem cells in preventing this abnormal inflammatory response. Sterile AF or saline was injected subcutaneously into the ears of wild-type, female X-CGD carrier, X-CGD, or X-CGD mice chimeric for varying numbers of either wild-type or RMGT-corrected neutrophils. Intradermal AF induced marked inflammation at both 3 and 30 d in the X-CGD mice, but not in the carriers or the wild-type mice. Similar to wild-type mice, chimeric X-CGD mice with $>20\%$ oxidase-positive neutrophils displayed a minimal and self-limited inflammatory response. Inflammation in chimeric (both wild-type and RMGT-corrected) mice with $<15\%$ oxidase-positive neutrophils was also improved compared to X-CGD mice, although still abnormal. This is the first evidence that partial correction of NADPH oxidase activity by gene therapy is likely to be beneficial in reducing or preventing the chronic inflammatory complications of CGD patients if sufficient numbers of RMGT-corrected neutrophils are obtained.

Key words: chronic granulomatous disease/inflammation/neutrophil/skin/transgenic knockout
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Chronic granulomatous disease (CGD) is an inherited disorder in which superoxide generation by the phagocyte NADPH oxidase is absent or markedly deficient (Dinauer, 2003). This enzyme generates highly reactive oxygen species necessary for phagocyte microbicidal activity. The most common form of CGD is X-linked (X-CGD) and results from mutations in the gp91^{phox} subunit of the NADPH oxidase flavocytochrome. CGD patients experience recurrent and often life-threatening bacterial and fungal infections from a wide variety of pathogens, predominantly *Staphylococcus aureus*, *Aspergillus* species, and gram-negative enteric bacilli. Chronic inflammatory granulomas are a distinctive hallmark of CGD, and skin granulomas are not infrequent in CGD patients (Segal *et al*, 2000). Chronic inflammatory lesions are likely to result from a combination of factors, including the incomplete degradation of cellular debris, which accumulates in the absence of respiratory burst-derived oxidants and/or a dysregulated inflammatory response (Gallin *et al*, 1983; Segal, 1996; Morgenstern *et al*, 1997; Ottonello *et al*, 2002; Petersen *et al*, 2002; Brown *et al*, 2003).

Abbreviations: AF, *Aspergillus fumigatus*; CGD, chronic granulomatous disease; RMGT, retroviral-mediated gene transfer; X-CGD, X-linked CGD

Our laboratory has established several models of granulomatous inflammation in X-CGD mice using sterilized *Aspergillus fumigatus* (AF) hyphae. Intratracheal instillation of sterilized AF into the lungs of X-CGD mice resulted in a chronic pneumonitis with granulomatous lesions, a phenomenon not observed in wild-type mice (Morgenstern *et al*, 1997). More recently, we described a novel model of cutaneous granuloma formation in murine X-CGD (Petersen *et al*, 2002). X-CGD mice injected subcutaneously with sterilized AF displayed significant acute (1–3 d) and chronic (30 d post-injection) inflammatory responses compared with wild-type and X-CGD carrier female mice. We proposed that this model may prove useful as a functional test to evaluate the efficacy of gene therapy protocols being developed for CGD.

Since CGD results from a mutation in a single gene in hematopoietic stem cells, it represents a candidate target disease for hematopoietic cell gene replacement therapy. Gene therapy for CGD could conceivably provide sufficient NADPH oxidase-reconstituted neutrophils to overcome, or at least reduce, the infectious and granulomatous complications of CGD. Retroviral-mediated gene transfer (RMGT) into CGD bone marrow cells has been shown, by our and other laboratories, to successfully correct respiratory burst oxidase activity in phagocytes *in vivo* and improve host defense against fungal and bacterial pathogens in murine models of CGD (Bjorgvinsdottir *et al*, 1997; Mardiney *et al*,

1997; Dinauer *et al*, 1999, 2001). The requirements for phenotypic correction varied with the pathogen. In general, more RMGT-corrected neutrophils were necessary to improve host defense following bacterial challenge than fungal challenge (Dinauer *et al*, 2001). The level of superoxide production per cell appeared to be a second important factor (Bjorgvinsdottir *et al*, 1997; Dinauer *et al*, 2001).

The efficacy of gene replacement therapy in ameliorating the chronic granulomatous complications of CGD have not yet been investigated. We therefore employed our model of cutaneous inflammation to address two main questions: First, how many RMGT-corrected neutrophils are necessary to prevent or reduce granulomatous inflammation induced by intradermal injection of sterile AF; and second, does the cellular level of neutrophil NADPH oxidase activity influence granuloma formation.

Results

Generation of chimeric RMGT-corrected and wild-type X-CGD mice X-CGD mice which were chimeric for varying levels of RMGT-corrected or normal neutrophils were generated by marrow transplantation, and the fraction of oxidase-positive neutrophils in each mouse determined by peripheral blood nitroblue tetrazolium (NBT) testing (Dinauer *et al*, 1999), a highly sensitive qualitative assay for NADPH oxidase activity. Note that wild-type neutrophils exhibit at least 3-fold more NADPH oxidase activity on a per-cell basis than MSCV-m91Neo-corrected neutrophils, as determined using a quantitative assay (Dinauer *et al*, 1999). Chimeric mice were stratified into groups based on the percentage of oxidase-positive neutrophils, as shown in Table I.

Acute phase (3 d) response to intradermal AF injection Three days after injection with 2.5 μg sterile AF or phosphate-buffered saline (PBS), ears were examined for clinical evidence of inflammation, as assessed by ear erythema and/or edema (Table I). No inflammation was observed in wild-type, carrier or X-CGD ears injected with PBS. As previously shown (Petersen *et al*, 2002), a 2.5 μg dose of AF did not elicit a response in the wild-type or carrier mice, but did so in all X-CGD mice. Only one of 12 chimeric mice with $>20\%$ oxidase-positive neutrophils, and none of the wild-type or carrier mice, showed any visible or palpable signs of inflammation 3 d after AF injection ($p<0.0001$, X-CGD *vs* chimeric mice with $>20\%$ oxidase-positive neutrophils). In contrast, 11 of 16 chimeric mice with $<15\%$ oxidase-positive neutrophils displayed acute inflammation of the AF-injected ear (Table I). The reduced incidence of clinical inflammation at 3 d between the group of X-CGD and chimeric mice with $<15\%$ oxidase-positive neutrophils approached statistical significance ($p=0.0525$).

The degree of ear inflammation 3 d after AF injection was quantitated by measuring the ear thickness (Fig 1*A*). As before (Petersen *et al*, 2002), X-CGD mice exhibited a ~ 2-fold increase in ear thickness 3 d after AF injection, whereas the ear thickness of wild-type mice and carrier females was unaffected. Chimeric mice with $>20\%$ NBT-positive neutrophils showed no increase in ear thickness ($p\leqslant 0.008$, chimeras *vs* X-CGD mice). In contrast, RMGT-corrected chimeric mice with $<15\%$ NBT-positive neutrophils still displayed a substantial increase in ear thickness at 3 d compared to wild-type control mice ($p=0.0004$). This measure of acute inflammation was slightly reduced compared to X-CGD mice, particularly in wild-type chimeras with $<15\%$ oxidase-positive neutrophils, which approached statistical significance compared to X-CGD mice

Table I. Mouse groupings and inflammatory reponses to intradermal sterile AF challenge

Genotype	N	% oxidase +PMN	Range	Oxidase +PMN ($\times 10^3$)	Range (# tested)	Inflammation at 3 d	Inflammation at 30 d	Inflammation by histology	Histologic grading
WT	10	100	ND	2.3 ± 1.3	1.3–3.2 (2)	0/10	0/10	0/10	0 ± 0
Carriers	6	~ 50	ND	1.2 ± 0.4	0.9–1.9 (5)	0/6	0/5	0/5	0 ± 0
X-CGD	12	0	ND	0	0 (2)	12/12[a]	12/12[a]	6/6	2.7 ± 1
Chimeras									
GTc 20%–35%	4	29.5 ± 2.4	26–31	ND	ND	0/4[b]	0/4[b]	0/4[b]	0 ± 0
GTc >35%	4	54.5 ± 8.5	43–61	ND	ND	0/4[b]	0/4[b]	0/4[b]	0 ± 0
WTc 20%–35%	4	27.8 ± 5.3	22–34	ND	ND	1/4[b]	1/4[b]	1/4[b]	0.25 ± 0.5
GTc <15%	12	10.2 ± 3.4	4–15	0.3 ± 0.2	0.05–0.6 (10)	9/12[c]	10/12[c]	1.4 ± 0.9	
WTc <15%	4	11 ± 4.2	6–16	0.3 ± 0.1	0.2–0.4 (4)	2/4[c]	2/4[c]	4/4	1.5 ± 0.6

RMGT-corrected and wild-type chimeric X-CGD mice were categorized by percentage of oxidase-positive neutrophils, as assessed by NBT testing. The number of oxidase-positive (+) neutrophils ($\times 10^3$) per μL of peripheral blood was calculated by multiplying the number of neutrophils per μl of peripheral blood by the percentage of NBT-positive neutrophils. The number of mice with clinical or histologic evidence of inflammation at 3 and 30 d, out of the total number of mice studied in each group, are as indicated. Criteria for the presence of clinical inflammation included visible erythema, edema, or ulceration, palpable boggy edema or presence of a firm papule/granuloma. Histologic evidence of inflammation in each biopsy specimen was graded on a 0–4+ scale, as described in the Materials and Methods.

[a]Total includes six X-CGD mice from our previous work (Petersen *et al*, 2002).

[b]$p\leqslant 0.0001$, X-CGD *versus* group of chimeras with $\geqslant 20\%$ oxidase-positive neutrophils.

[c]$p=0.0525$, X-CGD *versus* group of chimeras with $<15\%$ oxidase-positive neutrophils.

RMGT, retroviral-mediated gene transfer; X-CGD, X-linked chronic granulomatous disease; NBT, nitroblue tetrazolium; PMN, polymorphonuclear leukocytes; ND, not done; GTc, gene therapy chimera; WTc, wild-type chimera; WT, wild-type.

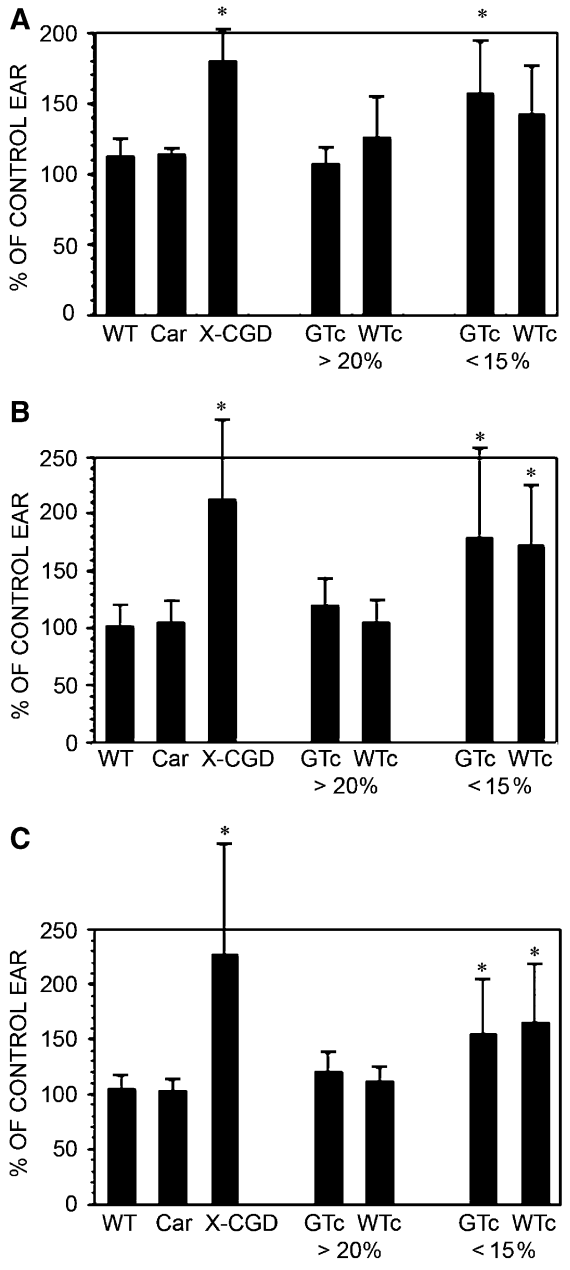

Figure 1
Transplantation with wild-type or gp91phox-transduced marrow reduces the exaggerated inflammatory response in X-linked chronic granulomatous disease (X-CGD) mice injected with intradermal *Aspergillus fumigatus* (AF). Sterile AF (2.5 μg) and phosphate-buffered saline (PBS) vehicle were injected into the dorsal ears of wild-type C57Bl6/J (WT), female X-CGD carrier (Car), X-CGD, gene therapy-corrected chimeric X-CGD (GTc), or wild-type chimeric X-CGD (WTc) mice, as described in Table I. Three days after injection inflammation was assessed by measuring ear thickness using calipers (*panel A*). Thirty days after injection the animals were sacrificed and 5 mM punch biopsies of the injection sites were obtained; the biopsy specimens were then measured for thickness (*panel B*) and weighed (*panel C*). The data are shown as the mean ± SD difference in the thickness or weight between the control PBS- and AF-injected ears. Asterisks (*) denote a statistically significant difference between WT controls and either X-CGD or X-CGD chimeric mice with <15% oxidase-positive neutrophils ($p \leqslant 0.008$, Mann–Whitney U test). Daggers (†) denote that X-CGD chimeric mice with >20% oxidase-positive neutrophils displayed significantly less inflammation compared to X-CGD control mice ($p \leqslant 0.04$, Student's *t* test).

($p = 0.057$). This suggests that the higher amount of superoxide produced per cell in wild type chimeras may reduce the degree of acute inflammation in this model.

Chronic phase (30 d) response to intradermal AF injection We next examined whether an inflammatory response was present 30 d after intradermal AF challenge. Three measures of chronic inflammation were assessed: gross examination; punch biopsy thickness and weight; and histology (Table I). In all X-CGD mice examined, a firm papule was palpable in the AF-injected ear, which was large, erythematous and ulcerated in some mice. No evidence of inflammation was palpable in the ears of wild-type or carrier mice injected with 2.5 μg of AF, as also reported previously (Petersen *et al*, 2002). A tiny, nonulcerated papule was palpable in the ear of one of the wild-type chimeric mice with 25% NBT-positive neutrophils. None of the RMGT-corrected chimeras with >20% NBT-positive neutrophils displayed clinical evidence of chronic inflammation ($p = 0.0001$, chimeric *vs* X-CGD mice). In comparison, a small nonulcerated papule was evident in nine of 12 RMGT-corrected chimeras with <15% NBT-positive neutrophils, and in two of four wild-type chimeras with <15% NBT-positive neutrophils (Table I). The reduced incidence of inflammation in chimeric mice with <15% oxidase-positive neutrophils compared with X-CGD mice approached but did not quite reach statistical significance ($p = 0.0525$).

Chimeric mice with >20% NBT-positive neutrophils showed no significant difference in ear thickness compared to wild-type mice at 30 d, in contrast to the substantial increase in ear thickness that persisted in X-CGD mice ($p \leqslant 0.03$; Fig 1*B*). At 30 d, however, ear thicknesses of both the wild-type and RMGT-corrected chimeras with <15% oxidase-positive neutrophils were still significantly greater than wild-type mice ($p = 0.0003$ and 0.014, respectively). The biopsy specimen weights mirrored the thickness measurements, as shown in Fig 1*C*). Chimeric mice with >20% NBT-positive neutrophils showed no significant increase in biopsy weight compared with wild-type mice at 30 d. In contrast, the biopsy weights of both the RMGT-corrected and wild-type chimeras with <15% oxidase-positive neutrophils were significantly greater than the wild-type controls ($p = 0.0006$ and 0.008, respectively). Biopsy weights were modestly decreased compared to the X-CGD controls, which approached statistical significance ($p = 0.08$ and 0.27, respectively).

Histologic specimens obtained 30 d after AF injection were examined for the presence or absence of chronic inflammation, and the extent of the inflammation was graded as described in the Materials and Methods. Results are shown in Table I, and representative photomicrographs are shown in Fig 2. Ears of wild-type mice injected 30 d previously with AF had a normal histologic appearance (Fig 2*A*). In marked distinction is a section of AF-injected X-CGD ear (Fig 2*B*), which shows a striking chronic inflammatory reaction with both neutrophil and mononuclear cell infiltration, as well as areas of granuloma formation, as described in our prior study (Petersen *et al*, 2002). The degree of inflammation ranged from 2+ to 4+ in the six X-CGD mice scored, with two mice having a score of 4+ inflammation. A reduced but still discernable chronic inflammatory process

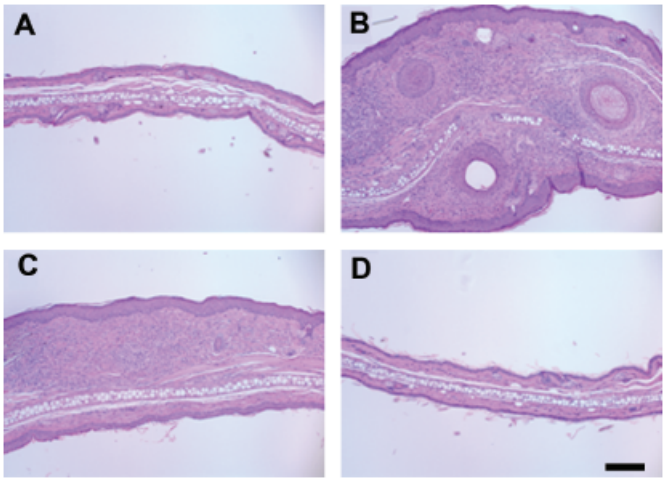

Figure 2
Transplantation with wild-type or gp91phox-transduced marrow reduces tissue inflammation and granuloma formation in X-linked chronic granulomatous disease (X-CGD) mice injected with intradermal (AF). Sterile AF (2.5 μg) and phosphate-buffered saline (PBS) vehicle were injected into the dorsal ears of wild-type C57Bl6/J, female X-CGD carrier, X-CGD, gene therapy-corrected chimeric X-CGD, or wild-type chimeric X-CGD mice. Thirty days after injection the animals were sacrificed and punch biopsies of the injection sites were obtained; after biopsy thicknesses and weights were obtained (Fig 1), biopsy specimens were processed for histologic evaluation. *Panel A* shows a representative ear biopsy section, stained with hematoxylin and eosin (H&E), from a wild-type mouse injected with AF, which displays normal histology. *Panel B* is a section from an X-CGD mouse, which shows an extensive chronic inflammatory reaction, with both neutrophilic and mononuclear cells, and evidence of fibrotic granuloma formation, as described previously (Petersen *et al*, 2002). The low-level (<15%) retroviral-mediated gene transfer (RMGT)-corrected chimera depicted in *panel C* shows evidence of chronic inflammation, but less than the X-CGD mouse section in *panel B*. Biopsies from ten of 12 low-level RMGT-corrected chimeras showed some degree of chronic inflammation as represented here; two mice, however, displayed no inflammation by histology. The ear biopsy section from a high-level (>20%) RMGT-corrected chimera (*panel D*) displays normal histology, similar to the wild-type mouse in *panel A*. *Scale bar* = 1250 μm.

was present in ten of 12 RMGT-corrected chimeric mice with <15% NBT-positive neutrophils (Fig 2*C*), and four of four wild-type chimeric mice. The small reduction in the incidence of histologic inflammation in the RMGT-corrected chimeras compared to X-CGD mice was not statistically significant ($p = 0.53$). No sections from a RMGT-corrected chimera with <15% NBT-positive neutrophils, however, displayed severe inflammation which warranted a score of 4+, and only one of 12 mice of this group was assigned a score of 3+. All four of the <15% wild-type chimeras demonstrated only focal inflammation, and were assigned scores of 1+ or 2+. In all RMGT-corrected and three of four wild-type chimeras with >20% NBT-positive neutrophils, the histologic appearance of AF-injected ears was normal at 30 d (Table I and Fig 2*D*). One wild-type chimeric mouse with 25% oxidase-positive cells had a small papule evident 3 and 30 d post-injection, with a single small focus of chronic inflammation upon histologic examination. There was otherwise no difference in the histologic appearance of biopsies obtained from wild-type chimeras *versus* RMGT-corrected chimeras with >20% oxidase-positive neutrophils.

Discussion

In this study, we investigated whether reconstitution of NADPH oxidase activity by transplantation of wild type or gene therapy-corrected bone marrow stem cells could reduce chronic inflammation and granuloma formation in murine X-CGD, using an established model in which mice are challenged with sterile AF hyphae. Achievement of only partial chimerism with RMGT-corrected cells is likely in the clinical setting (Barese *et al*, 2004). Thus, we studied mice with varying numbers of oxidase-corrected neutrophils. We found that moderate numbers of RMGT-corrected neutrophils (20%–35% NBT-positive) completely abrogated sterile AF-induced cutaneous inflammation and granuloma formation. In contrast, mice chimeric for lower levels of either RMGT-corrected (mean 10.2% ± 3.4%) or wild type (mean 11% ± 4.2%) neutrophils still exhibited exaggerated acute inflammation and persistent chronic inflammatory changes. Nevertheless, the incidence of this abnormal response was reduced compared to X-CGD mice, although this did not quite reach statistical significance, and the degree of chronic inflammation was also less severe compared to X-CGD mice, suggesting some beneficial effect. Thus, this is the first report demonstrating that partial correction of neutrophil NADPH oxidase activity by gene transfer can reduce exaggerated acute inflammation as well as the development of chronic granulomatous lesions in CGD (Figs 1 and 2), and defines a threshold for the percentage of oxidase-positive neutrophils necessary to prevent the abnormal inflammatory response observed in this model. This study further establishes a model system in which to study the clinical impact of gene therapy, as well as other immunomodulatory and anti-inflammatory strategies.

It remains unclear whether differences exist between the inflammatory responses of X-CGD mice chimeric for wild-type cells compared to RMGT-corrected cells, where cellular oxidase activity is ~3-fold lower than normal (Dinauer *et al*, 1999). Mice chimeric for >20% oxidase-positive neutrophils, either wild type or RMGT-corrected, lacked an abnormal inflammatory response to intradermal AF, similar to wild-type mice (Table I and Fig 1). We observed, however, subtle differences in the incidence and severity of inflammation 3 d after AF injection between mice chimeric for <15% RMGT-corrected compared to chimeras with wild-type neutrophils; no differences in these two groups, however, were evident at 30 d (Table I and Fig 1). Much larger numbers of mice would be required in order to demonstrate whether or not these subtle differences between wild type and gene therapy chimeras are statistically significant.

Whereas ~ 15% oxidase-positive (either RMGT-corrected or wild-type) murine neutrophils was the threshold for preventing AF-induced skin inflammation in this inbred mouse model, this boundary may not precisely translate to the human disease. The clinical severity of CGD can vary, likely due in part to disease-modifying genes (Dinauer, 2003). For example, in human CGD patients, polymorphisms in other host defense genes, such as myeloperoxidase and Fc receptor subtypes, may impact the frequency of chronic inflammatory complications, as has been reported for CGD patients with gastrointestinal inflammation (Foster *et al*, 1998). Nonetheless, our results, combined with

observations in CGD carriers and patients, outline guidelines for the partial correction of NADPH oxidase function which may reduce inflammatory as well as infectious (Dinauer *et al*, 2001) complications of CGD.

The means by which the lack of a respiratory burst results in increased inflammation and granuloma formation in CGD is not well understood. AF-induced inflammation may be due, at least in part, to the inability of the X-CGD neutrophils to efficiently degrade the hyphae material. Thus, oxidase-positive neutrophils are recruited into these lesions, but if present in insufficient numbers, cannot prevent the development of chronic lesions. Preliminary studies AF-induced chronic inflammatory skin lesions in X-CGD mice chimeric for small numbers of wild-type neutrophils indicate that the vast majority of neutrophils within the lesion are oxidase-negative (unpublished observations). Decreased apoptosis of CGD neutrophils (Ottonello *et al*, 2002; Brown *et al*, 2003), and increased levels of inflammatory mediators such as leukotriene B_4 (Petersen *et al*, 2002; Segal *et al*, 2002), may also contribute to an abnormal inflammatory response.

The sterile AF challenge model presented here may be specific only for pathogen-induced inflammation, and may not reproduce other aspects of CGD inflammation. To our knowledge, however, no other models of chronic granulomatous inflammation have been established using either the gp91^{phox}- or p47^{phox}-deficient CGD mice. The relationship of this model to the cutaneous lesions observed in women who are X-CGD carriers is also uncertain. Female carriers of X-CGD who, on average, have $\sim$50% oxidase-positive neutrophils, can exhibit a variety of skin manifestations, most commonly discoid lupus erythematosus-like lesions (reviewed in Dohil *et al* (1997) and Rupec *et al* (2000)). The pathogenesis of cutaneous lupus-like lesions in X-CGD carriers remains poorly understood, but it has been suggested that persistence of poorly degraded material may lead to aberrant activation of systemic immune, or even autoimmune, pathways (Rupec *et al*, 2000). It appears, however, that the etiology of the discoid lupus-like lesions differs from CGD granuloma formation, since female carriers of X-CGD do not develop granulomas. Moreover, if the persistence of poorly degraded material was the sole mechanism leading to discoid lupus-like lesions in CGD carriers, one would expect that CGD patients would develop discoid lupus-like lesions in greater number or in more severe forms than carriers, which they do not.

Further work is needed to elucidate the underlying mechanisms that contribute to the abnormal inflammation characteristic of chronic granulomatous disease, which may reveal novel means to downregulate the vigorous and persistent CGD inflammatory response and reduce patient morbidity. This work, however, clearly demonstrates that partial correction of phagocyte NADPH oxidase activity in a minority of cells by RMGT can be sufficient to reduce or prevent abnormal inflammatory responses in X-CGD.

Materials and Methods

Mice X-CGD (gp91$^{phox-/-}$) and carrier female (gp91$^{phox-/+}$) mice used in this study were backcrossed into the C57Bl6/J strain (Pollock *et al*, 1995; Dinauer *et al*, 1999). Wild-type C57Bl6/J mice (Jackson Lab, Bar Harbor, Maine) were maintained under pathogen-free conditions, and were fed autoclaved food and acidified water *ad libitum*. All protocols were approved by the institutional animal use and care committee at Indiana University School of Medicine.

Transduction of X-CGD bone marrow cells and transplantation of mice X-CGD marrow isolation, transduction with the MSCV-m91Neo retroviral vector and transplantation of X-CGD hosts with X-CGD or wild-type marrow cells was performed as described previously as per approved institutional protocols (Bjorgvinsdottir *et al*, 1997; Dinauer *et al*, 2001). X-CGD hosts were transplanted with a total of 2–3 $\times$ 10^6 marrow cells by tail vein injection using either MSCV-m91Neo-transduced X-CGD marrow cells (to generate high-level RMGT-corrected chimeric mice), mixtures of MSCV-m91Neo-transduced X-CGD marrow cells and fresh X-CGD marrow cells (low-level RMGT-corrected chimeric mice), or mixtures of wild-type C57Bl6/J and X-CGD marrow cells (wild-type chimeric mice). NADPH oxidase-positive neutrophils in chimeric X-CGD hosts were monitored by NBT (Sigma, St Louis, Missouri) testing of peripheral blood (Dinauer *et al*, 1999) on at least two occasions within 3 wk of sterile AF injection. Transplanted hosts were typically 4–6 mo post-transplant at the time of AF injection, and were paired with similarly aged control mice.

Subcutaneous injections and measurement of inflammation and granuloma formation Intradermal injections of sterilized AF hyphae were performed as described (Petersen *et al*, 2002). Mice were anesthetized with intraperitoneal Avertin (2.5% vol/vol in 0.9% NaCl, 0.015–0.025 mL per g body weight), and the dorsal aspect of one ear was injected with 50 μL of a solution containing 2.5 μg of AF, with the contralateral ear injected with PBS vehicle. At 3 and 30 d post-injection, all mice were examined for clinical evidence of inflammation at the injection sites. Three days after injection, ear inflammation was assessed by measuring ear thickness using spring-loaded calipers (Mitutoyo, Aurora, Illinois). At 30 d post-injection, the mice were sacrificed by Avertin overdose and CO_2 asphyxiation. A 5 mm punch biopsy was then taken of each ear, and each biopsy was weighed, measured for thickness, and processed for histologic evaluation. Biopsy specimens were evaluated for the presence or absence of inflammatory cells, and were graded on a 0–4 $+$ scale, with a score of 0 indicating no inflammation (e.g., as seen in ears injected with PBS), and a score of 4 denoting maximum inflammation observed in X-CGD controls, with the specimen being more than 2-fold thicker than a PBS-injected ear, with a neutrophil and chronic inflammatory cell infiltrate (with or without granuloma formation) present throughout the entire biopsy specimen. A minimum of four sections per biopsy specimen were examined and scored.

Statistical analyses InStat Version 3.05 for Windows (GraphPad Software, San Diego, California; www.graphpad.com) was used for all statistical analyses. The data are given as mean $\pm$ SD. The unpaired Student's *t* test (with or without Welch correction for unequal SD) was used for comparison of clinical observations (e.g., ear thickness and weight measurements) if the software calculated that the data were normally distributed; if not, the nonparametric Mann-Whitney U test was utilized. Fisher's exact test was used to determine if the incidence of inflammation between groups was significant.

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