Supplemental Data

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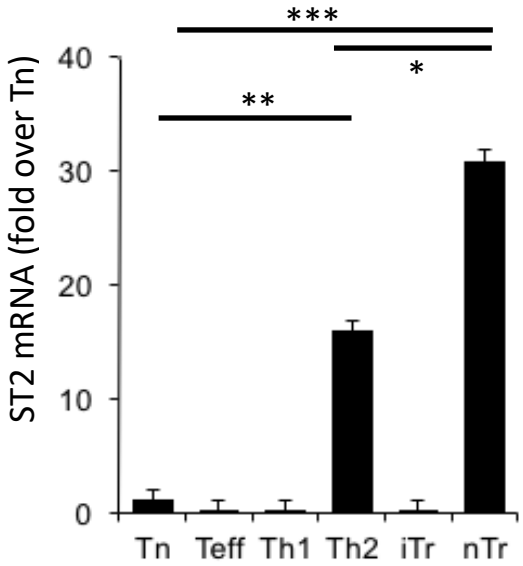
NOVEL INTERLEUKIN (IL)-2 AND IL-33 HYBRID CYTOKINE (IL233) AMELIORATES RENAL INJURY

Supplemental Figure 1.

Expression of ST2 on Tregs. Murine naïve $CD4^+$ T cells were isolated first by negative selection using the $CD4^+$ T-cell isolation kit (Miltenyi Biotech) following the manufacturer's directions, followed by Fluorescence activated cell sorting (FACS) of $CD3^+CD4^+CD44^{lo}$ cells at the UVA Flow Cytometry Core Facility. Subsequently, the naïve T-cells (Tn) cells were cultured in Complete Media (10^5cells/well in a round bottom 96-well plate) and stimulated with anti-CD3/CD28 T-cell activation beads (Invitrogen) for 4 days to generate activated T-cells (Teff) according to the manufacturer's protocol. In addition, Tn were cultured under Th1, Th2 and induced Treg (iTr) polarizing conditions as described[1] with minor modifications. Briefly, for Th1 differentiation, the Teff cultures were supplemented with IL-4 neutralizing antibody (10 μg/ml, clone 11B11, eBioscience), recombinant mouse IL-12 (rmIL-12; 10 ng/ml) and rm-interferon (IFN)-γ; 10 ng/ml, R&D Systems). For Th2 differentiation, the Teff cultures were cultured in the presence of anti-IFN-γ neutralizing antibody (10 μg/ml, clone XMG1.2, Biolegend) and rmIL-4 (10 ng/ml, R&D Systems). For iTr differentiation, the Teff cultures were supplemented with 1 ng/ml rmTGFβ and 50 ng/ml rmIL-2. The natural Tregs (nTr; $CD4^+$Foxp3-GFP^+ cells) were FACS-sorted from naïve Foxp3-gfp mice as before[2]. ST2 mRNA expression was found predominantly on Th2 cells and natural Tregs (nTr), but not on naïve (Tn), activated (Teff), Th1 T-cells or induced Tregs (iTr). The values represent fold change over Tn. P value: *<0.05; **<0.01 ***<0.001. n= 3.



Supplemental Figure 2.

Sequences of cytokines: Nucleotide and amino acid sequence for the synthetic gene and recombinant murine IL-2 (**A & B**), IL-33 (**C & D**) and IL-233 fusion cytokine (**E & F**). The coding sequences were synthesized by overlapping oligonucleotides and cloned in a modified pET44 vector (Novagen, EMD Millipore) for heterologous expression in *E. coli* as per standard molecular biology protocols. The italicized text represents IL-2 gene, underlined text represents IL-33 gene, the bold text represents linker sequence and the grey highlighted text represents coding sequence for TEV protease.

A. Synthetic gene for expression of murine IL-2:

GAATTCGAGAACCTGTACTTCCAGGGT*GCACCCACTTCAAGCTCCACTTCAAGCTCTACA*
GCGGAAGCACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGGAGCAGCTG
TTGATGGACCTACAGGAGCTCCTGAGCAGGATGGAGAATTACAGGAACCTGAAACTCCCC
AGGATGCTCACCTTCAAATTTTACTTGCCCAAGCAGGCCACAGAATTGAAAGATCTTCAG
TGCCTAGAAGATGAACTTGGACCTCTGCGGCATGTTCTGGATTTGACTCAAAGCAAAAGC
TTTCAATTGGAAGATGCTGAGAATTTCATCAGCAATATCAGAGTAACTGTTGTAAAACTA
AAGGGCTCTGACAACACATTTGAGTGCCAATTCGATGATGAGTCAGCAACTGTGGTGGAC
TTTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCATCTCAACAAGCCCTCAATAACTC
GAG

B. Amino acid sequence of the murine IL-2:

EFENLYFQG*APTSSSTSSSTAEAQQQQQQQQQQQQHLEQLLMDLQELLSRMENYRNLKLP*
RMLTFKFYLPKQATELKDLQCLEDELGPLRHVLDLTQSKSFQLEDAENFISNIRVTVVKL
KGSDNTFECQFDDESATVVDFLRRWIAFCQSIISTSPQ

C. Synthetic gene for the expression of murine IL-33:

GAATTCGAGAACCTGTACTTCCAGGGTTCTATCCAGGGTACTTCTCTGCTGACCCAGTCT
CCGGCTTCTCTGTCTACCTACAACGACCAGTCTGTTTCTTTCGTTCTGGAAAACGGTTGC
TACGTTATCAACGTTGACGACTCTGGTAAAGACCAGGAACAGGACCAGGTTCTGCTGCGT
TACTACGAATCTCCGTGCCCGGCTTCTCAGTCTGGTGACGGTGTTGACGGTAAGAAAGTT
ATGGTTAACATGTCTCCGATCAAAGACACCGACATCTGGCTGCACGCTAACGACAAAGAC
TACTCTGTTGAACTGCAACGTGGTGACGTTTCTCCGCCGGAACAGGCTTTCTTCGTTCTG
CACAAGAAATCTTCTGACTTCGTTTCTTTCGAATGCAAGAACCTGCCGGGTACTTACATC
GGTGTTAAAGACAACCAGCTCGCTCTGGTTGAAGAGAAAGACGAATCTTGCAACAACATC
ATGTTCAAACTGTCCAAAATCTAACTCGAG

D. Amino acid sequence of the murine IL-33:

EFENLYFQGSIQGTSLLTQSPASLSTYNDQSVSFVLENGCYVINVDDSGKDQEQDQVLLR
YYESPCPASQSGDGVDGKKVMVNMSPIKDTDIWLHANDKDYSVELQRGDVSPPEQAFFVL
HKKSSDFVSFECKNLPGTYIGVKDNQLALVEEKDESCNNIMFKLSKI

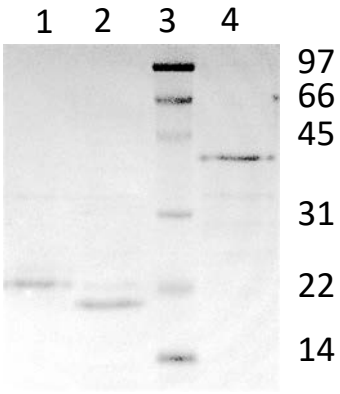
E. Synthetic gene for the expression of murine IL233 fusion protein:

GAATTCGAGAACCTGTACTTCCAGGGT*GCACCCACTTCAAGCTCCACTTCAAGCTCTACA*
GCGGAAGCACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGGAGCAGCTG
TTGATGGACCTACAGGAGCTCCTGAGCAGGATGGAGAATTACAGGAACCTGAAACTCCCC
AGGATGCTCACCTTCAAATTTTACTTGCCCAAGCAGGCCACAGAATTGAAAGATCTTCAG
TGCCTAGAAGATGAACTTGGACCTCTGCGGCATGTTCTGGATTTGACTCAAAGCAAAAGC
TTTCAATTGGAAGATGCTGAGAATTTCATCAGCAATATCAGAGTAACTGTTGTAAAACTA
AAGGGCTCTGACAACACATTTGAGTGCCAATTCGATGATGAGTCAGCAACTGTGGTGGAC
*TTTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCATCTCAACAAGCCCTCAA***GGTGGT**
GGCGGTTCTGGCGGTGGCGGTTCTGGTGGCGGTGGATCCTCTATCCAGGGTACTTCTCTG
CTGACCCAGTCTCCGGCTTCTCTGTCTACCTACAACGACCAGTCTGTTTCTTTCGTTCTG
GAAAACGGTTGCTACGTTATCAACGTTGACGACTCTGGTAAAGACCAGGAACAGGACCAG
GTTCTGCTGCGTTACTACGAATCTCCGTGCCCGGCTTCTCAGTCTGGTGACGGTGTTGAC
GGTAAGAAAGTTATGGTTAACATGTCTCCGATCAAAGACACCGACATCTGGCTGCACGCT
AACGACAAAGACTACTCTGTTGAACTGCAACGTGGTGACGTTTCTCCGCCGGAACAGGCT
TTCTTCGTTCTGCACAAGAAATCTTCTGACTTCGTTTCTTTCGAATGCAAGAACCTGCCG
GGTACTTACATCGGTGTTAAAGACAACCAGCTCGCTCTGGTTGAAGAGAAAGACGAATCT
TGCAACAACATCATGTTCAAACTGTCCAAAATCTAACTCGAG

F. Amino acid sequence of the murine IL233 fusion protein:

EFENLYFQG*APTSSSTSSSTAEAQQQQQQQQQQQQHLEQLLMDLQELLSRMENYRNLKLP*
RMLTFKFYLPKQATELKDLQCLEDELGPLRHVLDLTQSKSFQLEDAENFISNIRVTVVKL
*KGSDNTFECQFDDESATVVDFLRRWIAFCQSIISTSPQ***GGGGSGGGGSGGGGS**SIQGTSL
LTQSPASLSTYNDQSVSFVLENGCYVINVDDSGKDQEQDQVLLRYYESPCPASQSGDGVD
GKKVMVNMSPIKDTDIWLHANDKDYSVELQRGDVSPPEQAFFVLHKKSSDFVSFECKNLP
GTYIGVKDNQLALVEEKDESCNNIMFKLSKI

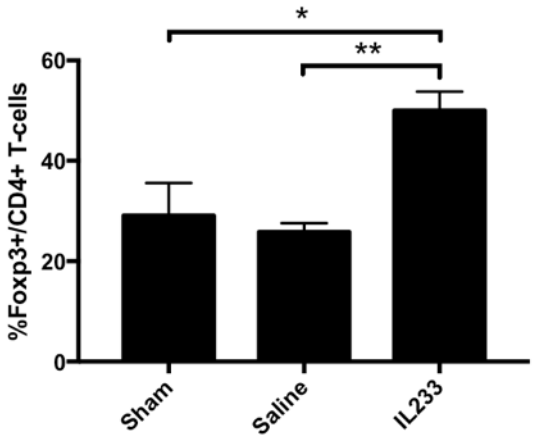
Supplemental Figure 3. Purified recombinant cytokines
Coomassie-stained 12% SDS-PAGE gel of 66 pmol of each purified recombinant protein demonstrates correct predicted molecular weights (MW). Lane 1: IL-33, Lane 2: IL-2, Lane 3: standards and Lane 4: IL233. MW indicated at right.



Supplemental Figure 4. Pretreatment with IL233 results in higher Treg proportion in the kidneys after ischemia-reperfusion injury (IRI).

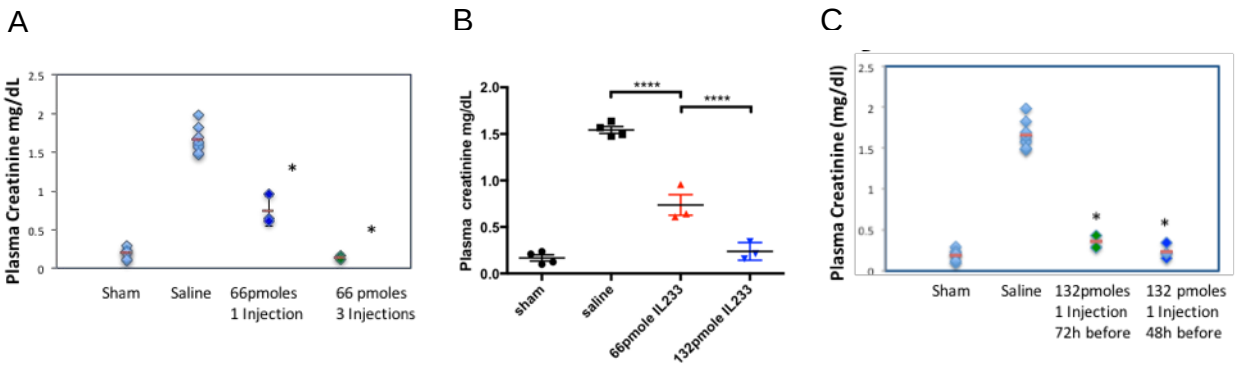
Mice were treated for 5 consecutive days with 66 pmol/mouse/day IL233 or saline (i.p.). On day 8 mice were subjected to 26 minutes of bilateral kidney ischemia followed by reperfusion for 24 hours. The kidneys were analyzed for the proportion and absolute number (Figure 4D) of Tregs. IL233 treated mice had a significantly greater proportion and numbers of $CD4^{+}Foxp3^{+}$ Tregs in the kidneys as compared to the saline-treated mice.

Values are mean ± SEM. P value: *<0.05, **<0.01. N=3 for sham, N=8 for saline and N=10 for IL233.

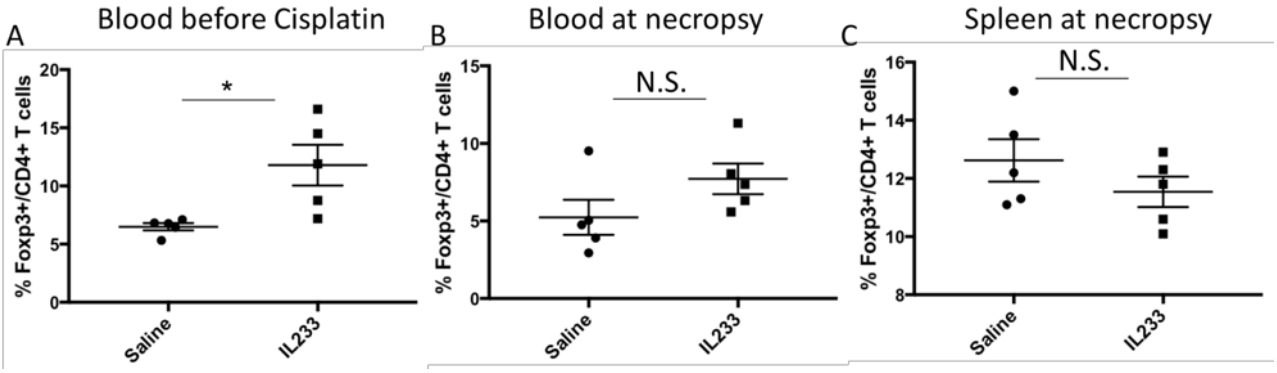


Supplemental Figure 5: Determining the lowest dose and frequency of injection for IL233-mediated protection from IRI.

A. Mice were injected with saline or 66 pmol/mouse/day of IL233 either for 1 or 3 days beginning 72 hours before conducting 26 minutes IRI, and mice were analyzed after 24 hours of reperfusion for plasma creatinine. The data show that 3 injections with IL233 before IRI was completely protective, but a single injection of 66 pmol IL233 offered partial protection. A single injection of doubling the dose of IL233 to 132 pmol/mouse completely protected the mice **(B)** when administered 48 or 72 hours before IRI **(C)**. Individual values and mean ± SEM are shown. P value: *<0.05; ****<0.0001.



Supplemental Figure 6. Treg proportion in IL233-mediated protection from cisplatin-induced nephrotoxic injury.
Mice were treated with saline or 66 pmol/day IL233 for 5 days and then injected with cisplatin 3 days after the last IL233 injection. The mice were euthanized 4 days after cisplatin treatment, and blood and spleens were analyzed for $Foxp3^{+}CD4^{+}$ T cells. Although Tregs were elevated in the blood of mice before cisplatin injection **(A)**, there was no significant difference in the proportion of Tregs in the blood **(B)** and spleens **(C)** of IL233-treated mice as compared to saline controls at necropsy on day 4 post-cisplatin treatment. **(D)** Histological analysis of H & E stained kidney sections showed variable but significantly increased areas of tubular necrosis in the saline-treated controls after cisplatin administration, but not in the IL233 mice. The tubular necrosis score is presented in Figure 7D. Scale bar = 50 μM (left) and 100 μM (right).
Symbols represent individual mice. Values are mean ± SEM. P value: N.S.>0.05, *<0.05. N=5.



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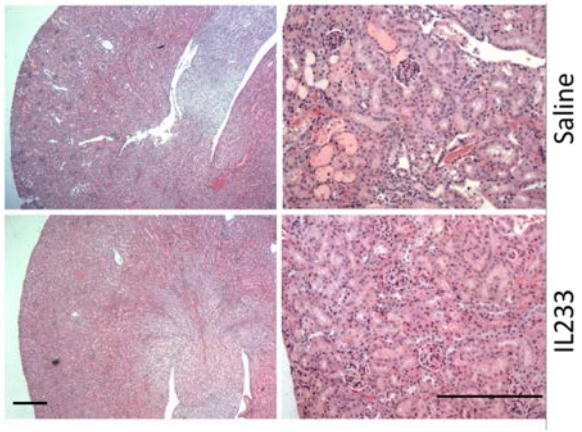
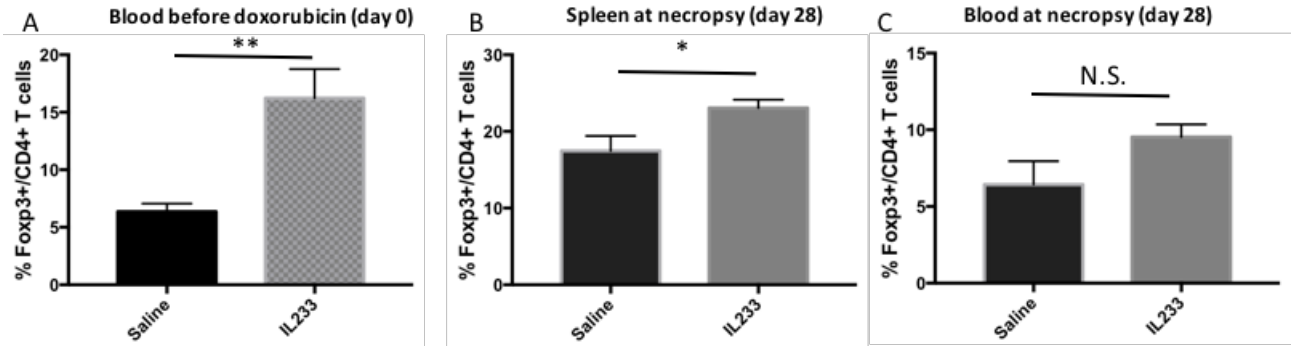
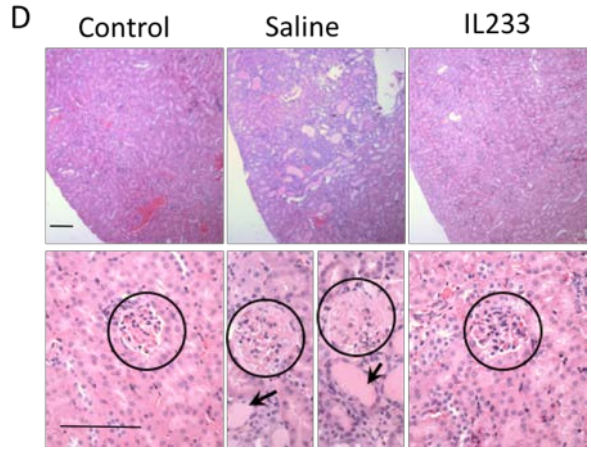


Supplemental Figure 7. Treg proportion in IL233-mediated protection from doxorubicin-induced nephrotoxic injury.

Mice were treated with saline or 66 pmol/day IL233 for 5 days and then were injected with 10 mg/kg doxorubicin 3 days after the last IL233 injection. The mice were euthanized after 4 weeks, and blood and spleens were analyzed for $Foxp3^{+}CD4^{+}$ T cells by flow cytometry. The proportion of $Foxp3^{+}CD4^{+}$ Tregs was elevated in the blood of mice before doxorubicin injection **(A)**, as well as in the spleens **(B)** of IL233-treated mice as compared to saline controls. **(C)** At necropsy, the $Foxp3^{+}CD4^{+}$ Tregs in the blood of IL233-treated mice displayed a non-significant trend in the increase in Tregs as compared to saline-treated mice. **(D)**. Histological analysis of H & E stained kidney sections mice pretreated with saline and then administered with doxorubicin showed significantly increased areas of tubular necrosis and proteinaceous casts (arrows) as well as glomerular hypertrophy, mesangial expansion (circle) as compared to saline only control, whereas the mice pre-treated with IL233 and then subjected to doxorubicin treatment did not show any such abnormalities. The quantified histopathlogical score is presented in Figure 8D. Scale bar = 50 μM. Values are mean ± SEM. P value: N.S.>0.05, *<0.05, **<0.01. N≥4.

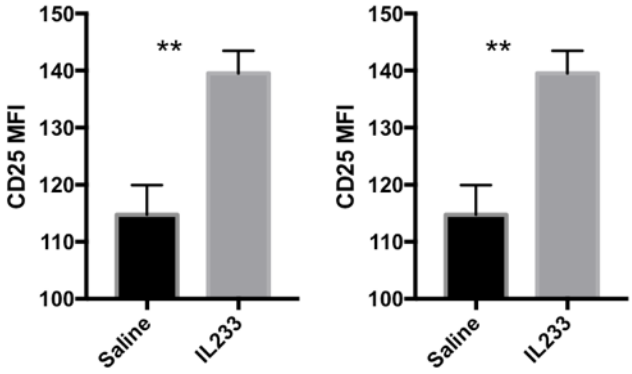




Supplemental Figure 8. Effect of IL233 treatment on IL-2 receptor (CD25) and IL-33 receptor (ST2) expression.

Mice were treated for 5 consecutive days with saline or 66 pmol/mouse/day of IL233. On day 8 mice were euthanized, and Tregs were analyzed for the expression of IL-2 receptor, α chain (CD25) and IL-33 receptor (ST2) by flow cytometry. $CD4^{+}Foxp3^{+}$ T-cells from the spleens of IL233-treated mice showed significantly higher expression of CD25 (left) and ST2 (right) as indicated by mean fluorescence intensity (MFI).

Values are mean ± SEM. P value: **<0.01. N≥8.

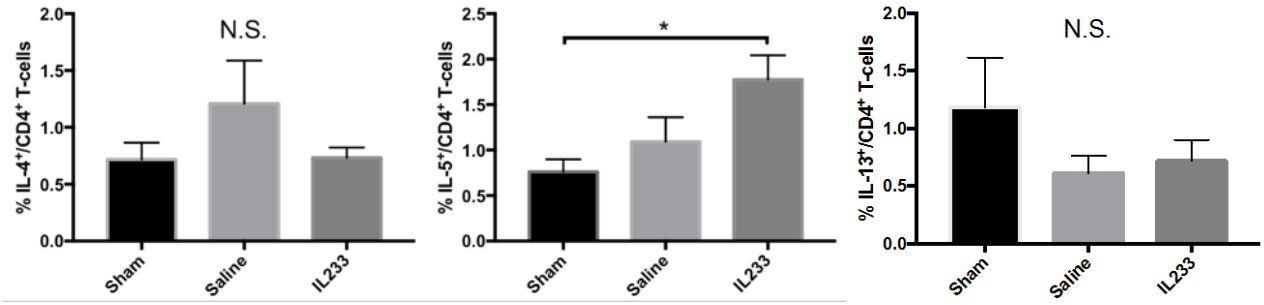


Supplemental Figure 9. IL233 treatment does not significantly influence Th2 response in mice undergoing IRI.

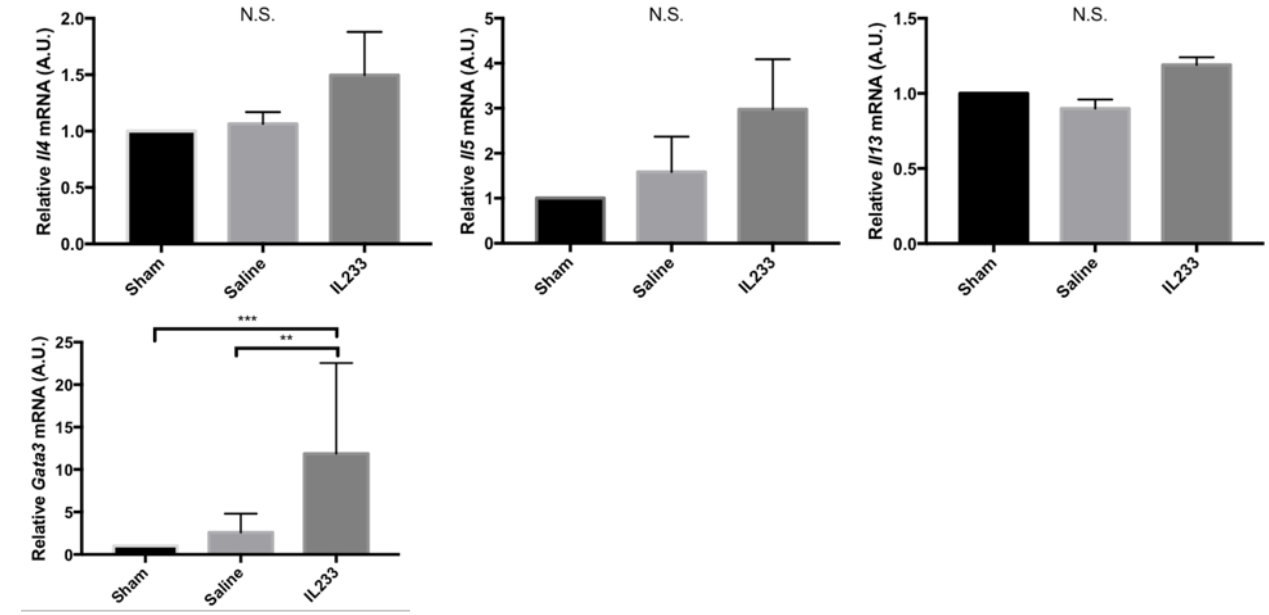
Mice were treated for 5 consecutive days with saline or 66 pmol/mouse/day IL233. On day 8 mice were subjected to 26 minutes of bilateral ischemia. After 24 hours of reperfusion, single cell suspensions of spleens were stimulated for 5 hours with phorbol myristate acetate (20 ng/ml) and ionomycin (1 μg/ml) in the presence of monensin (10 μg/ml). CD4-gated cells were analyzed for intracellular cytokine expression by flow cytometry (**A**). The data shows that compared to saline-treated mice, IL233 treatment did not significantly influence the Th2 response in terms of percent of IL-4+and IL-13+ CD4+ T-cells, although the proportion of IL-5+ T-cells was elevated in the IL233-treated mice as compared to untreated and sham-operated mice. (**B**) The kidneys of mice undergoing IRI were analyzed by semi-quantitative real-time PCR for Th2-related genes. Although there was a trend for increase in Th2 cytokine mRNA levels, it did not reach statistical significance. Interestingly, expression of *Gata3* mRNA was significantly up-regulated in the kidneys as compared to sham and saline-treated controls. However, this could also reflect an increase in ILC2, which also express *Gata3*.

Values are mean ± SEM. P value: N.S.>0.05, *<0.05, **<0.01, ***<0.001. N≥5.

A.



B.



References:

1. Vigne, S, Palmer, G, Martin, P, Lamacchia, C, Strebel, D, Rodriguez, E, Olleros, ML, Vesin, D, Garcia, I, Ronchi, F, Sallusto, F, Sims, JE, Gabay, C: IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+ T cells. *Blood,* 120**:** 3478-3487, 2012.
2. Sharma, R, Sung, SS, Ju, CY, Deshmukh, US, Fu, SM, Ju, ST: Regulatory T-Cell (Treg) hybridoma as a novel tool to study Foxp3 regulation and Treg fate. *Journal of autoimmunity,* 37**:** 113-121, 2011.