Introduction

Both in vitro and in vivo approaches are used for monoclonal antibody development. In vitro approaches, such as phage display or ribosomal display, select antibody sequences from an immunoglobulin variable chain cDNA library, while in vivo approaches use immunized animals as hosts and screen for monoclonal antibodies with conventional hybridoma techniques. Since the animal immune system is designed by nature for high affinity and highly-specific antibody development, the in vitro approach is obviously more cost effective than the in vivo approach.

Tolerance – the ability of the immune system to prevent responses to self antigens – makes it difficult to generate a strong immune response in mice with a mouse self-antigen or highly conserved human antigen [1]. Currently, specific knockout mice are used to overcome the immune tolerance associated with self-antigens. Generation of knockout mice for every mouse antigen is not very immunogenic in mice, making it difficult to generate antibodies using a conventional approach.

High titers of serum antibodies reacting to self-antigens are found in mouse human SLE-like models (NZB/W and MRL/lpr mice) without prior immunization with the corresponding self-antigens [3,4]. In fact, auto-immune NZB mice have been used successfully to generate antibodies against carbohydrate determinants in myelin-associated glycoprotein [5], capsular polysaccharides in group B Neisseria meningitides [6], and glycosphingolipid asialo-GM1 [7]. Recently, monoclonal antibodies against the highly conserved bovine recombinant prion protein have also been generated using NZB/W mice [8]. However, due to the multi-specificity and low affinity of auto-antibodies from NZB/W mice, there are still doubts whether therapeutic antibodies with high affinity and high specificity, as well as the desired biological activities, can be obtained from this type of mouse.

In this report, three pro-inflammatory cytokines, TNF-alpha, MIF and HMGB1 were used as test antigens in our efforts to exploit a new method to generate antibodies against highly conserved antigens. All three have been implicated as good drug targets for inflammation related diseases [9,10,11]. Human MIF and HMGB1 are representatives of highly conserved proteins and mouse TNF-alpha represents mouse self antigens. Our results demonstrate that monoclonal antibodies with high affinity and high specificity can be generated from NZB/W mice and that some of these antibodies possess neutralizing activity which is very useful in target validation and therapeutic antibody development.
Methods

Ethics Statement
Maintenance of mice and experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences.

Recombinant protein expression
Human MIF and mouse TNF-alpha were cloned into a PET-24a vector (Novagen) and expressed in E.coli strain BL21(DE3) and purified by affinity chromatography using Ni-NTA His bind resins (Novagen) according to the manufacturer’s instructions. All GST-tagged HMGB1 constructs were cloned into the expression vector pET41a, expressed in E.coli strain BL21(DE3) and purified with GST•BindTM Purification Kits (Novagen) according to the manufacturer’s protocol.

Immunization and hybridoma selection
Female BALB/c and NZB/W mice (12 weeks old) were injected subcutaneously with 50 μg of purified recombinant protein emulsified in complete Freund’s adjuvant. Two additional injections of 50 μg of antigen emulsified in incomplete Freund’s adjuvant were followed at bi-weekly intervals starting four weeks after the first immunization. Ten days after the second boost, the serum antibody titer was tested using ELISA. Two weeks after the second boost, the mice were given a final booster injection intraperitoneally with 50 μg protein. Three days after the last injection, spleen cells from the immunized mice were fused with myeloma Sp2/0 cells [12]. ELISA was employed for screening antigen-specific monoclonal antibodies.

ELISA
Antigen (10 μg/ml) was coated on microtiter plates overnight at 4°C. 100 μl of antisera diluted in PBST was added and plates were incubated for 2 hours at room temperature (RT). After washing, a 1:1000 dilution of HRP-conjugated goat anti-mouse Ig polyclonal antibody (R&D Systems) was added for 1 hour at RT. 100 μl substrate (TMB system) was added and plates were read at 450 nm. For dissociated constant (Kd) measurement, purified monoclonal antibodies were used as the primary antibody with a two-fold serial titration. The Kd was determined as the antibody concentration that can achieve 50% of the maximum ELISA reading. For antibody isotype characterization, HRP-conjugated rat anti-mouse IgG1, IgG2a or IgG2b monoclonal antibodies (BD reading. For antibody isotype characterization, HRP-conjugated) were added for 1 hour at RT. 100 μl of the RT reaction. 40 cycles of quantitative PCR was performed in a Rotor-Gene 6000 real time PCR machine (Corbett). Primers used in the IL-6 mRNA RT-PCR assay were as follows:

Mouse IL-6 primers for real-time PCR assay: Sense: 5'-AACCAGATGATGCACCTTGCAAGA
Anti-sense: 5'-GAGCATTGGAAATTGGGGTA

Mouse beta-actin primers for real-time PCR assay: Sense: 5'-GTTCTTTACGGATGTATGCT
Anti-sense: 5'-GGGTCTTTACGGATGTATGCT

LPS-induced sepsis model
6–8 week-old C57BL/6 mice, weighing between 18 and 20 g, were randomly divided into groups. Mice were injected intraperitoneally with indicated doses of Lipopolysaccharides (LPS) from E. coli strain 0111:B4, Sigma L2630) or LPS plus indicated doses of monoclonal antibodies. The number of surviving mice was recorded every six hours.

Statistical Analysis
Data were expressed as the means±SEM. Differences were analyzed using Dunnett’s multiple comparison tests, p values <0.05 were regarded as significant. Statistical significance of survival rate was determined using log-rank (Mantel-Cox) test.

Results
Macrophage migration inhibitory factor (MIF) is an important mediator of inflammatory responses and a drug target for sepsis and auto-immune diseases [10,14]. The human and mouse MIF proteins share 89% sequence identity. When NZB/W and BALB/C mice were immunized with a recombinant human MIF protein, only NZB/W mice generated good serum antibody titers (Fig. 1A). From the immunized NZB/W mice, 6 hybridoma clones (out of 136 clones screened) secreting antibodies specific to human MIF were obtained from NZB/W mice. Three of these, 4E10 (IgG2a), 10C3 (IgG2b) and 2A12 (IgG2b), are high-affinity antibody clones with dissociation constants (Kds) of 2.5 nM, 1.3 nM and 0.1 nM, respectively (measured by ELISA). In a competition assay, 10C3 and 4E10 did not compete with 2A12, while 4E10 partially competed with 10C3, suggesting that these three clones bind to different epitopes on MIF (Fig. 1B). Of the three clones, 10C3
blocked in vitro MIF-induced nitric oxide secretion in the macrophage cell line Raw264.7 (Fig. 1C). Furthermore, 10C3 showed in vivo activity when it rescued LPS-induced lethality in a mouse sepsis model (Fig. 1D). HMGBl is a proven therapeutic target in experimental models of ischemia, acute respiratory distress syndrome, rheumatoid arthritis, sepsis, and cancer [9,15]. Due to the fact that the human and mouse HMGBl proteins are highly conserved (98% identity) and that HMGBl-deficient mice die shortly after birth, it is hard to develop monoclonal antibodies against HMGBl. In this study, neither wild type nor NZB/W mice developed sufficient serum antibody titer when immunized with GST-tagged HMGBl protein. We introduced a universal T cell epitope from a Mycobacterium tuberculosis antigen [16] into the C-terminus of HMGBl and the resulting recombinant protein was used as an antigen for immunization. The T cell epitope greatly enhanced the immune response of NZB/W mice to HMGBl (Fig. 2A). Out of 328 hybridoma clones screened, 17 hybridoma clones that secrete monoclonal antibodies specific for HMGBl were identified. Four of these were high affinity clones, with Kds ranging from 0.5 nM to 10 nM (measured by ELISA). Two clones bound to the HMGBl box A, the other two could only bind to the box A+B, failing to recognize the box A or box B (Fig. 2B). The binding specificities of clone 3B1 and 3E8 were further confirmed by western blot (Fig. 2C, left panel). When whole cell lysates were used, antibodies from clones 3C5, 3E8 and 3B1 specifically recognized the endogenous HMGBl in Hela cell lysates (Fig. 2C, right panel). Antibodies from the three clones also blocked HMGBl-induced IL-6 mRNA up-regulation in Raw264.7 cells (Fig. 2D), making these antibodies good candidates for therapeutic antibodies. In a LPS-induced sepsis model, one of the anti-HMGBl clones, 3E8, was tested for in vivo-neutralizing activity. 3E8 protected mice effectively from lethal doses of LPS treatment (Fig. 2E). 3B1, a box A specific clone, had similar effects in protecting mice from LPS induced sepsis (data not shown).

Generation of reagent antibodies suitable for in vivo use is essential in the target validation phase of antibody drug discovery projects. Rabbit polyclonal or even rat monoclonal antibodies will trigger an immune response in mice, and can render the antibody ineffective as early as the second week of treatment. In contrast, antibodies like mouse anti-mouse TNF-alpha are expected to avoid host responses and are desirable for efficacy assessments in mouse disease models and early safety studies. We immunized NZB/W mice with recombinant mouse TNF-alpha and successfully generated 20 hybridoma clones (out of 748 clones screened) secreting antibodies against mouse TNF-alpha. One antibody clone bound to mouse TNF-alpha with a high affinity (Kd = 1.4 nM, measured by Surface Plasmon Resonance on a Biacore3000, Fig. 3A), and good specificity (the epitope mapped to amino acids 19–40 of the TNF-alpha mature chain, data not shown).
Figure 2 Generation of high affinity, biologically active anti-HMGB1 antibodies from NZB/W mice. (A) A T cell-specific tag fused to HMGB1 further increased immune response in NZB/W mice. NZB/W mice were immunized with GST-HMGB1 or HMGB1-MT. Serum titers were measured by ELISA using HMGB1-His-coated plates. Serum titer was defined as the highest dilution of serum at which the A450 ratio (A450 of sample/A450 of negative serum) was greater than 2.0. (B) Binding affinity and specificity of anti-HMGB1 antibodies. Antibody binding affinity was measured by ELISA using HMGB1-His-coated plates and the Kd of each antibody is indicated in the figure. Antibody specificity was measured by ELISA using the GST-HMGB1 Box (A), GST-HMGB1 Box (B) or GST-HMGB1 Box (A+B) as antigens. (C) Binding specificity of anti-HMGB-1 antibodies. Left panel: 0.3 μg of purified recombinant proteins (A+B:HMG1-MT, A: GST-boxA, B:GST-boxB) were loaded and separated by electrophoresis on 10% SDS-PAGE gels. Western blots were carried out with 1% dry milk as the blocking buffer and 2 μg/ml purified antibodies as the primary antibody. The blots were then labeled with goat anti-mouse Ig-HRP and ECL substrate. 3 μg of purified recombinant proteins were separated by electrophoresis and stained with Coomassie Brilliant Blue for loading control. Right panel: 1 × 10⁶ Hela cells were lysed in 1 ml cell lysis buffer and mixed with 5 × SDS sample buffer. 20 μl each of the above samples was separated by electrophoresis on 10% SDS-PAGE gels. Western blots were carried out with 5% BSA as the blocking buffer and 1 μg/ml biotinylated antibodies as the primary antibody. The blots were then labeled with streptavidin-HRP and ECL substrate. (D) anti-HMGB1 antibodies blocked HMGB1-induced IL-6 up-regulation. Raw264.7 cells were stimulated with 1 μg/ml HMGB1 for 8 hours, with or without 50 μg/ml of the antibodies indicated. Total mRNA was extracted with Trizol, and IL-6 mRNA levels were measured by real-time RT-PCR. Data are expressed as the means±SEM (n = 3). ** indicating p<0.01 when compared to samples treated with HMGB1 only. (E) Anti-HMGB1 mAb 3E8 protects mice from LPS-induced sepsis. 8 week-old C57BL/6 female mice, 8 mice/group, were injected intraperitoneally with 25 mg/kg LPS, along with three different doses of anti-HMGB1 antibody 3E8 or isotype control antibodies. The number of surviving mice was recorded every six hours.

doi:10.1371/journal.pone.0006087.g002
shown), and could neutralize mouse TNF-alpha activity by blocking TNF-alpha’s cytotoxic effect on L929 cells (Fig. 3B). It also showed good in vivo activity in the LPS-induced sepsis model (Fig. 3C).

**Discussion**

Our work has demonstrated that immunization of NZB/W mice can be used as an effective and universal method to develop antibodies against self and highly conserved antigens.

The NZB/W mouse strain spontaneously develops antibodies against nucleic acids and self-nuclear proteins at 5–6 months of age. Using standard immunization protocols, we were able to induce strong antibody responses in this strain against highly conserved human antigen MIF or mouse self-antigen TNF-alpha before the onset of auto-immune disease. This is consistent with the notion that B cell tolerance is generally defective in NZB/W mice [17]. However, previous work has demonstrated that self-reactive T-cell cells assisting autoantibody production in NZB/W mice do not result from a generalized defect in T-cell tolerance, and that thymic and peripheral tolerance to most self-antigens are intact in NZB/W mice [18]. This could explain the weak antibody response observed here against HMGB1, a nuclear protein expressed at high level in all cells. To by-pass this obstacle, we introduced a strong T-cell epitope to the C terminus of recombinant HMGB1 protein. This T cell stimulatory epitope is from the 38-kDa lipoprotein of the *Mycobacterium tuberculosis*. Previous studies showed that this peptide can stimulate T cell proliferation in mice of H-2b, H-2d, and H-2k haplotypes [19]. Unlike regular fusion partners such as KLH, BSA or GST, this epitope does not elicit B cell response on its own, avoiding the disadvantages of autoreactive B cells in competing for T cell help [16]. Our data demonstrated that tagging the antigen with a T cell-specific epitope can further boost the immune response, and this response is stronger than that induced by a GST-HMGB1 fusion protein.

The NZB/W mouse strain has also been used to develop antibodies against bovine prion protein (PrP) [8]. Multiple hybridoma clones which secreted antibodies that bound specifically to different epitopes of PrP were isolated from NZB/W mice.
immunized with KLH-conjugated PrP. However, the binding affinity of these PrP-specific antibodies was not reported [8]. In our case when cytokines with clear therapeutic indications were chosen as test antigens, the goal was to develop high affinity antibodies with neutralization ability. We were able to demonstrate that antibodies generated with our method are mostly within the 1-10 nM Kd affinity range, sufficiently high for therapeutic purposes.

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Acknowledgments

We thank Mr. Chunchun Liu for his help with FACS data analysis and Ms. Yuanyuan Chen for her help on Biacore analysis.

Author Contributions

Conceived and designed the experiments: JT. Performed the experiments: HZ YW WW JJ YL QW YW. Analyzed the data: JT. Wrote the paper: JT.