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Supplementary Materials for

Lymph node fibroblastic reticular cell transplants show robust therapeutic efficacy in high-mortality murine sepsis

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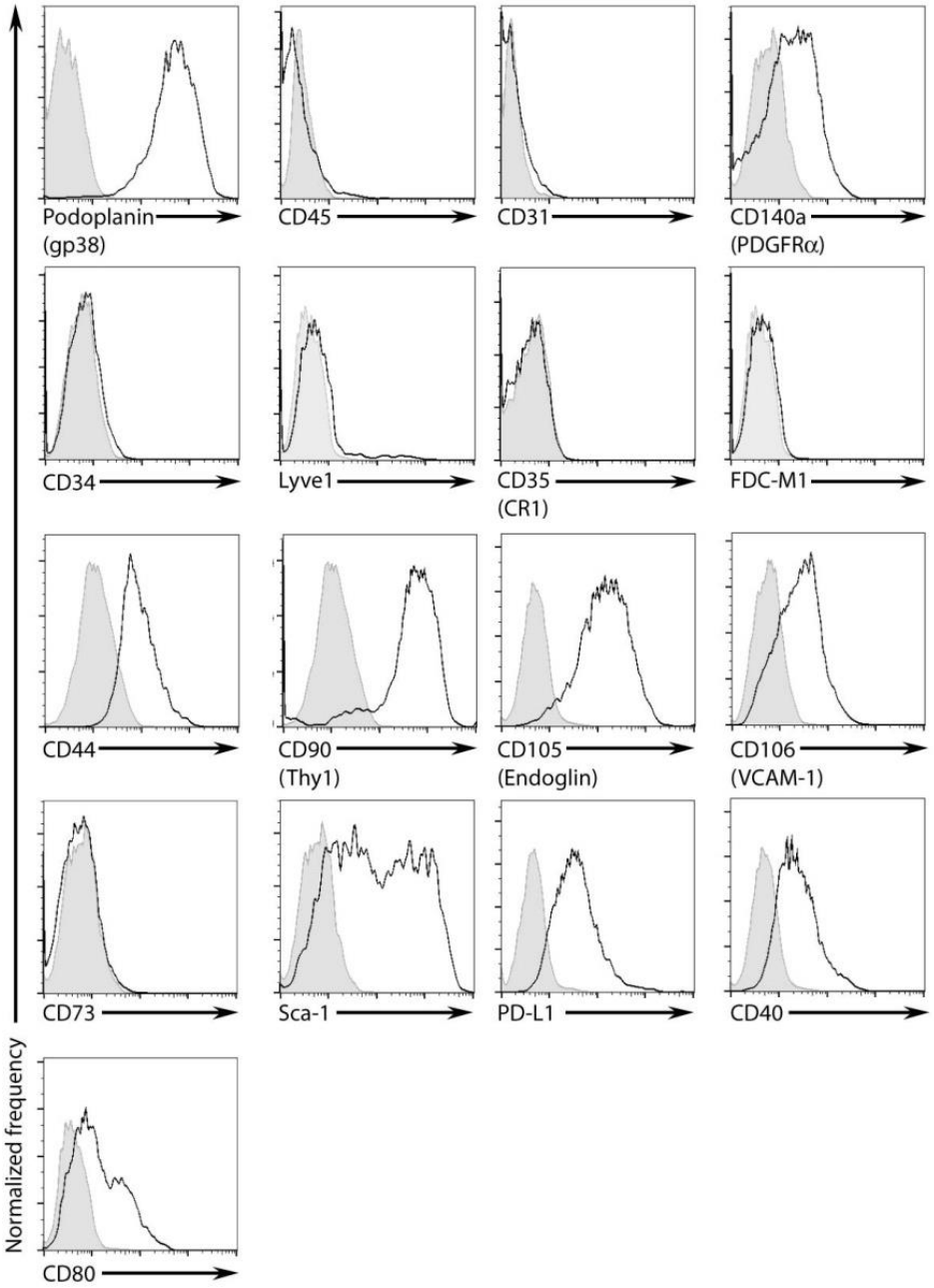
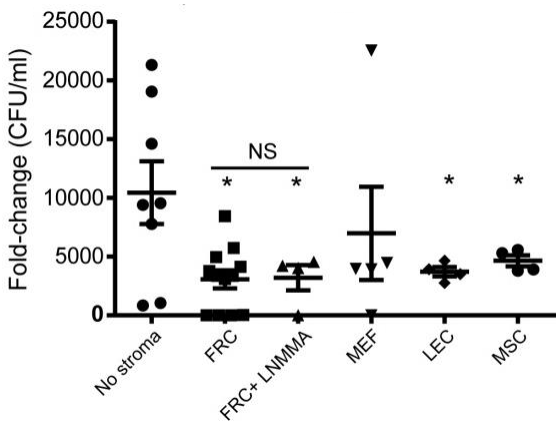
The PDF file includes:

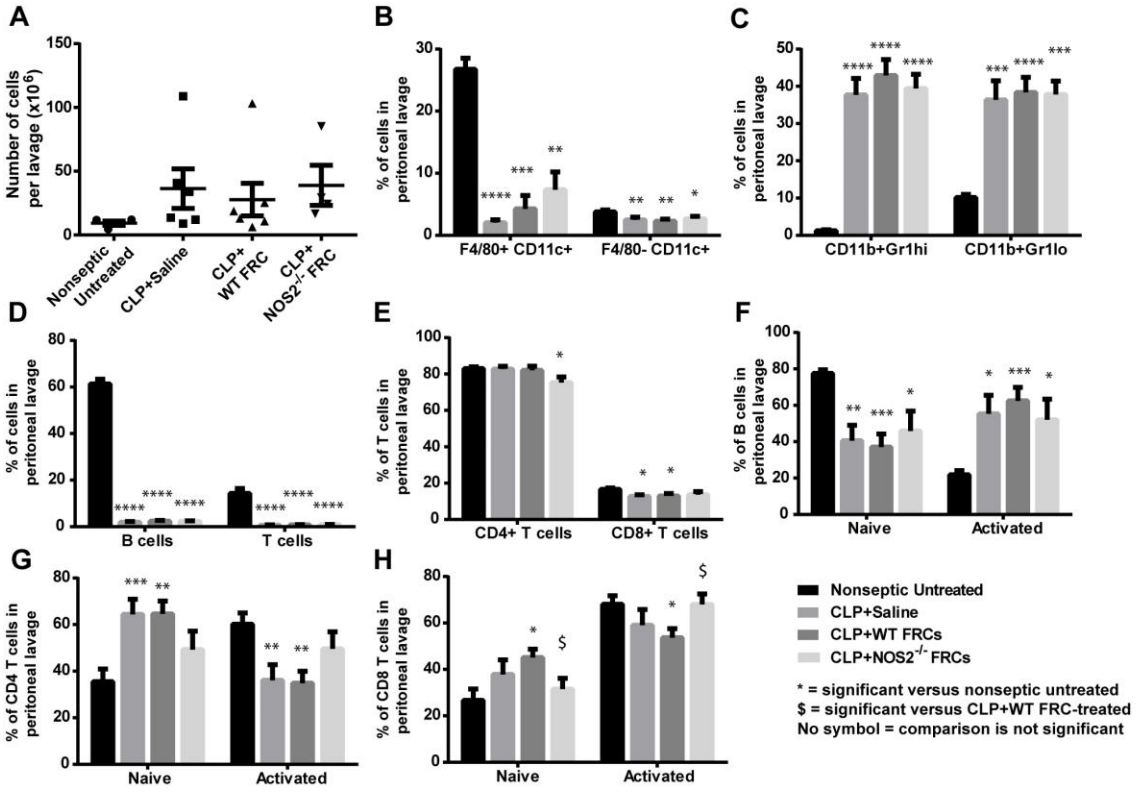
Supplementary Materials:



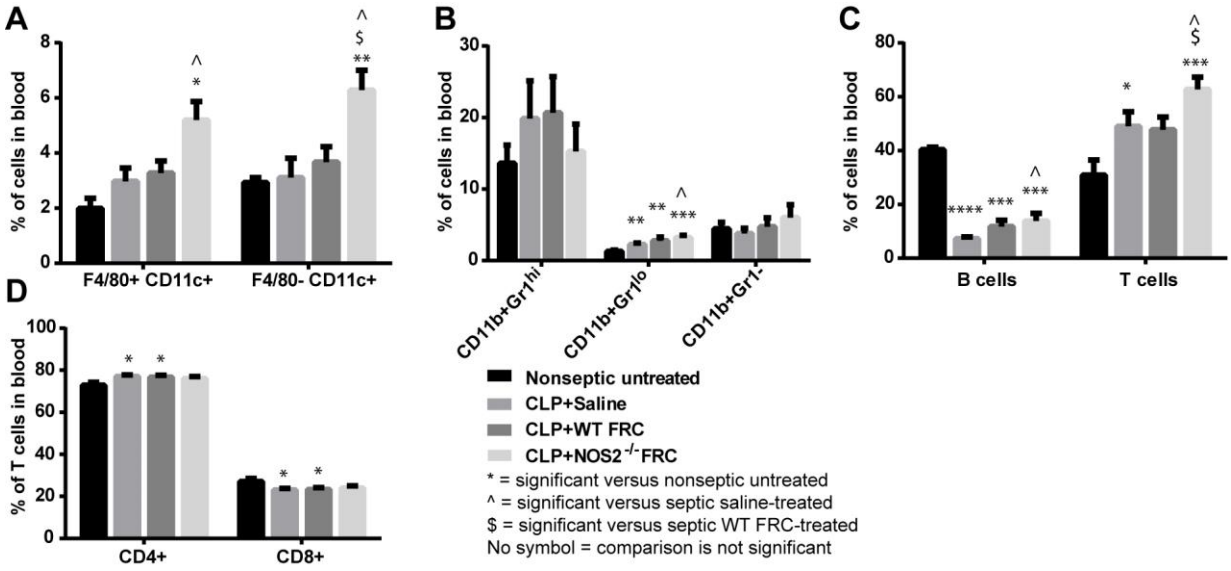
Supplementary Figure 1: Phenotype of ex vivo-expanded FRCs. FRCs were isolated from 4 to 6 week old C57Bl6 mice, enzymatically digested, and grown under standard culture conditions in complete alpha-MEM for 1 to 2 weeks before trypsin harvest, and depletion of $CD45^+$ and $CD31^+$ cells. Flow cytometric assessment was performed for various surface markers (shown with a black line) compared to relevant negative staining controls (shown with a shaded curve). Plots represent a minimum of 2 independent experiments.



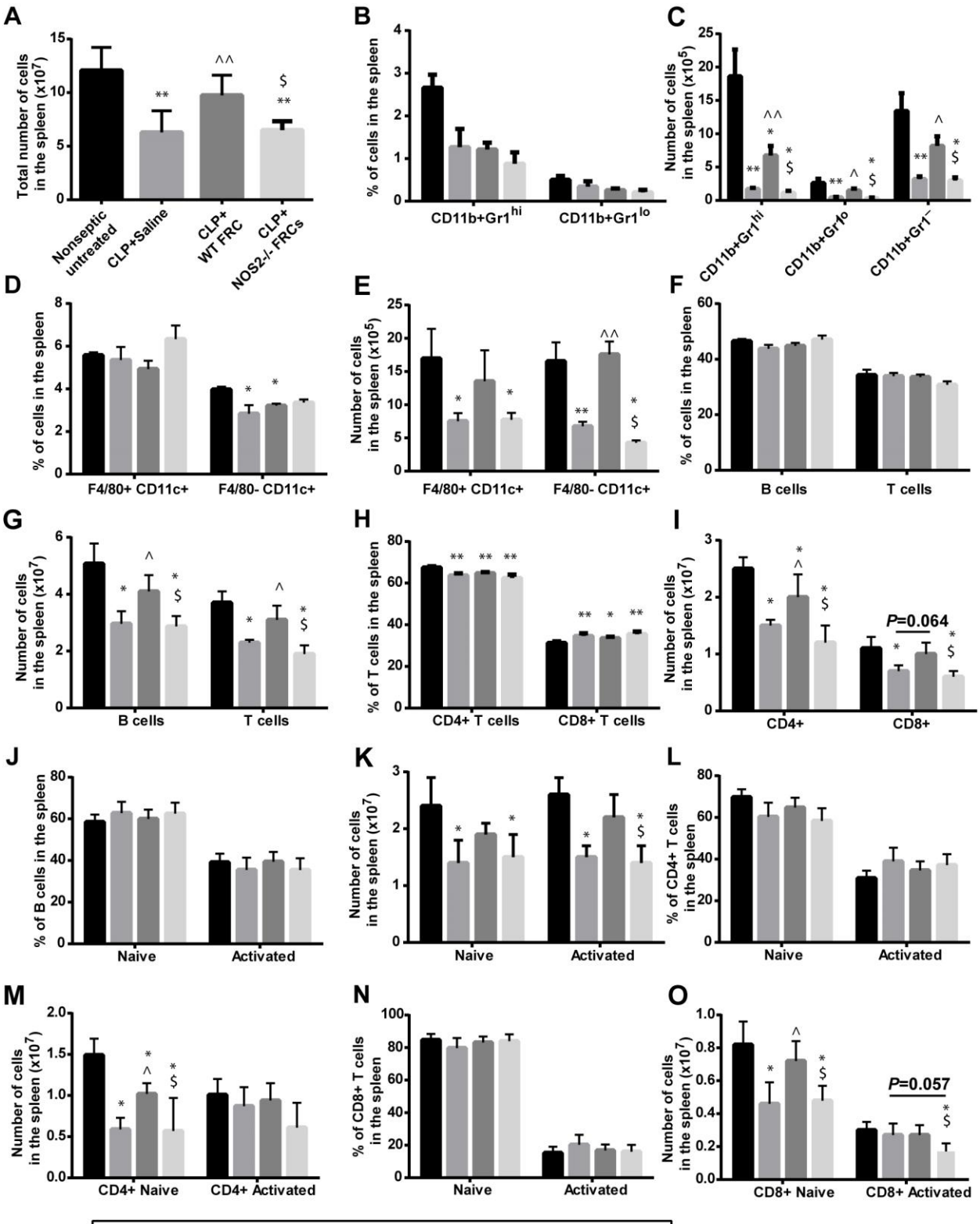
Supplementary Figure 2: FRCs inhibit growth of *E. coli* **in vitro in a NOS2-independent manner.** $5x10^4$ stroma were plated onto the upper chamber of 0.4μm 24 plate transwells (Costar) and left overnight to adhere to surface. E.coli (strain XL-1) was grown with shaking for 16 h in LB broth and added to wells of tissue culture plates. Transwells containing adherent stroma were co-cultured with *E. coli* in RPMI-1640 +10%FBS in the lower chamber for 16 h. Bacteria were counted using a standard CFU assay on LB agar plates using 5 dilutions. Fold-change in bacterial growth per sample is shown, and was calculated using the final CFU and the CFU of seed stock. **P*<0.05 for each test group compared to the no stroma control, Mann-Whitney U test. *n*= 4-12 from 5 independent experiments, mean+SEM shown.



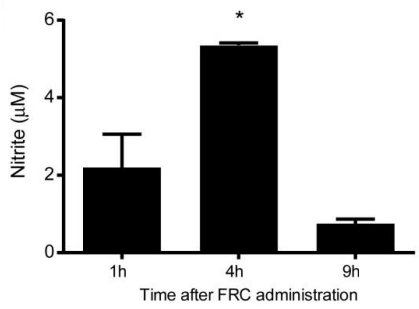
Supplementary Figure 3: Flow cytometric analysis of leukocyte subsets in peritoneal lavage fluid after FRC therapy. Balb/c mice aged 4 to 6 weeks received CLP sepsis, followed 4 h later by a single dose ($1x10^6$) of allogeneic C57Bl6-derived FRCs, $NOS2^{-/-}$ FRCs or saline (vehicle alone control). Nonseptic untreated mice are also shown. Septic mice received standard of care, including antibiotics administered from the time of FRC treatment, and fluid resuscitation after surgery. After 16 h, tissues were harvested for flow cytometric assessment, compared to nonseptic untreated controls. (**A**) Total number of peritoneal leukocytes isolated from lavage fluid is shown. (**B**) Percentage (%) of CD11c+ and F4/80+ myeloid subsets in peritoneal lavage fluid is shown. (**C**) Percentage (%) of Ly6G and CD11b-expressing subsets in peritoneal lavage fluid is shown. (**D**) Percentage (%) of leukocytes in peritoneal lavage fluid identified as B and T cells is shown; (**E**) Percentage (%) of peritoneal T cells that are $CD4^+$ or $CD8^+$ is shown; (**F**) Percentage (%) of peritoneal B cells that are naïve or activated is shown; (**G**) Percentage (%) of peritoneal $CD4^+$ T cells that are naïve or activated is shown; (**H**) Percentage (%) of peritoneal $CD8^+$ T cells that are naïve or activated is shown; Bars depict mean + SEM, and represent n=4 to 10 mice per group from 2 to 4 independent experiments. Statistical analysis was performed using a 2-tailed Mann-Whitney U test. *P<0.05 **P<0.01 ***P<0.001 ****P<0.0001, FRC-treated and saline-treated groups compared to nonseptic untreated mice. \$$P$<0.05 $NOS2^{-/-}$ FRC-treated mice compared to FRC-treated mice. Data from untreated, saline and FRC-treated groups are also depicted in fig. 4B-E.



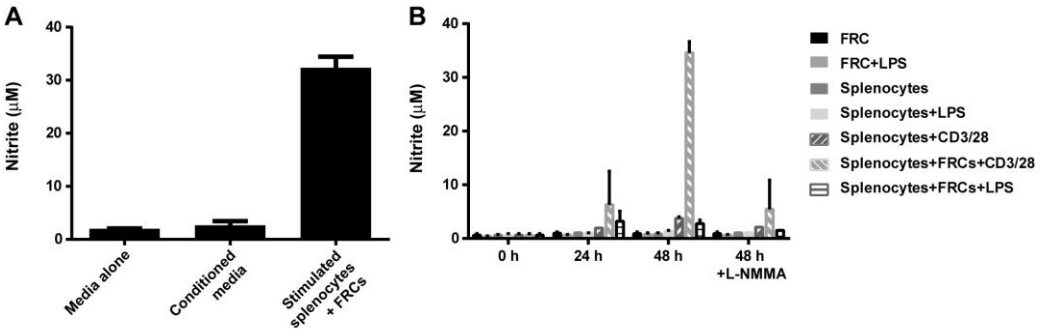
Supplementary Figure 4: Flow cytometric analysis of leukocyte subsets in blood after FRC therapy. Balb/c mice aged 4 to 6 weeks received CLP sepsis, followed 4 hours later by a single dose ($1x10^6$) of allogeneic C57Bl6-derived FRCs, $NOS2^{-/-}$ FRCs or saline (vehicle alone control). Nonseptic untreated mice are shown for comparison. Septic mice received standard of care, including antibiotics administered from the time of FRC treatment, and fluid resuscitation after surgery. After 16 hours, tissues were harvested for flow cytometric assessment, compared to nonseptic untreated controls. (**A**) Percentage (%) of blood leukocytes that are $CD11c^+$ and $F4/80^+$ myeloid subsets is shown. (**B**) Percentage (%) of $F4/80^-CD11c^-$ blood leukocytes that express Ly6G and CD11b is shown. (**C**) B and T lymphocyte subsets (%) from blood are shown. (**D**) Percentage (%) of T cell subsets that are $CD4^+$ or $CD8^+$ is shown. Bars depict mean + SEM, and represent n=4 to 10 mice per group from 2 to 4 independent experiments. Statistical significance was assessed using a 2-tailed Mann-Whitney U test. *P<0.05 **P<0.01 ***P<0.001 ****P<0.0001, FRC- and saline-treated groups compared to nonseptic untreated mice. ^P<0.05 FRC-treated groups compared to saline-treated mice. \$$P$<0.05 $NOS2^{-/-}$ FRC-treated mice compared to FRC-treated mice. Data from untreated, saline and FRC-treated groups are also depicted in fig. 5A-C.


A
Total number of cells in the spleen (x10^7)
Nonseptic untreated
CLP+Saline
CLP+ WT FRC
CLP+ NOS2-/- FRCs
B
% of cells in the spleen
CD11b+Gr1hi
CD11b+Gr1lo
C
Number of cells in the spleen (x10^5)
CD11b+Gr1hi
CD11b+Gr1lo
CD11b+Gr1–
D
% of cells in the spleen
F4/80+ CD11c+
F4/80- CD11c+
E
Number of cells in the spleen (x10^5)
F4/80+ CD11c+
F4/80- CD11c+
F
% of cells in the spleen
B cells
T cells
G
Number of cells in the spleen (x10^7)
B cells
T cells
H
% of T cells in the spleen
CD4+ T cells
CD8+ T cells
I
Number of cells in the spleen (x10^7)
P=0.064
CD4+
CD8+
J
% of B cells in the spleen
Naive
Activated
K
Number of cells in the spleen (x10^7)
Naive
Activated
L
% of CD4+ T cells in the spleen
Naive
Activated
M
Number of cells in the spleen (x10^7)
CD4+ Naive
CD4+ Activated
N
% of CD8+ T cells in the spleen
Naive
Activated
O
Number of cells in the spleen (x10^7)
P=0.057
CD8+ Naive
CD8+ Activated
Nonseptic Untreated
CLP+Saline
CLP+WT FRCs
CLP+NOS2-/- FRCs
* = significant versus nonseptic untreated
^ = significant versus septic saline-treated
$ = significant versus septic WT FRC treated
No symbol = comparison is not significant

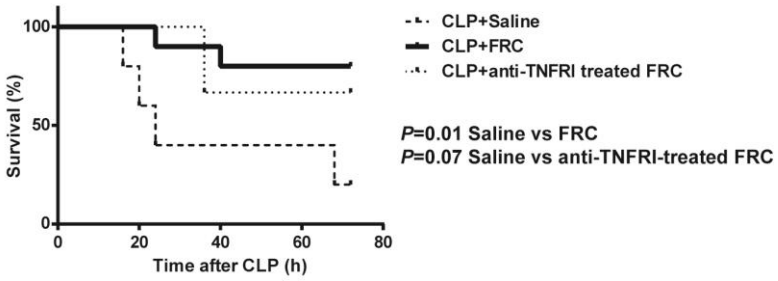
Supplementary Figure 5: Flow cytometric analysis of leukocyte subsets in spleen after FRC therapy. Balb/c mice aged 4 to 6 weeks received CLP sepsis, followed 4 hours later by a single dose ($1x10^6$) of allogeneic C57Bl6-derived FRCs, $NOS2^{-/-}$ FRCs or saline (vehicle alone control). Nonseptic untreated mice are shown for comparison. Septic mice received standard of care, including antibiotics administered from the time of FRC treatment, and fluid resuscitation after surgery. After 16 hours, tissues were harvested for flow cytometric assessment, compared to nonseptic untreated controls. (**A**) Total number of leukocytes in the spleen. (**B**) Percentage (%), and (**C**) number of myeloid cell subsets in the spleen differentiated by CD11b and Gr1 expression are shown. (**D**) Percentage (%), and (**E**) number of myeloid cell subsets in the spleen differentiated by F4/80 and CD11c expression are shown. (**F**) Percentage (%) and (**G**) number of splenic B cells and T cells are shown. (**H**) Percentage (%) and (**I**) number splenic T cells that are $CD4^+$ or $CD8^+$ are shown. (**J**) Percentage (%) and (**K**) number of splenic B cells that are naïve or activated are shown. (**L**) Percentage (%) and (**M**) number of splenic $CD4^+$ T cells that are naïve or activated are shown. (**N**) Percentage (%) and (**O**) number of splenic $CD8^+$ T cells that are naïve or activated are shown. Bars depict mean + SEM, and represent n=4 to 10 mice per group from 2 to 4 independent experiments. Statistical significance between saline and FRC treated septic mice was assessed using a 2-tailed Mann-Whitney U test. *P<0.05 **P<0.01, FRC- and saline-treated groups compared to nonseptic untreated mice. ^P<0.05 ^^P<0.01 FRC-treated groups compared to saline-treated mice. \$$P$<0.05 $NOS2^{-/-}$ FRC-treated mice compared to FRC-treated mice. Data from untreated, saline and FRC-treated groups are also depicted in fig. 5D-F.



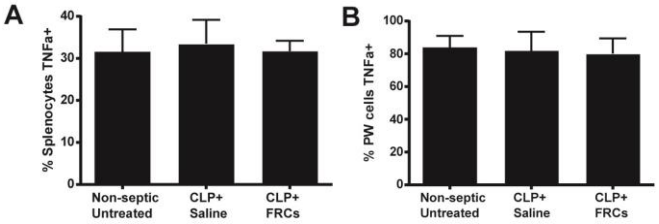
Supplementary Figure 6: Nitrite measurement in serum after FRC therapy. Balb/c mice aged 4 to 6 weeks received CLP sepsis, followed 4 h later by a single dose ($1x10^6$) of allogeneic C57Bl6-derived FRCs or saline (vehicle alone control). Septic mice received standard of care, including antibiotics administered from the time of FRC treatment, and fluid resuscitation after surgery. Untreated nonseptic mice are also shown. Septic mice received standard of care, including antibiotics administered from the time of FRC treatment, and fluid resuscitation after surgery. Mice received $1x10^6$ FRCs i.p. 4 hours after CLP surgery. Serum samples were taken at stated timepoints after FRC administration and nitrite concentration calculated and shown, using the Griess assay (Promega) according to the manufacturer's instructions. *P<0.05, Kruskal-Wallis test for non-parametric data. n=2 to 4, mean+SEM shown.



Supplementary Figure 7: FRCs do not secrete NO in vitro in response to LPS. (**A**) $5x10^4$ FRCs were plated and allowed to adhere overnight. Media was replaced and collected again after 48 h and termed conditioned media (CM). For the stimulated sample (positive control), $1x10^6$ RBC-lysed splenocytes were added to adherent FRCs together with 0.5μg/ml anti-CD3, 0.5μg/ml anti-CD28. Supernatant was collected after 48 h. Media alone was used as a negative control. Nitrite levels are shown and were measured using a Griess assay (Promega) according to the manufacturer's instructions. Mean+SEM shown from 2 independent experiments. (**B**), $5x10^4$ FRCs were plated and allowed to adhere overnight. Splenocytes were isolated from C57Bl/6 mice and red cell lysed, and $1x10^6$ leukocytes added to wells. Where appropriate, 1μg/ml LPS, 0.5μg/ml anti-CD3, 0.5μg/ml anti-CD28, and 400μM LNMMA (iNOS inhibitor) were added to wells. Supernatant was collected at timepoints indicated and frozen until required. Nitrite levels are shown and were measured using a Griess assay (Promega) according to the manufacturer's instructions. Mean+SEM shown from 2 to 4 independent experiments.



Supplementary Figure 8: TNFRI does not mediate the survival benefit seen in FRC-treated mice with CLP sepsis. Balb/c mice aged 4 to 6 weeks received CLP sepsis, followed 4 h later by a single dose ($1x10^6$) of allogeneic C57Bl6-derived FRCs pre-treated with an anti-TNFRI blocking antibody or isotype control for 20 min, washed 3 times, and injected in saline. Saline-treated (vehicle-treated) mice are shown for comparison. All septic mice received standard of care, including antibiotics administered from the time of FRC treatment, and fluid resuscitation after surgery. Mice were monitored for survival, which is shown, and groups were statistically compared using the Log-Rank (Mantel-Cox) test; n=5 to 10 from a minimum of 2 independent experiments. Data include some mice also depicted in Fig. 1D.



Supplementary Figure 9: TNFα production by restimulated peritoneal and splenic leukocytes. Balb/c mice aged 4 to 6 weeks received CLP sepsis, followed 4 h later by a single dose ($1x10^6$) of allogeneic C57Bl6-derived FRCs, or saline (vehicle alone control). Untreated (healthy non-septic) mice are shown for comparison. Septic mice received standard of care, including antibiotics administered from the time of FRC treatment, and fluid resuscitation after surgery. (**A**) Splenocytes, and (**B**) leukocytes from peritoneal lavage fluid were taken from untreated or septic mice 16 h after CLP, and stimulated in vitro with PMA/ionomycin, with GolgiStop (BD Biosciences), to capture cytokine production. % $TNF\alpha^+$ cells is shown. Data depict *n*=5 mice; mean+SD shown.

Supplementary table 1: GenBank accession number for microarray analyses of cultured FRCs.

Accession	Title
GSE60111	Culture-expanded mouse lymph node fibroblastic reticular cells
GSM1465080	FRC_LN_Cult_rep1
GSM1465081	FRC_LN_Cult_rep2