Electronic supplementary materials (ESM)

Extracellular HMGB1 Exacerbates Autoimmune Progression and Recurrence of Type 1 Diabetes by impairing Regulatory T Cell Stability

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ESM Methods

Production and purification of an HMGB1 neutralising antibody

Hybridoma cells (2E11P) capable of secreting an HMGB1 monoclonal neutralising antibody were transferred from the Vanderbilt University (Nashville, Tennessee, USA). The cell line was routinely tested and authenticated negative for mycoplasma contamination. Briefly, hybridoma cells were injected intraperitoneally into BALB/c mice to induce the large-scale production of mAb. The antibody was purified by HiTrap Protein A HP (17-0402-01; GE, Piscataway, NJ, USA). To remove any contaminated endotoxin, mAbs were passed through Pierce High-Capacity Endotoxin Removal Spin Columns (88276; Pierce Biotechnology, Rockford, IL, USA) as instructed.

Measurement of insulin autoantibodies

Serum was collected from NOD mice with new-onset diabetes before treatment randomization. IAAs were measured using a Mouse IAA ELISA Kit (ml002189-J; mlbio, Shanghai, China), and analyzed as previously described [1].

ELISA

The protein levels of TNF-α, IFN-γ, IL-1β, IL-4, IL-17A, and IL-10 were determined using
the ELISA kits obtained from BD Biosciences (San Diego, CA, USA) with previously established techniques [2]. The level of TGF-β was determined using an ELISA kit obtained from eBioscience (San Diego, CA, USA). Serum insulin was measured using a Rat/Mouse Insulin ELISA kit (EZRMI-13K; Millipore, Billerica, Merck KGaA, USA). Circulating HMGB1 levels were detected in mice and humans using a Mouse/Rat HMGB1 ELISA Kit (ARG81310; arigo Biolaboratories, Hsinchu City, China) and a Human HMGB1/HMG-1 ELISA Kit (NBP2-62766; Novus Biologicals, CO, USA), respectively.

**Histological and immunohistochemical analysis and immunofluorescence staining**

Tissue sections were subjected to H&E staining [3], immunohistochemical staining (HMGB1, CD4 and CD8) [4], and immunofluorescence staining (insulin and glucagon) [5] as previously described. Insulitis scores were assessed in a blinded fashion by two examiners as follows: 0, normal islets; 1, peri-insulitis; 2, islets with lymphocyte infiltration in <50% of the area; and 3, islets with lymphocyte infiltration in >50% of the area or completely destroyed [6].

**Flow cytometry**

To evaluate surface markers, the cells were stained in PBS containing 2% BSA and FITC-conjugated anti-CD4 (557307), APC-Cy7-conjugated anti-CD8a (557654), PE-conjugated anti-CD25 (553075), PE-conjugated anti-CD44 (553134) and APC-conjugated anti-CD62L.
antibodies. FOXP3 staining was conducted with APC-conjugated anti-FOXP3 (560401) antibodies according to the manufacturer’s instructions for the Mouse FOXP3 Buffer Set obtained from BD Biosciences (560409; San Diego, CA, USA). To analyze intracellular cytokine expression, cells were stimulated with ionomycin (1 mM), phorbol12-myristate 13-acetate (PMA) (50 ng/ml), and GolgiStop for 4–6 h. At the end of stimulation, the cells were stained with PE-Cy7-conjugated IFN-γ (505826) and PE-conjugated IL-17A (559502) antibodies according to the manufacturer’s instructions for Intracellular Staining Permeabilization Wash Buffer (421002; Biolegend, San Diego, CA, USA) [2]. The antibody against IFN-γ was purchased from eBioscience (San Diego, CA, USA), while all other antibodies were ordered from BD Biosciences (San Diego, CA, USA).

Cell purification and culture

To induce naïve CD4+ T cell differentiating into FOXP3+ cells, the isolated naïve cells (5 × 10^6 cells per ml) were seeded into plates coated with 10 ng/ml anti-CD3 and 10 ng/ml anti-CD28 antibodies, supplemented with 5 ng/ml TGF-β and 10 ng/ml IL-2 (R&D Systems, Minneapolis, MN, USA) for 3 days, and then the populations of differentiated cells were expanded for up to an additional 2 days in the presence of 5 ng/ml TGF-β and 10 ng/ml IL-2 [7]. The impact of the HMGB1–RAGE/TLRs–PI3K–AKT–mTOR axis on Treg stability was determined by applying inhibitors, including 1μM wortmannin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 4 μM perifosine (Selleck Chemicals, Houston, TX, USA), 100nM rapamycin (HY-10219; MedChemExpress, Shanghai, China),
500 nM FPS-ZM1 (HY-19370; MedChemExpress), 100 μM C29 (HY-100461; MedChemExpress), and 100nM TAK-242 (HY-11109; MedChemExpress), with the addition of human recombinant HMGB1 (rHMG1) at 500ng/ml (1690-HMB-050; R&D Systems, Minneapolis, MN, USA).

**Methylation analysis of TSDR**

Briefly, enriched Tregs were cultured with anti-CD3/CD28 and IL-2 for 3 days in the presence of 500ng/ml rHMGB1 or not, and genomic DNA was prepared by a DNeasy Blood and Tissue Kit (69504; Qiagen, Redwood, CA, USA). Bisulfite conversion of DNA was conducted with a Methylamp™ DNA Modification Kit (P-1001-1; Epigentek, Farmingdale, NY, USA) as instructed. Intron 1 of Foxp3 (corresponding to CNS2) was amplified with a primer set as described (forward, 5′-TAT TTT TTT GGG TTT TGG GAT ATT A-3′ and reverse, 5′-AAC CAA CCA ACT TCC TAC ACT ATC TAT-3′). PCR products were ligated into pGEM-T Easy vectors (A1380; Promega, Madison, WI, USA) and sequenced (10 sequences per sample).

**Real-time PCR and Western blot analysis**

Total RNAs were extracted from Tregs using the RNAiso plus reagents (9109; TaKaRa, Japan) as instructed. Real-time PCR was conducted to assess Treg function-related genes using an ABI prism 7500 Sequence Detection System (Applied Biosystems, San Francisco,
CA, USA). Actb was used for normalization, and the relative expression levels for each target gene were calculated using the $2^{\Delta\Delta Ct}$ method.

For Western blot analysis, Treg lysates were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% nonfat milk for 1 h and then incubated with primary antibodies overnight at 4°C. Antibody against RAGE (1:1000 dilution, ab3611) were purchased from Abcam (Cambridge, MA, USA); antibodies against TLR2 (1:100 dilution, sc-10739), TLR4 (1:100 dilution, sc-16240), and GAPDH (1:100 dilution, sc-25778) were ordered from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and antibodies against PI3K (1:1000 dilution, 4257s), phosphorylated PI3K (p-PI3K, Tyr458) (1:1000 dilution, 4228s), AKT (1:1000 dilution, 4685s), phosphorylated AKT (p-AKT, Ser473) (1:1000 dilution, 4060s), mTOR (1:1000 dilution, 2983s), and phosphorylated mTOR (1:1000 dilution, 2971s) were derived from Cell Signaling Technology (Danvers, MA, USA). After incubation with an HRP-conjugated secondary antibody, the reactive bands were visualized using an enhanced chemiluminescence system as reported [8].

**In vitro suppression assays and T cell-transfer model of colitis**

In vitro suppression assays:

CD4⁺CD25⁻ cells (conventional T cells) and CD4⁺CD25⁺ cells were purified to obtain
responder cells and Tregs, respectively. Tregs were preincubated with rHMGB1 (500 ng/ml) for 24 h. CFSE-labeled CD4^+CD25^- cells were cultured in triplicates in round-bottom 96-well plates along with anti-CD3/CD28 stimulation. The inhibitory effect of Tregs on the proliferation of responder cells was tested by directly addition different ratios of Tregs into 2 × 10^5 responder cells. After 3 days of co-culture, cell proliferation was assessed by flow cytometry following CFSE dilution.

T cell-transfer model of colitis:

Colitis in NOD-scid mice was induced. Briefly, CD4^+CD25^+ Tregs and naïve CD4^+CD44^low/^CD62L^+CD25^- T cells from NOD mice were prepared with isolation kits. Tregs were preincubated with or without rHMGB1 (500ng/ml) for 24 h and then washed in sterile PBS. Each NOD-scid recipient mice was given by intravenous injection of 2 × 10^5 naïve T cells alone or together with 5 × 10^4 Tregs. Body weight of mice was monitored weekly. The severity of colonic inflammation was graded by two pathologists in a blinded fashion using well-described four-point scales [9]: 0, normal tissue; 1, one or a few multifocal mononuclear cell infiltration in the lamina propria, along with minimal epithelial hyperplasia and slight to no mucus depletion from goblet cells; 2, more frequent lesions, and typical changes including several multifocal, mild inflammatory cell infiltration in the lamina propria. Mucin depletion and mild epithelial hyperplasia are also present. Inflammation rarely involves the submucosa and small epithelial erosions are occasionally observed; 3, lesions are more frequent or involve a large area of the mucosa. Inflammation is moderate and
usually involves the submucosa but is rarely transmural. Crypt abscesses are sometimes seen, and inflammatory cells are a mixture. Moderate epithelial hyperplasia and mucin depletion are present. Ulcers are accidently observed; lesions often involve majority of the intestinal section. Inflammation is intense, and is sometimes transmural. Few mucin-containing cells and epithelial hyperplasia characterized by crowding of epithelial cells in elongated glands are present. Ulcers and crypt abscesses are seen and foci of fibrinoid necrosis are present in the submucosa contiguous to crypt abscesses and ulcerations.
ESM Results

Purification and confirmation of the HMGB1 neutralising antibody

We first purified the HMGB1-neutralising antibody from peritoneal dropsy obtained from BALB/c mice using protein A columns. The purified antibody was further passed over polylysine columns to remove any contaminating endotoxin. The purity of the antibody was high as determined by SDS-PAGE (ESM Fig. 1a). The purified monoclonal antibody showed high specificity and titers against HMGB1, as shown on the western blots (ESM Fig. 1b). To test its neutralising potency, rHMGB1 was added into the RAW264.7 cell cultures, which increased TNF-α secretion by 20-fold, while addition of above purified antibody 2 h before rHMGB1 stimulation, TNF-α secretion was reduced by 3-fold (ESM Fig. 1c), indicating that the neutralising antibody is highly potent to block the HMGB1 stimulatory effect.
References:

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| Gene | Forward (5’-3’) | Reverse (5’-3’) |
|------|----------------|----------------|
| Ctl4 | GCTTCCTAGATTACCCCTTCC | CGGGCATGGTTCTGGATCA |
| Pdcd1 | ACCCTGGTCATTCCTTGGG | CATTGCTCCTCTGACACTG |
| Tgfβ1 | CTCCGTCGCTGTCTAGTGC | GCCTAGTTTGGACAGGATG |
| Il10 | GCTCTTACTGACTGGCATGAG | GCAGCCTCTAGGACAGGATG |
| Il12a | CTGTGCCTTGGTAGCATCTAG | GCAGAGTCTCGCCATTATGAC |
| Ebi3 | CTTACAGGCTCGGTGTGGG | GTGACATTAGCATGTAGGGA |
| Actb | GGCTGTATTCCTCCCTCATG | CCAGTTGGTAACAATGCCAT |
| ESM Table 2  Clinical characteristics of the participants |
|---------------------------------------------------------|
| Control participants | Participants with T1D |
| Number | 34 | 30 |
| Age (years) | 29.47±2.190 | 19.29±3.118 |
| Sex (M/F) | 11/23 | 18/12 |
| HbA1c (mmol/mol) | 37.57±3.176 | 83.29±12.99 |
| HbA1c (%) | 5.586±0.2907 | 9.759±1.184 |
| FPG (mmol/l) | 5.916±0.1576 | 8.410±1.158 |
| 2HG (mmol/l) | 6.114±0.5822 | 25.44±1.637 |
| FPI (pmol/l) | 68.92±9.16 | 15.33±7.21 |
| 2HI (pmol/l) | 258.63±42.66 | 23.06±14.69 |
| Diabetes duration (months) | NA | 6.4±2.9 |
| Number of subjects with IAA⁺ | NA | 19 |
| Number of subjects with GADA65⁺ | NA | 17 |
| Number of subjects with IAA⁺GADA65⁺ | NA | 6 |

HbA1c, glycosylated hemoglobin; FPG, fasting plasma glucose; 2HG, 2-hour postprandial blood glucose; FPI, fasting plasma insulin; 2HI, 2-hour postprandial blood insulin.
ESM Figures

ESM Fig. 1 HMGB1-neutralising antibody was produced from peritoneal dropsy. (a) Coomassie staining of the anti-HMGB1 antibody purified through HiTrap Protein A HP. (b) Western blot analysis of anti-HMGB1 antibody purified through HiTrap Protein A HP. (c) RAW264.7 cells were used to test the neutralising effect of the antibody. rHMGB1-stimulated RAW264.7 cells secreted high levels of TNF-α, whereas blockade of HMGB1 by the neutralising antibody significantly inhibited TNF-α secretion. All in vitro studies were conducted 3 times. Values are expressed as mean ± SEM. Statistical difference was analysed by unpaired Student’s t test; ***p<0.001. MW, molecular weight
ESM Fig. 2 No functional beta cells were found in recipient pancreatic tissues of NOD females 4 weeks after they got diabetes. The figure is representative of 3 mice studied. Scale bar, 100μm. Original magnification ×200

ESM Fig. 3 Cytokine production by PLN mononuclear cells was detected after anti-CD3 mAb stimulation in vitro. n=5 in each study group. Values are expressed as mean ± SEM. Statistical difference was analysed by unpaired Student’s t test
ESM Fig. 4 The purity of isolated naïve CD4⁺ T cells (a) and CD4⁺CD25⁺ Tregs (b) from the spleen of NOD mice by MACS
ESM Fig. 5 The transgenic reporter mice were generated using the indicated strategy. There is a Cre reporter allele that has a $\text{loxP}$-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato) inserted into the Gt(ROSA)26Sor locus in 007914-B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$ J mice. We cross the 008694-NOD/ShiLt-Tg(Foxp3-EGFP/cre)1cJbs/J mice with the 007914-B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$ J mice. In the resultant mice, Foxp3-GFP-Cre$^+$ Tregs excise the $\text{loxP}$-flanked STOP cassette to drive constitutive transcription of the gene encoding RFP from the R26 promoter, which permanently marks the FOXP3$^+$ T cells and their progeny.
**ESM Fig. 6** Representative FACS plots (a) and frequencies (b) of CD44 and CD62L expression by CD4\(^+\) T cells in colon lamina propria 9 weeks after adoptive transfer. 

\(n=4\)/group. Values are expressed as mean ± SEM. Statistical difference was analysed by unpaired Student’s \(t\) test.
ESM Fig. 7 The frequencies of Tregs were shown in the indicated conditions. (a) Frequencies of FOXP3 expression by CD4⁺ PBMC from diabetic (n=7) and control participants (n=4). (b) Percentages of FOXP3⁺ cells within the CD4⁺ PBMC during incubation of HC-PBMC with HC-serum or T1D-serum in the presence of HMGB1 neutralising antibody or not. n=5 per group. Values are expressed as mean ± SEM. Statistical difference was analysed by unpaired Student’s t-test. HC, healthy control; T1D, type 1 diabetes.