Bone Marrow-Derived Cells Are Required for the Induction of a Pulmonary Inflammatory Response Mediated by CD40 Ligation

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The expression of inflammatory mediators by various cells following *in vitro* CD40 ligation is well known. However, knowledge of the role and interaction with these cells in the establishment and maintenance of *in vivo* immune-mediated inflammation is limited. In this report, a chimeric mouse model based on CD40 knockout and wild-type mice was used to assess the role of bone marrow (BM)-derived and non-BM-derived cells in a CD40-mediated pulmonary inflammation response. CD40+ BM-derived cells were required for initial cell recruitment, pulmonary edema, and weight loss associated with this response. The structural CD40+ non-BM-derived cells of the lung, such as fibroblasts, epithelial cells, and endothelial cells, could not by themselves establish any level of pulmonary inflammation. However, both the CD40+ BM-derived cells and the structural CD40+ non-BM-derived cells of the lung were required to maximize the level of pulmonary inflammation. Both B cells and T cells played a contributing role in macrophage recruitment and pulmonary edema but neither contributed to the inflammation-associated weight loss. These experiments indicate that CD40+ BM-derived cells were critical to the induction of pulmonary inflammation and that alveolar macrophages, B cells, and T cells contributed to selective aspects of the response. (Am J Pathol 1999, 154:919–926)

The CD40 receptor is constitutively expressed on a variety of bone marrow (BM)¹ and non-BM^{2–4} derived cells. These cell types include B cells,⁵ dendritic cells,⁶ monocytes,⁷ macrophages, epithelial cells,^{3,8} and endothelial cells.⁹ The CD40-ligand CD154 is expressed predominantly on activated CD4+ T cells in a transient manner,¹⁰ although eosinophils,^{11,12} basophils, mast cells,¹³ and CD8+ T cells¹⁴ are also capable of activation-induced CD154 expression. Repetitive CD40-CD154 interactions involving these various cells during their response to immunological stimuli result in an enhancement of their cellular activation level.^{15,16} In the context of innate immunity, this interaction triggers cytotoxic activity and inflammatory mediator production,^{7,17,18} which may con-

tribute to the immunopathology associated with autoimmune and inflammatory diseases.

The CD40-CD154 interaction is a well known critical component in the activation of adaptive immune responses.^{19,20} Of potentially equal importance, but less well defined, is the *in vivo* role of CD40-CD154 interaction in innate immunity and its association with the development of immune-mediated inflammation. *In vitro* activation of endothelial cells and lung fibroblasts by CD40 ligation results in the up-regulated expression of cellular adhesion molecules and increased production of proinflammatory cytokines.^{21,22} Ligation of CD40 on human monocytes *in vitro* results in enhanced tumoricidal activity, induction of a state of activation, and production of proinflammatory cytokines.⁷ These findings have led to speculation that CD40+ cells contribute to the activation and regulation of immune-mediated inflammation.²³ The extent to which these *in vitro* findings have been extrapolated to *in vivo* models has remained incomplete. *In vivo* modeling of CD40 involvement in inflammation is also warranted by the potential for application of a CD154 plasmid construct as a transgenic adjuvant in vaccination therapies.²⁴ Consideration must be given to the nonspecific stimulation of unrelated CD40+ cells that encounter the CD154-transfected cells. The consequences of unrestricted CD40-CD154 interactions have been demonstrated in a CD154 transgenic mouse model.²⁵ *In vivo* models to study the role of CD40-CD154 interactions in the establishment and maintenance of inflammation are needed to further understand how this interaction could impact immune-mediated inflammatory diseases.

We have recently shown that instilling soluble CD154 into the lungs of mice results in an inflammatory response similar to that which occurs during human pulmonary immune responses.²⁶ Our *in vivo* model of CD40 ligation is useful for the investigation of immune-mediated pulmonary inflammation. In this paper, we report on the involvement of different CD40+ cells in establishing and maintaining a pulmonary inflammatory response induced by a specific anti-CD40 monoclonal antibody (mAb). Our evidence demonstrates that CD40+ BM-derived cells in the lung (macrophages, B cells, and possibly dendritic cells)

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were critical to the establishment of this response, but were not by themselves capable of sustaining the full extent of the inflammation. CD40+ non-BM-derived cells of the lung (endothelial cells, epithelial cells, and fibroblasts) were not capable of establishing this response in the absence of CD40+ BM-derived cells. In the absence of B cells, macrophage recruitment and pulmonary edema associated with this CD40 ligation-dependent inflammation were diminished; they were diminished further in the absence of B and T cells. The results indicate that CD40+ BM-derived cells were critical to the establishment of CD40-induced pulmonary inflammation and that CD40+ non-BM-derived cells of the lung were also required to maximize the inflammatory response.

Materials and Methods

Mice

Male and female C57BL/6 wild-type (+/+) mice, approximately 9 weeks of age, were obtained from the Trudeau Institute animal breeding facility for use in this study. CD40^{-/-} breeder pairs were obtained from Dr. R. Geha²⁷ and bred at the Trudeau Institute animal breeding facility. Male CD40 knockout (-/-) mice used in this study were approximately 10–12 weeks of age. Breeding pairs of μ MT mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the Trudeau Institute breeding facility. Male μ MT mice used in this study were approximately 10–12 weeks of age. Ten-week-old male SCID mice on a C57BL/6 background were obtained directly from The Jackson Laboratory for this study.

Production of Anti-CD40 Antibody

The hybridoma cell line which secretes the rat IgG anti-CD40 mAb, 1C10, was obtained by permission from DNAX Corporation (Palo Alto, CA). The cell line was cultured in RPMI 1640 media (Gibco BRL, Grand Island, NY) supplemented with 3–10% fetal calf serum, 200 U/ml penicillin, 200 ug/ml streptomycin, and 20 mmol/L HEPES buffer. The cells were grown in a CellMax artificial capillary cell culture system (Spectrum Labs, Laguna Hills, CA) for three months, during which time tissue culture supernatant was recovered regularly. The antibody was purified using an AvidChrom IgPure column (Unisyn Technologies, Hopkinton, MA). The control mAb used in these procedures was a rat IgG mAb specific for horseradish peroxidase (HRPN) which was grown and purified in the same manner as the anti-CD40 mAb.

Chimeric Mice

Chimeric mice were made by reconstituting the BM compartment of +/+ or CD40^{-/-} mice following irradiation. Recipient mice were irradiated (9.5 Gy) and then immediately reconstituted by i.v. injection of 10^7 +/+ or CD40^{-/-} donor BM cells. The animals were then housed for 1 month, under sterile conditions when required, to

allow re-establishment of their BM compartment. After this time, peripheral blood samples were taken from the reconstituted animals. B cells were stained for the presence or absence of CD40 using FITC-anti-CD40 and phycoerythrin-anti-B220 (PharMingen, San Diego, CA) and analyzed by flow cytometry.

Experimental Design

Anti-CD40 mAb was given in 60-ug doses by intratracheal administration to mice under light halothane anesthesia. The mAb was given daily for 3 consecutive days and then every second day for 6 days. The mice were then exsanguinated by perforation of the abdominal aorta following deep halothane anesthetization. The difference between the starting and final body weight of each mouse was recorded as the change in body weight over the duration of the experiment. The lungs of the mice were lavaged as previously described.²⁸ Briefly, the trachea was exposed, cannulated, and washed incrementally with five 1-ml volumes of Hanks' balanced salt solution:EDTA (3 mmol/L). Using this lung lavage technique, at least 85% of the lavage fluid was retrieved. The total number of cells recovered in the lung lavage fluid was recorded and an aliquot of the cells obtained from each animal was stained using Diff-Quik (Baxter, Miami, FL). Measurements of the serum albumin levels found in the lung lavage fluids have been used as indicators of tissue injury and subsequent transudation of proteins from the bloodstream.²⁹ Serum albumin levels found in the lung lavage fluids were determined using an albumin colorimetric assay (Sigma Diagnostics, St. Louis, MO). Color absorbency was read at 630 nm and albumin concentration was reported as ug/ml of lung lavage fluid. Lung tissue sections were cut from lungs fixed in phosphate-buffered 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin for histological assessment.

Flow Cytometry

Cells obtained from lung lavage were stained with the following fluorochrome-conjugated mAbs: phycoerythrin-anti-B220, cyochrome-anti-CD4, biotinylated-anti-CD8, phycoerythrin-anti-CD44 (PharMingen, San Diego, CA), streptavidin-allophycocyanin (Caltag Laboratories, Burlingame, CA), FITC-anti-IgG, A, M (Organon Teknika, West Chester, PA), and FITC-anti-CD62L (Trudeau Institute, Saranac Lake, NY). The cells were washed and resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin-0.1% sodium azide. The cells were stained for 30 minutes at 4°C. Confinement of the analysis to the lymphocyte population was accomplished by establishing forward and side scatter settings to exclude other cell populations. Surface marker phenotypes were detected on a FACS caliber cytofluorometer (Becton Dickinson, San Jose, CA) and analyzed using CellQuest software (Becton Dickinson).

Table 1. Cellular Infiltration into Lungs of Mice Following Anti-CD40 mAb Treatment

Mice	Antibody treatment	Total macrophages ($\times 10^6$)	Total lymphocytes ($\times 10^6$)	Total PMN ($\times 10^6$)
+/+*	Anti-CD40	1.57 ± 0.79	8.19 ± 3.37	5.20 ± 1.67
CD40 ^{-/-}	Anti-CD40	$0.25 \pm 0.13^{\dagger}$	$0.01 \pm 0.01^{\dagger}$	$0.10 \pm 0.05^{\dagger}$
+/+	Anti-HRPN	0.32 ± 0.11	0.20 ± 0.08	2.10 ± 0.67
CD40 ^{-/-}	Anti-HRPN	0.66 ± 0.42	0.11 ± 0.11	2.04 ± 0.97

Measurements are means $\pm$ SD, $n = 6$ mice/group except where noted otherwise.

* $n = 5$ mice/group.

[†]Statistically significant differences were observed relative to the +/+ mice treated with anti-CD40 mAb, $P < 0.05$.

Statistics

Data are expressed as the mean $\pm$ standard deviation (SD). The results reported here are from one experiment that was representative of two independent experiments. The sample size of each group is as stated for each experiment. Differences between the designated groups were determined using Mann-Whitney rank sum analysis or a pairwise Dunn's ANOVA on the difference of rank means. Differences were considered significant if $P < 0.05$.

Results

Specificity of the Anti-CD40 mAb

The specificity of the anti-CD40 mAb and its ability to induce a pulmonary inflammatory response are shown

(Table 1). Treatment of +/+ mice with anti-CD40 mAb induced a significant cellular infiltration that was not observed in similarly treated CD40^{-/-} mice. A substantial accumulation of cells in the alveolar space as well as significant levels of perivascular and peribronchiolar cuffing were evident in the +/+ mice treated with anti-CD40 mAb (Figure 1a). Treatment of CD40^{-/-} mice with anti-CD40 mAb (Figure 1b) and treatment of +/+ mice (Figure 1c) and CD40^{-/-} mice with anti-HRPN mAb elicited none of the above indications of pulmonary histopathology. A significant transudation of serum albumin into the alveolar space (Figure 2a) and the development of a wasting syndrome characterized by body weight loss (Figure 2b) were observed only in the +/+ mice treated with anti-CD40 mAb. In fact, a slight weight gain was observed over the duration of the experiment in the CD40^{-/-} mice treated with anti-CD40 mAb and in the +/+ mice treated with the control anti-HRPN mAb.

Figure 1. Lung histopathology following anti-CD40 mAb treatment. Lung sections from (A) +/+ mice treated with anti-CD40 mAb, (B) CD40^{-/-} mice treated with anti-CD40 mAb, and (C) +/+ mice treated with control anti-HRPN mAb. Magnification, $\times 100$.

Figure 2. A: Serum albumin levels in lung lavage fluids of +/+ or CD40^{-/-} mice following treatment with anti-CD40 or anti-HRPN mAb. Statistically significant reductions were found relative to +/+ mice treated with anti-CD40 mAb, $P < 0.05$. Sample size was 5–6 mice per group. B: Change in body weight of +/+ or CD40^{-/-} mice following treatment with anti-CD40 or anti-HRPN mAb. Statistically significant difference was found relative to +/+ mice treated with anti-CD40 mAb, $P < 0.05$. Sample size was 5–6 mice per group.

Treatment of Chimeric Mice with Anti-CD40 mAb

Anti-CD40 mAb treatment of the +/+ mice that received +/+ BM-derived cells elicited an intense pulmonary cellular infiltrate (Table 2). Similar treatment of the +/+ or CD40^{-/-} mice given CD40^{-/-} BM-derived cells or of the CD40^{-/-} mice given +/+ BM-derived cells resulted in reduced pulmonary cellular infiltration levels. However, the total numbers of lymphocytes and polymorphonuclear cells (PMNs) recovered from the CD40^{-/-} mice that received +/+ BM-derived cells were significantly greater than those recovered from +/+ mice or CD40^{-/-} mice that received CD40^{-/-} BM-derived cells. Thus, the cellular infiltration was greatest in the +/+ mice given +/+ BM-derived cells, slightly elevated in the CD40^{-/-} mice given +/+ BM-derived cells, and nearly undetectable in either the +/+ mice or CD40^{-/-} mice given CD40^{-/-} BM-derived cells.

T and B cell infiltration occurred only in mice that were given +/+ BM-derived cells; the +/+ recipients had significantly more lymphocytes than the CD40^{-/-} recipients (Table 3). Little detectable lymphocyte infiltration occurred in the mice given CD40^{-/-} BM-derived cells

regardless of whether the recipients were +/+ or CD40^{-/-} mice. In mice that received +/+ cells, the level of CD8⁺ T cell infiltration was greater than that of the CD4⁺ T cells. In both the CD4⁺ and CD8⁺ T cell subsets, the activation phenotype CD44^{high}/CD62L^{low} predominated.

Significant serum albumin levels were noted in the lung lavage fluids of the +/+ mice that received +/+ BM-derived cells (Figure 3a). The only other mice to show detectable albumin levels in their lung lavage fluids were the CD40^{-/-} mice that received +/+ BM-derived cells. In a similar manner, only those mice reconstituted with +/+ BM-derived cells showed a significant loss in body weight (Figure 3b).

Treatment of μ MT and SCID Mice with Anti-CD40 mAb

Macrophage infiltration into the lungs of anti-CD40 mAb-treated μ MT and SCID mice was substantially reduced relative to similarly treated +/+ mice (Table 4). As expected, the lymphocyte infiltration level into the lungs of the anti-CD40 mAb-treated SCID mice was significantly

Table 2. Cellular Infiltration into Lungs of Wild-type/CD40 Chimeric Mice Treated with Anti-CD40 mAb

Mice	Donor BM-derived cells	Antibody treatment	Total macrophages ($\times 10^6$)	Total lymphocytes ($\times 10^6$)	Total PMN ($\times 10^6$)
+/+ mice	+/+	Anti-CD40	4.81 $\pm$ 4.54	23.70 $\pm$ 12.27	10.19 $\pm$ 4.05
	CD40 ^{-/-}	Anti-CD40	0.25 $\pm$ 0.19	0.10 $\pm$ 0.08 [†]	0.24 $\pm$ 0.15 [†]
CD40 ^{-/-} mice	+/+	Anti-CD40	0.49 $\pm$ 0.34*	3.00 $\pm$ 0.98*	3.36 $\pm$ 0.76*
	CD40 ^{-/-}	Anti-CD40	0.14 $\pm$ 0.08	0.02 $\pm$ 0.01	0.19 $\pm$ 0.07

Measurements are means $\pm$ SD, $n = 5$ to 7 mice/group.

*Statistically significant differences were observed relative to the +/+ cells $\rightarrow$ +/+ mice group, $P < 0.05$

[†]Statistically significant difference was observed relative to +/+ cells $\rightarrow$ CD40^{-/-} mice group, $P < 0.05$.

Table 3. Phenotype of Infiltrating Lymphocyte Population ($\times 10^6$) Following Anti-CD40 mAb Treatment

Mice	Donor BM-derived cells	B cells	CD4+	CD4+		CD8+	CD8+	
				CD44 ^{high}	CD62L ^{low}		CD44 ^{high}	CD62L ^{low}
+/+	+/+	7.50 $\pm$ 4.11	2.95 $\pm$ 1.65	2.61 $\pm$ 1.55	2.44 $\pm$ 1.55	6.35 $\pm$ 2.84	5.36 $\pm$ 2.50	4.51 $\pm$ 2.40
CD40 ^{-/-}	CD40 ^{-/-}	0.01 $\pm$ 0.00 [†]	0.05 $\pm$ 0.03 [†]	0.06 $\pm$ 0.03 [†]	0.05 $\pm$ 0.02 [†]	0.03 $\pm$ 0.03 [†]	0.03 $\pm$ 0.03 [†]	0.03 $\pm$ 0.02 [†]
	+/+	0.27 $\pm$ 0.11*	0.81 $\pm$ 0.19*	0.75 $\pm$ 0.19*	0.67 $\pm$ 0.20*	1.50 $\pm$ 0.53*	1.38 $\pm$ 0.51*	1.20 $\pm$ 0.50*
	CD40 ^{-/-}	0.00 $\pm$ 0.00 [†]	0.02 $\pm$ 0.01 [†]	0.02 $\pm$ 0.01 [†]	0.02 $\pm$ 0.01 [†]	0.01 $\pm$ 0.00 [†]	0.01 $\pm$ 0.00 [†]	0.01 $\pm$ 0.00 [†]

Measurements are means $\pm$ SD, $n = 5$ to 7 mice/group.

*Statistically significant differences were observed relative to the +/+ cells $\rightarrow$ +/+ mice group, $P < 0.05$

[†]Statistically significant differences were observed relative to +/+ cells $\rightarrow$ CD40^{-/-} mice group, $P < 0.05$.

reduced compared to the +/+ mice. Although the PMN influx in the anti-CD40 mAb-treated μ MT mice was significantly greater than that for the +/+ mice in this experiment, the PMN influx in the repeat experiment was equivalent among the three strains of mice. The levels of serum albumin in the lung lavage fluids of the treated μ MT and SCID mice were both significantly reduced relative to the similarly treated +/+ mice, with that for the SCID mice being significantly lower than that for the μ MT mice (Figure 4a). The +/+, μ MT, and SCID mice all lost equivalent amounts of body weight (Figure 4b).

Discussion

We recently established a model of an immune-mediated pulmonary inflammatory response based on the specific ligation of the CD40 receptor by soluble CD154.²⁶ However, difficulties in quantifying and purifying the soluble CD154 made the use of this inflammagen impractical. These difficulties were overcome by replacing the soluble CD154 with a specific anti-CD40 mAb. In the results presented here, the specificity of the anti-CD40 mAb for the CD40 receptor and its ability to induce a pulmonary inflammation response similar to that elicited by soluble CD154 were demonstrated by the treatment of +/+ and CD40^{-/-} mice with the mAb. The lack of inflammation in the CD40^{-/-} mice treated with either the anti-CD40 or

anti-HRP mAb indicated the absence of any nonspecific effects that these antibodies might possess. Histological evidence from the lung sections of +/+ mice treated with anti-CD40 mAb showed extensive perivascular and peribronchiolar cuffing as well as occlusion of the alveolar space by cellular infiltrates. These same histopathological features are evident in the lungs of a CD154 transgenic mouse model.²⁵ In our model, +/+ mice treated with the anti-CD40 mAb also displayed a substantial intra-alveolar accumulation of erythrocytes in addition to the leukocytic infiltrate (not shown). None of these histopathological features were evident in the CD40^{-/-} mice treated with the anti-CD40 mAb nor in the +/+ mice treated with the control anti-HRP mAb. Thus, the pulmonary inflammation response was induced due to specific ligation of the CD40 receptor by the anti-CD40 mAb.

The chimeric mouse model differentiates between the levels of necessity for either the CD40+ BM- or non-BM-derived cells of the lung in the induction and maintenance of a pulmonary inflammatory response. The intensity of the cellular influx, body weight loss, and albumin levels in the lung lavage fluids of +/+ mice that received +/+ BM-derived cells was significantly greater than that observed for +/+ mice that received CD40^{-/-} BM-derived cells as well as for CD40^{-/-} mice that received either +/+ or CD40^{-/-} BM-derived cells. However, the

Figure 3. A: Serum albumin levels in lung lavage fluids of chimeric mice following treatment with anti-CD40 mAb. Statistically significant differences relative to +/+ cells $\rightarrow$ +/+ mice group, $P < 0.05$, statistically significant difference relative to +/+ cells $\rightarrow$ CD40^{-/-} mice group, $P < 0.05$. Sample size was 5–7 mice per group. B: Change in body weight of chimeric mice following treatment with anti-CD40 mAb. Statistically significant differences relative to +/+ cells $\rightarrow$ +/+ mice group, $P < 0.05$, statistically significant differences relative to +/+ cells $\rightarrow$ CD40^{-/-} mice group, $P < 0.05$. Sample size was 5–7 mice per group.

Table 4. Cellular Infiltration into Lungs of Wild-type, μ MT, and SCID Mice Treated with Anti-CD40 mAb

Mice	Antibody treatment	Total macrophages ($\times 10^6$)	Total lymphocytes ($\times 10^6$)	Total PMN ($\times 10^6$)
+/+	Anti-CD40	1.80 ± 0.73	5.36 ± 2.46	1.37 ± 0.52
μ MT	Anti-CD40	1.02 ± 0.48	3.81 ± 1.83	$3.79 \pm 1.34^*$
SCID	Anti-CD40	$0.85 \pm 0.54^*$	$0.16 \pm 0.07^*$	1.99 ± 0.91

Measurements are means $\pm$ SD, $n = 7$ to 8 mice/group.

*Statistically significant differences were observed relative to the +/+ mice, $P < 0.05$.

lesser intensity of the pathological sequelae observed in the CD40 $^{-/-}$ mice that received +/+ BM-derived cells was still significantly greater than for the +/+ or CD40 $^{-/-}$ mice that received CD40 $^{-/-}$ BM-derived cells. The evidence from these BM chimeras suggested that CD40 $^{+}$ BM-derived cells such as macrophages, B cells, and, possibly, dendritic cells were responsible for the establishment of this inflammatory response. The lesser, albeit significant, response of the CD40 $^{-/-}$ mice that received +/+ BM-derived cells suggested that CD40 $^{+}$ non-BM-derived cells maximized the response despite being unable to establish it by themselves. The equivalent lack of intensity of the inflammatory responses in the +/+ and CD40 $^{-/-}$ mice that received CD40 $^{-/-}$ BM-derived cells is consistent with this hypothesis. The +/+ mice that received +/+ BM-derived cells and the CD40 $^{-/-}$ mice that received CD40 $^{-/-}$ BM-derived cells responded to the anti-CD40 mAb treatment in a manner similar to that seen with the +/+ and CD40 $^{-/-}$ mice, respectively (Table 1 versus Table 2, Figure 2a versus Figure 3a). This suggested that the irradiation and reconstitution procedure did not affect the response to the anti-CD40 mAb treatment.

The actual contribution of the CD40 $^{+}$ non-BM-derived cells of the lung to the inflammatory response may be as a source of required inflammatory mediators such as soluble cytokines or chemokines and adhesion molecules. Ligation of CD40 on fibroblasts and endothelial cells *in vitro* results in the production of these inflammatory mediators.^{18,21,22,30} The elicitation of inflammatory mediators from these CD40 $^{+}$ non-BM-derived cells could be reliant on a series of sequential signals. Soluble

cytokines and chemokines elicited from CD40-activated BM-derived cells could induce an increase in the level of expression of constitutive CD40 on non-BM-derived cells in the lung.^{15,16} The enhanced CD40 signaling, in conjunction with that by inflammatory cytokines, may in turn elicit an activated state in the CD40 $^{+}$ non-BM-derived cells. The absence of either of these signaling mechanisms could result in a diminished response or its ablation, depending on which signal is lacking.

In the CD40 $^{-/-}$ mice that received +/+ BM-derived cells, direct activation of B cells and indirect T cell activation via CD40-induced macrophage products may account for the recruitment of these cells into the alveolar space. However, the inability to induce a sufficient level of CD40-dependent expression of proinflammatory mediators from the non-BM-derived cells of the lung may have limited their recruitment. The absence of a lymphocytic infiltrate in the mice that received CD40 $^{-/-}$ BM-derived cells would suggest the inability either to activate these BM-derived cells or to produce inflammatory mediators from the CD40 $^{+}$ non-BM-derived cells in the lung. In the chimeric mice that received +/+ BM-derived cells, the characteristics of the T cell infiltrates were similar. The number of infiltrating CD8 $^{+}$ T cells was substantially greater than the number of CD4 $^{+}$ T cells. In both the CD4 $^{+}$ and CD8 $^{+}$ subsets, the majority of infiltrating cells exhibited the activation phenotype CD44^{high}/CD62L^{low}. The significance of this finding is a subject of current investigation.

The observation that significant numbers of B cells and T cells were recovered from the lung lavage fluids of mice that received +/+ BM-derived cells led to the possibility

Figure 4. A: Serum albumin levels in lung lavage fluids from +/+, μ MT, and SCID mice following anti-CD40 mAb treatment. Statistically significant reduction relative to +/+ treated mice, $P < 0.05$, statistically significant reduction relative to μ MT treated mice, $^*P < 0.05$. Sample size was 7–8 mice per group. B: Change in body weight of +/+, μ MT, and SCID mice following anti-CD40 mAb treatment. Sample size was 7–8 mice per group.

that B cells and T cells may have been actively recruited into and may have contributed to the CD40-induced response, rather than having been bystanders. In μ MT and SCID mice, the level of macrophage infiltration following treatment with anti-CD40 mAb was reduced relative to that seen in similarly treated +/+ mice. This suggested that B cells and/or T cells may have been associated with the recruitment of additional macrophages in this response. That the loss of B cells, or of both B cells and T cells, did not result in a decrease in the PMN influx suggested either that PMN recruitment was independent of lymphocyte recruitment or that it preceded lymphocyte recruitment in this response. The production of neutrophil chemoattractants by CD40 activation of resident alveolar macrophages could have been responsible for PMN recruitment. *In vitro* priming of macrophages by CD40 ligation results in the production of neutrophil chemoattractants and the up-regulation of macrophage CD40 surface expression.¹⁶ Increased CD40 expression is also observed on alveolar macrophages recovered from sarcoidosis patients.³¹ It has been suggested that chemokine products from *in vivo* primed alveolar macrophages may be directly involved in initial neutrophil recruitment in inflammatory diseases of the lung.^{32–34} In view of the evidence in the literature, our results suggest that resident and newly recruited alveolar macrophages activated by CD40 ligation were involved in recruiting PMNs into the lung during this immune-mediated pulmonary inflammation.

Evidence from the CD40 chimeras implied that the presence of +/+ BM-derived cells were critical in the induction of pulmonary edema. The significantly reduced serum albumin levels found in the lung lavage fluids of μ MT and SCID mice suggested that both B cells and T cells were involved in the edema associated with this response. That CD40-induced edema and PMN infiltration were still induced in SCID mice suggested that a cause-and-effect relationship between these cells and pulmonary damage could be associated with the CD40-mediated activation of alveolar macrophages. The priming of alveolar macrophages is associated with their production and release of neutrophil chemotactic proteins, and the pulmonary transudation of albumin in response to lung trauma.³² Our results comport with the evidence that suggests an active role for primed alveolar macrophages, in conjunction with neutrophil and lymphocyte sequestration, in the establishment and maintenance of CD40-induced pulmonary edema.

As with the development of pulmonary edema, the CD40-induced wasting syndrome was associated with the receipt of +/+ BM-derived cells by either +/+ or CD40^{–/–} mice. However, the lack of any differences in the change in body weight between the +/+, μ MT, and SCID mice suggests that T cells and B cells do not influence the CD40-induced wasting syndrome. The CD40-mediated activation of macrophages and possibly the subsequent neutrophil involvement were likely responsible for the associated weight loss in this inflammatory response.

In summary, our CD40 chimeric mouse model of immune-mediated pulmonary inflammation suggests that

an induction sequence initially involving CD40+ BM-derived cells and followed by induction of CD40+ non-BM-derived cells is required to establish and maintain the CD40-dependent inflammatory response to its full extent in the lung. The pathological sequelae associated with this response can all be linked to CD40-dependent activation of BM-derived cells. Not all of these sequelae were associated with B cell and T cell activation. However, activation of alveolar macrophages by CD40 ligation is most likely responsible for the events preceding the induction of these sequelae. The level of neutrophil recruitment in the chimeric and knockout models and their association with the intensity of each of the sequelae imply that these cells are required in the inflammatory response. The use of *in vivo* CD40-induced pulmonary inflammation models establishes a link between the cause and effect seen in *in vitro* cell activation studies and the pathological observations *in vivo* with pulmonary inflammation syndromes. Further studies on the involvement of specific cell subsets and their inducible products are underway.

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References

1. Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C, Liu YJ, Rousset F, Saeland S: The CD40 antigen and its ligand. *Annu Rev Immunol* 1994, 12:881–922
2. Fries KM, Sempowski GD, Gaspari AA, Blieden T, Looney RJ, Phipps RT: CD40 expression by human fibroblasts. *Clin Immunol Immunopathol* 1995, 77:42–51
3. Galy AH, Spits H: CD40 is functionally expressed on human thymic epithelial cells. *J Immunol* 1992, 149:775–782
4. Hollenbaugh D, Ochs HD, Noelle RJ, Ledbetter JA, and Aruffo A: The role of CD40 and its ligand in the regulation of the immune response. *Immunol Rev* 1994, 138:23–37
5. Laman JD, Claassen E, Noelle RJ: Functions of CD40 and its ligand, gp39 (CD40L). *Crit Rev Immunol* 1996, 16:59–108
6. Van Den Berg TK, Habold J, Renardel C, de Lavalette EA, Dijkstra CD, Klaus GGB: Properties of mouse CD40: differential expression of CD40 epitopes on dendritic cells and epithelial cells. *Immunology* 1996, 88:294–300
7. Alderson, MR, Armitage RJ, Tough TW, Strockbine L, Fanslow WC, Spriggs MK: CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. *J Exp Med* 1993, 178:669–674
8. Atsuta J, Sterbinsky SA, Pitt J, Schwiebert LM, Bochner BS, Schleimer RP: Phenotyping and cytokine regulation of the BEAS-2B human bronchial epithelial cell: Demonstration of inducible expression of the adhesion molecules VCAM-1 and ICAM-1. *Am J Respir Cell Mol Biol* 1997, 17:571–582
9. Hollenbaugh D, Mischel-Petty N, Edwards CP, Simon JC, Denfeld RW, Kiener PA, Aruffo A: Expression of functional CD40 by vascular endothelial cells. *J Exp Med* 1995, 182:33–40
10. Yellin MJ, Sippel K, Inghirami G, Covey LR, Lee JJ, Sinning J, Clark EA, Chess L, Lederman S: CD40 molecules induce down-modulation, and endocytosis of T cell surface T cell-B cell activating molecule/CD40L. *J Immunol* 1994, 152:598–608
11. Ohkawara Y, Lim KG, Xing Z, Glibetic M, Nakano K, Dolovich J, Croitoru K, Weller PF, Jordana M: CD40 Expression by human peripheral blood eosinophils. *J Clin Invest* 1996, 97:1761–1766

12. Gauchat JF, Henchoz S, Fattah D, Mazzei G, Aubry JP, Jomotte T, Dash L, Page K, Solari R, Aldebert D, Capron M, Dahinden C, Bonnefoy JY: CD40 ligand is functionally expressed on human eosinophils. *Eur J Immunol* 1995, 25:863–865
13. Gauchat JF, Henchoz S, Mazzei G, Aubry JP, Brunner T, Blasey H, Life P, Talabot D, Flores-Romo L, Thompson J, Kishi K, Butterfield J, Dahinden C, Bonnefoy JY: Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 1993, 365:340–343
14. Hermann P, Van-Kooten C, Gaillard C, Banchereau J, Blanchard D: CD40 ligand-positive CD8⁺ T cell clones allow B cell growth and differentiation. *Eur J Immunol* 1995, 25:2972–2977
15. Karmann K, Hughes CCW, Schechner J, Fanslow W, Pober JS: CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc Natl Acad Sci USA* 1995, 92:4342–4346
16. Kiener PA, Moran-Davis P, Rankin BM, Wahl AF, Aruffo A, Hollenbaugh D: Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. *J Immunol* 1995, 155:4917–4925
17. Sempowski GD, Chess PR, Moretti AJ, Padilla J, Phipps RP, Blieden TM: CD40 mediated activation of gingival and periodontal ligament fibroblasts. *J Periodont* 1997, 68:284–292
18. Yellin MJ, Winikoff S, Fortune SM, Baum D, Crow MK, Lederman S, Chess L: Ligation of CD40 on fibroblasts induces CD54 (ICAM-1) and CD106 (VCAM-1) up-regulation and IL-6 production and proliferation. *J Leukoc Biol* 1995, 58:209–216
19. Grewal IS, Flavell RA: CD40 and CD154 in cell-mediated immunity. *Adv Immunol* 1998, 16:111–135
20. Foy MT, Aruffo A, Bajorath J, Buhlmann JE, Noelle RJ: Immune regulation by CD40 and its ligand gp39. *Annu Rev Immunol* 1996, 14:591–617
21. Sempowski GD, Chess PR, Phipps RP: CD40 is a functional activation antigen and B7-independent T cell costimulatory molecule on normal human lung fibroblasts. *J Immunol* 1997, 158:4670–4677
22. Dechanet J, Grosset C, Taupin JL, Merville P, Banchereau J, Ripoche J, and Moreau JF: CD40 ligand stimulates proinflammatory cytokine production by human endothelial cells. *J Immunol* 1997, 159:5640–5647
23. Stout RD, Suttles J: The many roles of CD40 in cell-mediated inflammatory responses. *Immunol Today* 1996, 17:487–492
24. Mendoza RB, Cantwell MJ, Kipps TJ: Cutting edge: Immunostimulatory effects of a plasmid expressing CD40 ligand (CD154) on gene immunization. *J Immunol* 1997, 159:5777–5781
25. Clegg CH, Rulffes JT, Haugen HS, Hoggatt IH, Aruffo A, Durham SK, Farr AG, Hollenbaugh D: Thymus dysfunction and chronic inflammatory disease in gp39 transgenic mice. *Int Immunol* 1997, 9:1111–1122
26. Wiley JA, Geha R, Harmsen AG: Exogenous CD40 ligand induces a pulmonary inflammation response. *J Immunol* 1997, 158:2932–2938
27. Hollander GA, Castigli E, Kulbacki R, Su M, Burakoff SJ, Gutierrez-Ramos JC, Geha RS: Induction of alloantigen-specific tolerance by B cells from CD40-deficient mice. *Proc Natl Acad Sci USA* 1996, 93:4994–4998
28. Harmsen AG: Role of alveolar macrophages in lipopolysaccharide-induced neutrophil accumulation. *Infect Immun* 1988, 56:1858–1863
29. Pendino KJ, Meidhof TM, Heck DE, Laskin JDD, Laskin DL: Inhibition of macrophages with gadolinium chloride abrogates ozone-induced pulmonary injury and inflammatory mediator production. *Am J Respir Cell Mol Biol* 1995, 13:125–132
30. Yellin MJ, Brett J, Baum D, Matsushima A, Szabolcs M, Stern D, Chess L: Functional interactions of T cells with endothelial cells: the role of CD40L-CD40-mediated signals. *J Exp Med* 1995, 182:1857–1864
31. Nicod LP, Isler P: Alveolar macrophages in sarcoidosis coexpress high levels of CD86 (B7.2), CD40, and CD30L. *Am J Respir Cell Mol Biol* 1997, 17:91–96
32. Fan J, Marshall JC, Jimenez M, Shek PN, Zagorski J, Rotstein OD: Hemorrhagic shock primes for increased expression of cytokine-induced neutrophil chemoattractant in the lung: role in pulmonary inflammation following lipopolysaccharide. *J Immunol* 1998, 161:440–447
33. Hashimoto S, Pittet J-F, Hong K, Folkesson H, Bagby G, Kobzik L, Frevert C, Watanabe K, Tsurufuji S, Wiener-Kronish J: Depletion of alveolar macrophages decreases neutrophil chemotaxis to *Pseudomonas* airspace infections. *Am J Physiol* 1996, 270:L819–L828
34. Gupta S, Feng L, Yoshimura T, Redick J, Fu SM, Rose Jr. CE: Intra-alveolar macrophage-inflammatory peptide 2 induces rapid neutrophil localization in the lung. *Am J Respir Cell Mol Biol* 1996, 15:656–663