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Numan Javed, Guang Xue, Ailing Lu, Yue Xing, Yoichiro Iwakura, et al.. Cross reactivity of *S. aureus* to murine cytokine assays: A source of discrepancy.. Cytokine, Elsevier, 2016, 81, pp.101-108. 10.1016/j.cyto.2016.03.007 . pasteur-01441010

**HAL Id: pasteur-01441010**

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Submitted on 10 Mar 2021

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# Cross reactivity of *S. aureus* to murine cytokine assays: A source of discrepancy

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## **Abstract**

*Staphylococcus aureus* is one of the versatile Gram positive bacteria causing a range of diseases. Upon challenge, host immune cells recognize *S. aureus* and mount diverse immune responses including production of pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ . These cytokines are important mediators of inflammation which can be detected via various immunological methods such as enzyme linked immunosorbent assay (ELISA) and immunoblotting. In the current study, we found that a number of clinical isolates as well as laboratory strains of *S. aureus* exhibited cross reactivity with ELISA antibodies for murine IL-1 $\beta$  and TNF- $\alpha$  assays. This cross reactivity generates exaggerated false positive signals which can be a source of discrepancy for the understanding of real immune responses against *S. aureus* infection by host immune cells.

## 1. Introduction

*Staphylococcus aureus* (*S. aureus*) is one of the notorious bacteria which cause an array of infections ranging from superficial to invasive ones. This bacterium is acquiring resistance against antibiotic of first choice such as methicillin, so that its significance is increasing for causing community associated and nosocomial infections [1]. Methicillin resistant *S. aureus* (MRSA) is a superbug harboring Staphylococcal cassette chromosomes mec (SCCmec) which is becoming pandemic almost all around the world [2]. Yet there is no effective vaccine against MRSA yet, which indicates that our knowledge about immune response against this bacterium is still inadequate.

Host innate immune defense serves as a significant arsenal against *S. aureus* challenge. And one of the most important components of innate immune system is the macrophage. Irrespective of the species, macrophages interact with *S. aureus* [3]. Macrophages recognize conserved structures called pathogen associated molecular patterns (PAMPs) from microbes such as *S. aureus* via pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and Nucleotide-binding Oligomerization Domain receptors (NOD-like receptors, NLRs) [4]. A subset of NLRs such as NLRP3 or some DNA sensors such as absent in melanoma 2 (AIM2) can recruit adaptor protein ASC as well as pro-caspase-1, thus assemble a big protein complex called inflammasome [5]. The activation of an inflammasome leads to caspase-1 maturation, which facilitates the cleavage of pro-IL-1 $\beta$  into mature IL-1 $\beta$ , and the executor protein GSDMD further promotes IL-1 $\beta$  secretion and cell pyroptosis [6]. *S. aureus*  $\alpha$ -hemolysin has the ability of activating the NLRP3 inflammasome in human and murine monocytic cells [7]. Similarly, it has also been reported that AIM2 is critical for host survival and inflammatory mediator induction during acute CNS infection with *S. aureus* [8].

IL-1 $\beta$  is a master mediator of the inflammatory process which can be measured via sandwich ELISA. In this method, monoclonal antibody against IL-1 $\beta$  is used, which can detect both mature IL-1 $\beta$  and pro-IL-1 $\beta$ . The selectivity of immunoassays like ELISA results from the specificity of the antigen–antibody reaction. In many cases, however, the specificity compromised the ability of other molecules with structurally similar or identical epitopes to bind to the same antibody. This competition is commonly referred to as cross-reactivity. The use of monoclonal antibodies has greatly increased the specificity of immunoassays. Similarly, the use of two-site (or

sandwich) assays that require two distinct epitopes on the antigen to be recognized greatly decreases cross-reactivity but may not fully eliminate this potential problem [9].

In the current study, we demonstrate that this sandwich type ELISA exhibited cross reactivity with some unknown epitopes from various strains of *S. aureus*. This cross-reactivity signal was initially taken as IL-1 $\beta$  production from *S. aureus* infected mouse macrophages. Further experiments with IL-1 $\beta$  deficient murine primary cells revealed that it was a non-specific signal reacting to the ELISA antibodies from *S. aureus*. In addition, antibodies from the mouse TNF- $\alpha$  ELISA kit also reacted with *S. aureus*. Therefore, our data demonstrate that *S. aureus* has the ability of cross reacting with murine IL-1 $\beta$  and TNF- $\alpha$  ELISA antibodies, which generate false positive signals. Thus all reported data for *S. aureus* induced murine IL-1 $\beta$  or TNF- $\alpha$  secretion monitored via ELISA as indication of host immune responses should be revalidated with alternative approaches carefully due to the cross reactivity problem reported in our present work.

## 2. Materials and methods

### 2.1 Bacterial Strains & Growth Conditions

Various strains of *S. aureus* including clinical isolates MR4 & MS3 (Bacterial Culture Bank of Department of Microbiology & Molecular Genetics (MMG) Quaid-e-Azam Campus, University of the Punjab, Lahore. Pakistan), MW2 (kindly provided by Dr. Baolin Sun from University of Science and Technology of China), Newman (kindly provided by Feng Shao from National Institute of Biological Science, Beijing, China), 65.8 T (kindly provided by Collection de l'Institut Pasteur, Centre de ressources biologiques de l'Institut Pasteur, Paris, France) were grown overnight in Brain Heart Infusion (BHI) broth at 37°C. The bacteria were washed three times at 4000 rpm for 10 min at 4°C and suspended in phosphate buffered saline (PBS) at a concentration of  $1 \times 10^9$  colony forming units/ml by adjusting OD 1.00 at 600 nm, also confirmed by retrospective plate counting of inoculated dilutions of the suspension on LB agar.

### 2.2 Genotyping of Bacteria

Bacterial genomic DNA was isolated from overnight cultured bacteria in BHI broth by chloroform/isoamyl alcohol method as described [10]. Briefly, *S. aureus* were grown overnight; 1.5ml of culture was centrifuged at maximum speed in sterile aliquot tube for 2 minutes. The supernatant was discarded and the pellet was resuspended in 300 ul lysis solution. The pellet was mixed thoroughly in this solution and was incubated for 30 minutes at 65°C in water bath. After this incubation, equal volume of 24:1 chloroform/isoamyl alcohol was added and mixed gently then centrifuged at 12000 rpm for 10 minutes. The upper aqueous layer was transferred to a fresh tube with equal volume of isopropanol and one tenth of sodium acetate solution was mixed gently until a stringy white DNA precipitate was formed. Then mixture was centrifuged for 10 minutes at room temperature. The supernatant was discarded and 70% ethanol was added to the pellet. Finally the mixture was centrifuged for 2 minutes and supernatant was discarded. The pellet was allowed to dry and was resuspended in 100µl TE buffer and stored at -20°C. All the strains were screened for 16SrRNA, *nuc* and *mecA* genes using standard PCR conditions. The oligonucleotide primers were synthesized and purchased from Shanghai Sunny Biotechnology Co.,Ltd. The primers used in this study are: Staph 756F (5'-AAC TCT GTT ATT AGG GAA GAA CA-3') and Staph 750R (5'-CCA CCT TCC TCC GGT TTG TCA CC-3') for

*Staphylococcus* genus-specific 16SrRNA; Nuc1 (5'-GCG ATT GAT GGT GAT ACG GTT-3') and Nuc2 (5'-AGC CAA GCC TTG ACG AAC TAA AGC-3') for specie-specific *nuc* and Mec A1(5'-GTA GAA ATG ACT GAA CGT CCG ATA A-3') and MecA2 (5'-CCA ATT CCA CAT TGT TTC GGT CTA A-3') for *mecA* gene [11].

### **2.3 Antimicrobial Sensitivity Testing**

Antimicrobial screening was also done for all the strains by standard Kirby-Bar agar diffusion method using oxacillin and vancomycin (5µg) discs on Muller Hinton Agar [12].

### **2.4 Mice**

*Il1α<sup>-/-</sup>* and *Il1β<sup>-/-</sup>* mice had been described before [13], *Myd88<sup>-/-</sup>*, *Trif<sup>-/-</sup>*, *Trif/Myd88<sup>-/-</sup>* and *Caspase1/11<sup>-/-</sup>* (*Casp1/11<sup>-/-</sup>*) mice were from the Jackson Laboratories [14]. *Nlrp3<sup>-/-</sup>* and *Asc<sup>-/-</sup>* mice had been reported before [15-16], wild type (WT) mice were from Shanghai Laboratory Animal Center. All mice are on C57BL/6 genetic background. All experimental procedures were performed complying with national guidelines and were approved by the animal care and use committee at Institut Pasteur of Shanghai.

### **2.5 Preparation & infection of Murine BMDMs**

BMDMs were prepared as described [17]. Briefly, bone marrow cells were flushed from the femurs and tibias of mice and cultured in IMDM medium (Gibico) supplemented with 10% FBS and 30% L cell supernatant (containing M-CSF) for 5 days in a humidified incubator with 5% CO<sub>2</sub> at 37°C. Mature BMDMs were plated in 96 or 6 well plates overnight, then infected with *S. aureus* with indicated MOIs for 6 hours, spin at high speed in order to sediment the bacteria. Afterward, culture supernatants or cell lysates were subjected to further analysis.

### **2.6 Cell free system for bacteria**

For cross reactivity determination, the same numbers of bacteria were inoculated in mammalian cell free IMDM medium without penicillin and streptomycin for 6 hours under the same conditions as BMDMs culture.

## **2.7 ELISA**

The quantification of mouse IL-1 $\beta$  and TNF- $\alpha$  in culture supernatants was performed through ELISA method according to manufacturer's instructions (eBioscience, mouse IL-1 $\beta$  ELISA kit Catalog Number: 88-7013; eBioscience, mouse TNF- $\alpha$  ELISA kit Catalog Number: 88-7324). Similarly, the cross reactive signals were measured in host cell free system containing only bacteria.

## **2.8 Western Blotting**

After stimulation with bacteria, the supernatant from BMDM culture was harvested and cells were lysed with SDS loading buffer. Total proteins from culture supernatant were concentrated using trichloroacetate & methanol, protein samples were separated on a 10–15% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane (Merck Millipore Ltd, Germany) by electro blotting. Membranes were blocked overnight at 4°C using 5% fat free milk in TBST before incubation with the primary antibody. Rabbit anti mouse antibodies to caspase-1 (SC-514) or IL-1 $\beta$  (SC-7884) (Santa Cruz Biotechnology, Inc. CA, USA) were used as primary antibodies. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary goat anti rabbit antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Immuno reactive proteins were detected using Enhanced Chemiluminiscence (ECL) reagent (Amersham, USA).  $\beta$ -Actin (Santa Cruz Biotechnology) was used as internal control to validate the amount of protein loaded onto the gels. For the validation of cross reactivity of antibodies other than SC7884, Capture & detection antibodies (eBioscience ELISA system) were used as primary antibodies for which chicken anti mouse and horseradish peroxidase (eBioscience ELISA system) served as secondary antibodies respectively.



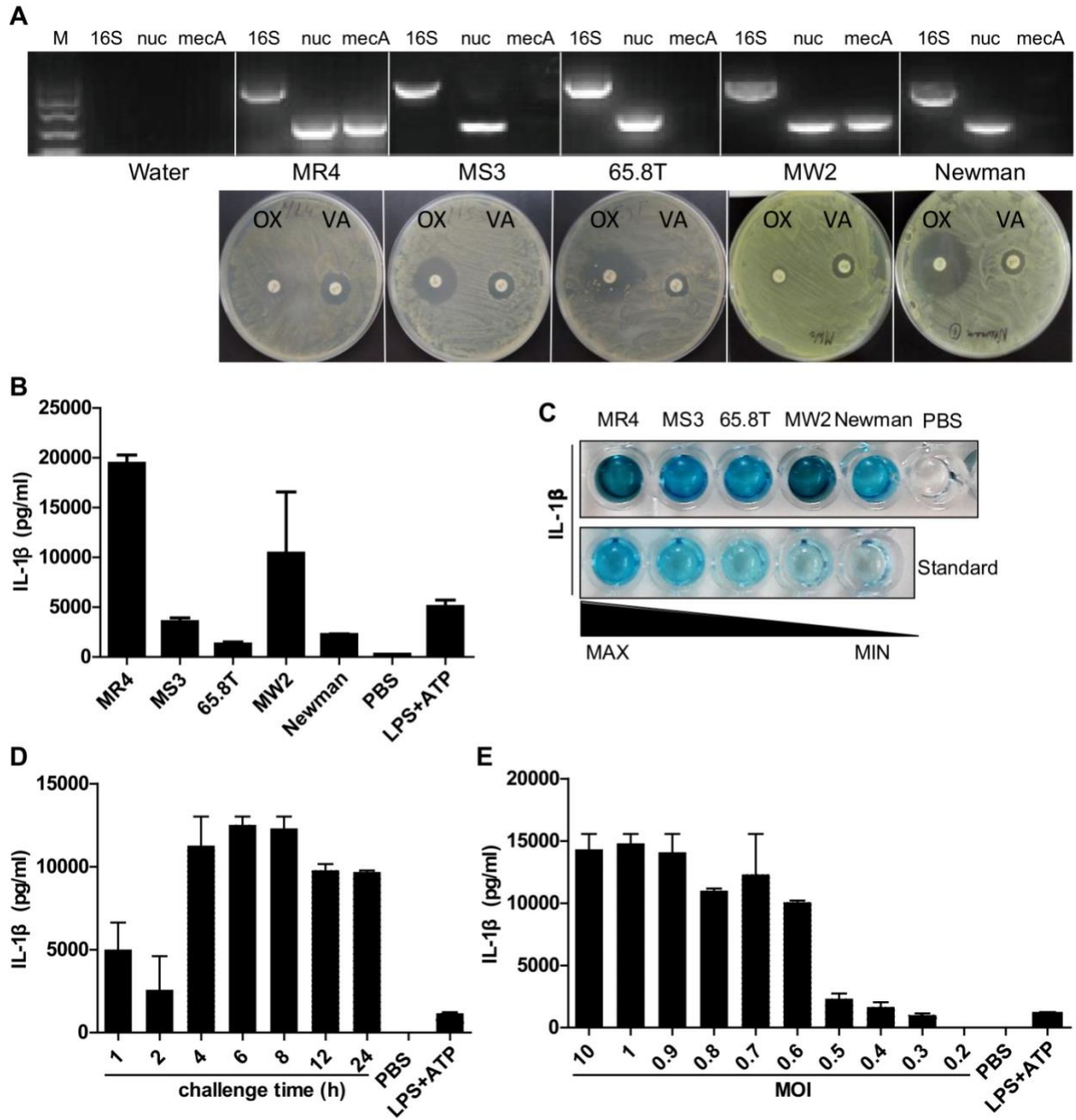
### 3. Results and Discussion

#### 3.1 *S. aureus* induced strong signals in ELISA for mouse IL-1 $\beta$ from BMDMs

In order to study the immune response against *S. aureus*, we used the clinical isolates named as MR4 and MS3 to challenge mouse BMDMs. In addition we also used various laboratory strains of *S. aureus* such as 65.8T, MW2 and Newman as controls. For the characterization of these strains we performed genotyping by amplifying the genomic DNA from all the aforementioned strains of *S. aureus* conserved 16s rRNA gene and species determinant *nuc* gene. All the isolates exhibited these two genes which determine its genus and specie validity (Fig. 1A). Furthermore, for the determination of antimicrobial sensitivity, we probed the antibiotic resistance harboring *mec A* gene. Both MR4 and MW2 carry this gene which indicated that these strains belong to methicillin resistant *S. aureus* (MRSA), while MS3, 65.8T and Newman strains did not show positive signal for this gene which determined their phenotype as Methicillin sensitive *S. aureus* (MSSA) (Fig. 1A). The phenotypes of these strains were further discriminated through standard Kirby-Bar antimicrobial sensitivity testing [10], wherein the MRSA strains MR4 and MW2 were resistant to both Oxacillin (OX) and Vancomycin (VA), while MSSA strains MS3, 65.8T and Newman were sensitive to Oxacillin but not Vancomycin (Fig. 1A).

In order to monitor a potential activation of inflammasome by certain clinical isolates of *S. aureus*, we tested the ability of MR4 to induce IL-1 $\beta$  production from WT mouse BMDMs. Interestingly, a very strong signal of IL-1 $\beta$  was induced upon BMDMs incubation with MR4 at MOI=1 for 6 hours (Fig. 1B). During the performance of ELISA for IL-1 $\beta$ , there was a robust change in color on adding 1X 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution after Avidin-Horseradish Peroxidase (HRP) step, by all the strains of *S. aureus* in comparison with serially doubled diluted standard. The color intensity was comparable with the absolute quantified signal of IL-1 $\beta$  by MR4, MS3, 65.8T, MW2 and Newman strains (Fig. 1C). For the validation of this finding, we decided to check IL-1 $\beta$  signal after various time points upon infection with MR4. It was obvious that the IL-1 $\beta$  signal in ELISA appeared as early as 1 hour after infection and clearly increased after 4 hours, and stayed high for 24 hours (Fig. 1D). In addition, we also checked the ability of various MOIs of MR4 for this IL-1 $\beta$  induction. Our data indicated that this response of MR4 showed a clear dose dependency. MOI as low as 0.6 still induced a clear IL-1 $\beta$  signal (Fig. 1E). Taken together, our data revealed that MR4 was a strong

inducer of IL-1 $\beta$  signal in ELISA when interacting with murine BMDMs, but it is odd that such a strong signal has been evident only after 1 hour of bacterial incubation.



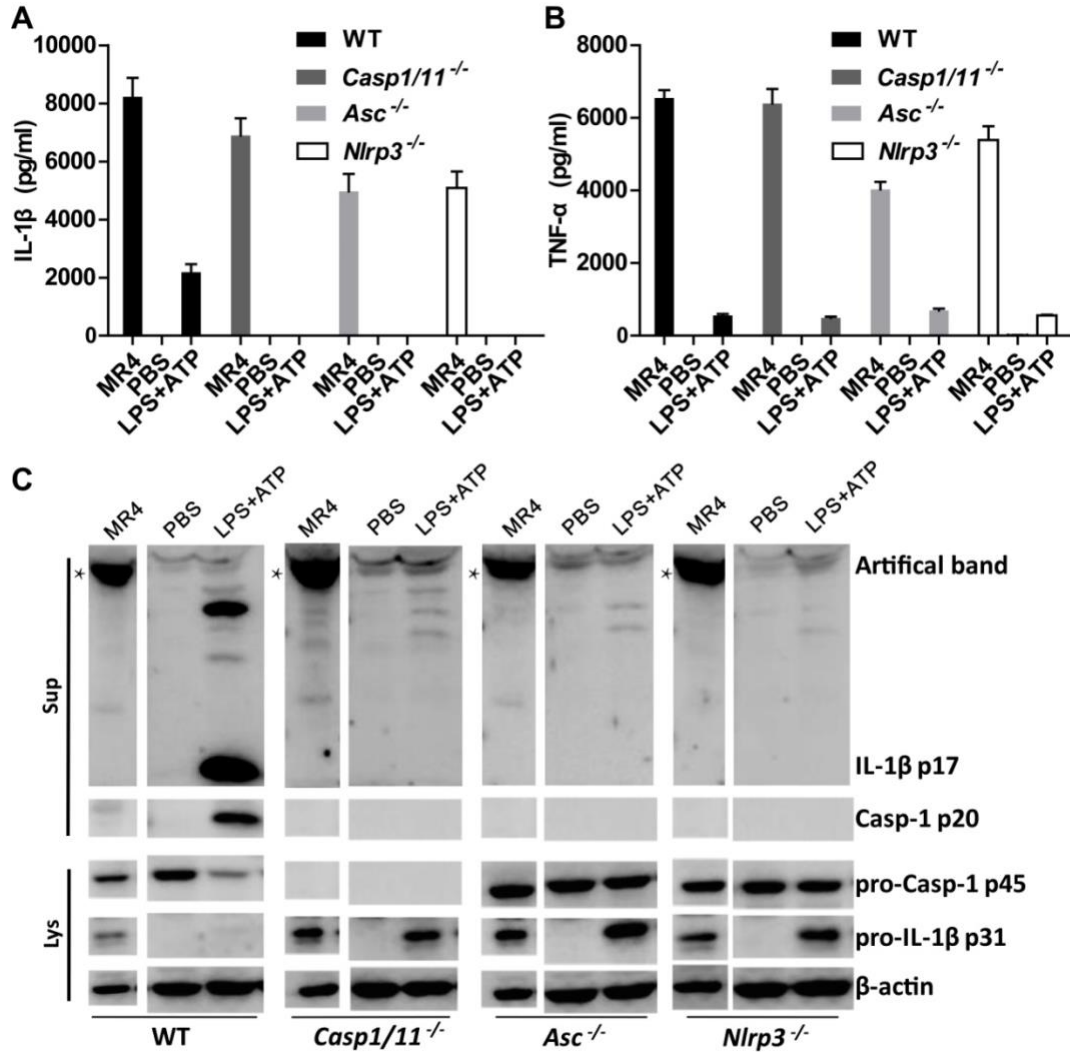
**Fig 1: *S. aureus* induced strong signals for mouse IL-1 $\beta$  from BMDMs culture.** (A), Amplification of 16SrRNA (genus specific), *nuc* (species specific) and *mecA* antibiotic resistance harboring gene from various isolates of *S. aureus* by PCR. Antimicrobial sensitivity testing of MR4, MS3, 65.8T, MW2 & Newman strains was done using Oxacillin (OX) & Vancomycin (VA) discs by Kirby Bar method. (B), Murine BMDMs from WT mouse were stimulated with MR4 & MS3 at MOI=1, PBS and LPS+ATP served as

controls. IL-1 $\beta$  secretion was monitored with ELISA. (C), Appearance of color in IL-1 $\beta$  ELISA wells containing supernatant from WT-BMDM infected with MR4, MS3, 65.8T, MW2, Newman & PBS (Mock) group on adding IX TMB substrate solution after Avidin-HRP step in comparison with serially double diluted standard. (D), Time course for the induction of IL-1 $\beta$  signals by MR4 in BMDMs. (E), MOI titration of MR4 for the induction of IL-1 $\beta$  signals in BMDMs.

### 3.2 Inflammasome independent induction of IL-1 $\beta$ signals in ELISA by MR4

After observing such a strong IL-1 $\beta$  induction by MR4 from macrophages, we speculated that this could be due to activation of certain inflammasome. As Kebaier *et al* has already reported that *S. aureus* has the ability to induce IL-1 $\beta$  production via the NLRP3 inflammasome in a murine model of pneumonia [18], so we used *Nlrp3*<sup>-/-</sup> BMDMs for investigating the mechanism of IL-1 $\beta$  induction by MR4. Surprisingly, we found that MR4 mediated IL-1 $\beta$  signal was totally independent of NLRP3 at all (Fig. 2A). Similarly, there was no abolishment of the IL-1 $\beta$  signal in ELISA when we further use *Caspase-1/11*<sup>-/-</sup> or *Asc*<sup>-/-</sup> BMDMs in the above mentioned experiments (Fig. 2A). We also quantified the level of TNF- $\alpha$  via ELISA from the same samples for IL-1 $\beta$  ELISA assay, which gave approximately the same levels of signals as IL-1 $\beta$  (Fig. 2B and 2A). Moreover, the cleavage product of pro-IL-1 $\beta$  (mature IL-1 $\beta$ ) was not detected from WT & *Nlrp3*<sup>-/-</sup> BMDMs infected with MR4 through Western blotting (Fig. 2C). On the hand, we found a unique strong artificial band having the size of approximately 55 kDa which could be a source of strong signals for MR4 in ELISA (Fig. 2C). From previous work, Franchi *et al* described that *S. aureus* lack the ability of activating caspase-1 in murine macrophages at different bacteria-macrophage ratios for up to 24h of culture from immunoblotting analysis [19]. Similarly, there was no signal of mature IL-1 $\beta$  detected via western blotting either [19]. Furthermore, another work has also reported that upon challenging to murine mammary glands, *S. aureus* did not process the caspase-1 but strong signals for IL-1 $\beta$  were generated in ELISA [20]. Caspase-1 activation from MR4 infected cells was also totally negative in comparison with the positive control which was LPS+ATP treated cells. In the latter, a clear caspase-1 activation and mature IL-1 $\beta$  secretion was observed, which was totally dependent on the NLRP3 inflammasome (Fig. 2C). Similarly, MR4 exhibited the same artificial band while interacting with *Caspase-1/11*<sup>-/-</sup> or *Asc*<sup>-/-</sup> BMDMs without cleavage of IL-1 $\beta$  and caspase-1 (Fig. 2C). Thus,

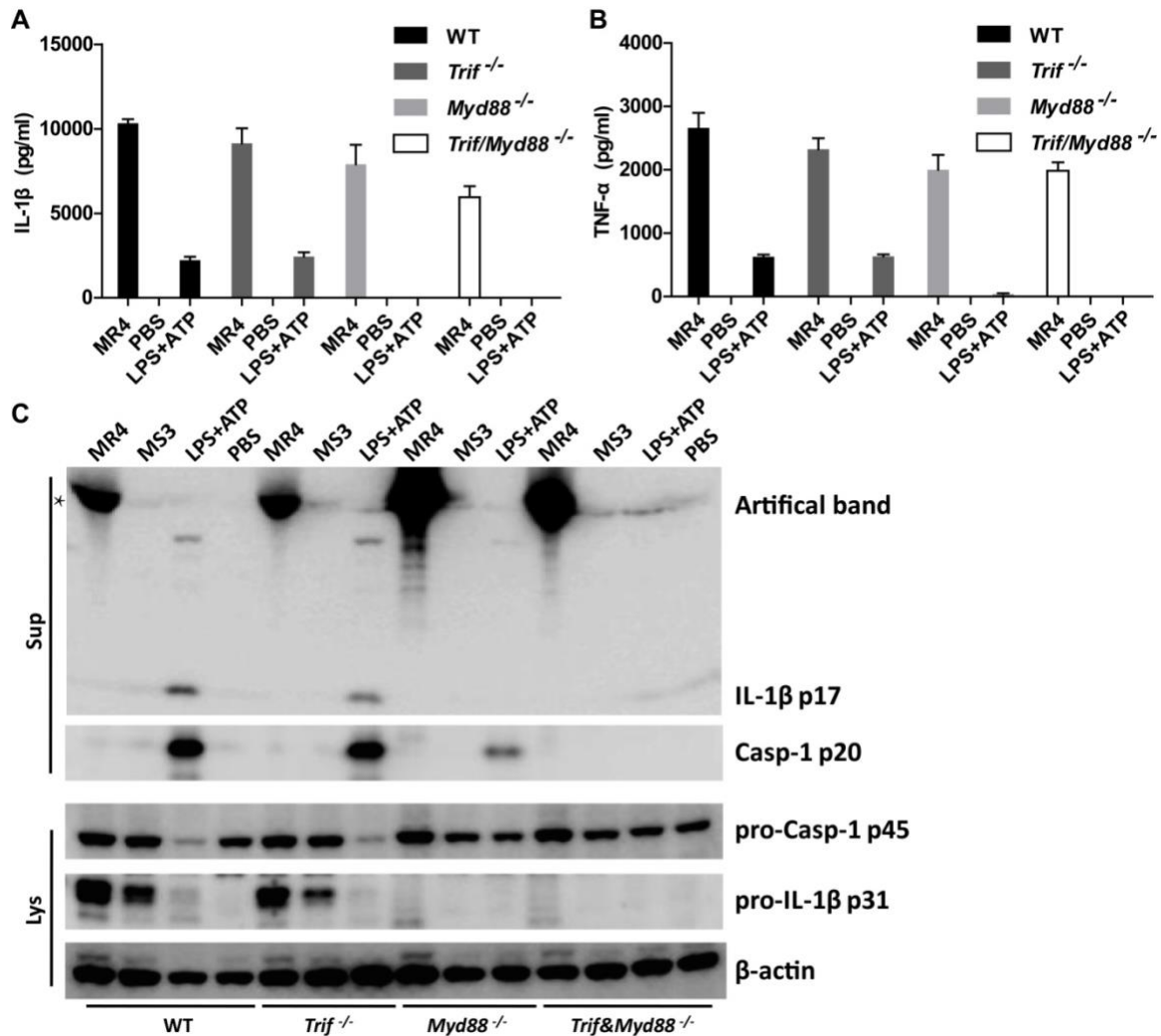
all these data suggested that the IL-1 $\beta$  signal quantified via ELISA was not an indication of mature IL-1 $\beta$  (p17) accumulation, although MR4 induced a clear synthesis of pro-IL-1 $\beta$  in all genotype of BMDMs (Fig. 2C).



**Fig 2: Inflammation independent induction of IL-1 $\beta$  and TNF- $\alpha$  signals by MR4 from murine BMDMs.** (A-B), BMDMs from WT, *Caspase-1/11*<sup>-/-</sup>, *Asc*<sup>-/-</sup> and *Nlrp3*<sup>-/-</sup> mice were stimulated with MR4 at MOI=1, PBS and LPS+ATP served as controls. Cell Culture supernatant was quantified for (A) IL-1 $\beta$  induction, (B) TNF- $\alpha$  response via ELISA. (C), BMDMs from WT, *Caspase -1/11*<sup>-/-</sup>, *Asc*<sup>-/-</sup> & *Nlrp3*<sup>-/-</sup> mice were stimulated with MR4 at MOI=1, PBS and LPS+ATP served as controls. Cell Culture supernatant (Sup) was analyzed for artificial band (band followed by star), cleavage of IL-1 $\beta$  and caspase-1. Cell lysates (Lys) was also analyzed for pro-caspase-1, pro-IL-1 $\beta$ , and  $\beta$ -actin respectively via immunoblotting.

### 3.3 TLR signaling independent induction of IL-1 $\beta$ signals by MR4

After knowing the fact that MR4 has a strong ability to induce pro-IL-1 $\beta$  synthesis, which is independent from the activity of NLRP3 inflammasome but dependent on NF- $\kappa$ B signaling, we rationalized that the pro-IL-1 $\beta$  induction should be dependent on TLR signaling as TLR2 is the receptor for PGN from *S. aureus* [21]. To verify this possibility, we applied *Trif*<sup>-/-</sup>, *Myd88*<sup>-/-</sup> and *Trif/Myd88*<sup>-/-</sup> BMDMs for infection with MR4. It was really astonishing, as we still observed the similar levels of IL-1 $\beta$  signals as from all other genotypes of cells test before (Fig. 1-2) upon MR4 infection of macrophages in ELISA (Fig. 3A). Similarly, there was no any change in the level of TNF- $\alpha$  induction from any gene deficient cell either (Fig. 3B). Moreover, in immunoblotting we observed the same artificial band for MR4 as found earlier in inflammasome component gene knockout BMDM, while activation of caspase-1 and mature IL-1 $\beta$  release was only in LPS+ATP treated cells, not from MR4 or MS3 infected cells at all (Fig. 3C). These data suggest that the IL-1 $\beta$  or TNF- $\alpha$  signal induction by MR4 from BMDMs was also TLR independent.

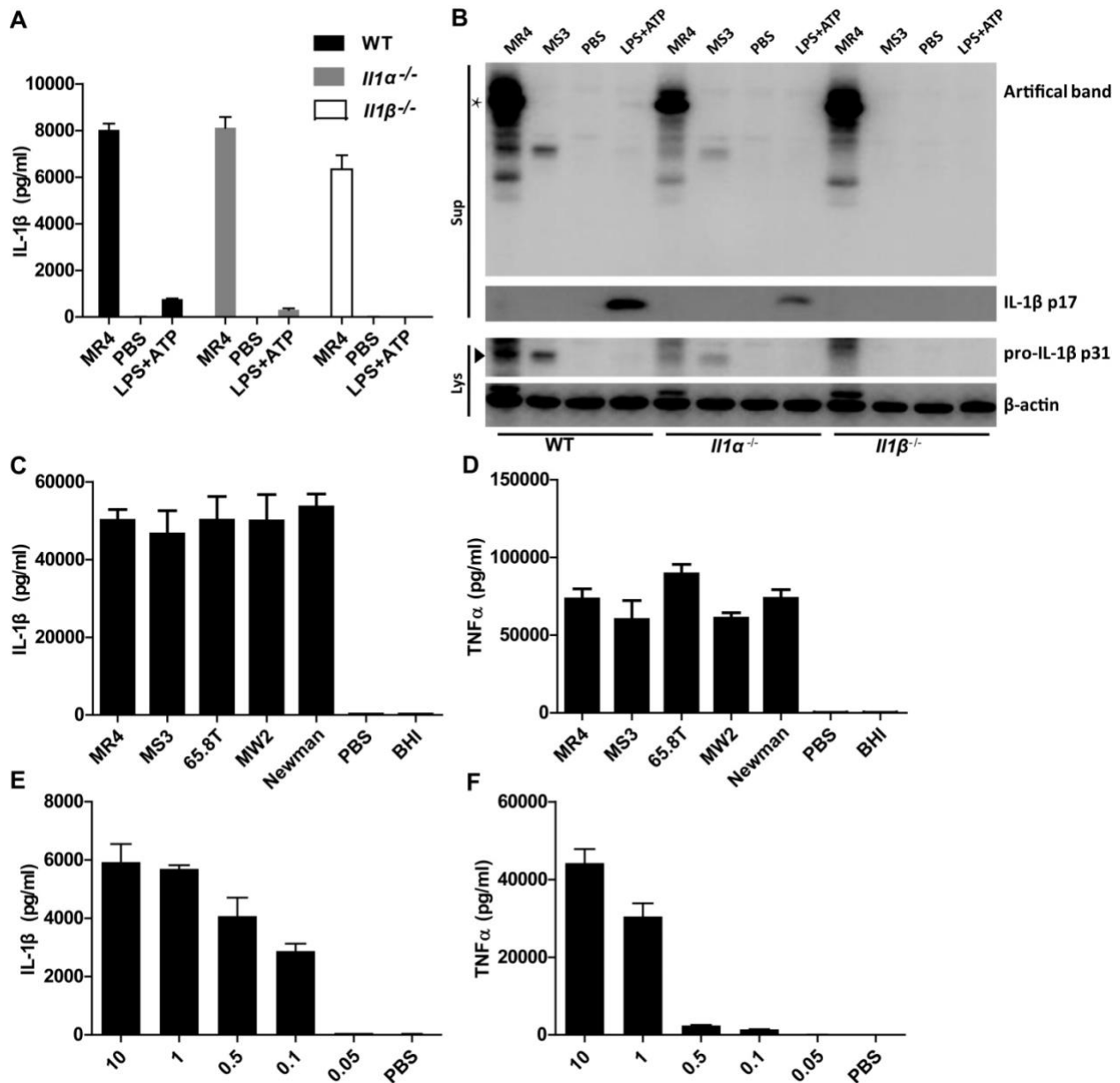


**Fig 3: TLR signaling independent induction of signals by MR4 in murine BMDMs.** (A-B), BMDMs from WT, *Trif*<sup>-/-</sup>, *Myd88*<sup>-/-</sup> and *Trif/Myd88*<sup>-/-</sup> mice were stimulated with MR4 at MOI=1, LPS+ATP and PBS served as controls. Cell culture supernatant was quantified for (A) IL-1 $\beta$ , (B) TNF- $\alpha$  induction by this strain via ELISA. (C), BMDMs from WT, *Trif*<sup>-/-</sup>, *Myd88*<sup>-/-</sup> and *Trif/Myd88*<sup>-/-</sup> mice were stimulated with MR4 and MS3 at MOI=1, LPS+ATP and PBS served as controls. Cell culture supernatant (Sup) was analyzed for artificial band (band followed by star), cleavage of IL-1 $\beta$  and caspase-1. Cell lysates (Lys) was probed for pro-caspase-1, pro-IL-1 $\beta$ , and  $\beta$ -actin respectively through western-blotting.

### 3.4 *Il1 $\beta$* <sup>-/-</sup> BMDMs failed to abolish the IL-1 $\beta$ signal induced by MR4

To finally validate the IL-1 $\beta$  signal induced by MR4 from BMDMs, we applied genetically modified mice for experiment. Surprisingly, *Il1 $\beta$* <sup>-/-</sup> BMDMs still gave a strong signal for IL-1 $\beta$  in

ELISA upon MR4 infection, the same as from WT or *Il1 $\alpha$* <sup>-/-</sup> macrophages (Fig. 4A). As expected, with immunoblotting we could not detect any mature IL-1 $\beta$  signal from *Il1 $\beta$* <sup>-/-</sup> BMDMs upon any treatment but MR4 also exhibited the same artificial band as shown in earlier experiments (Fig 2&3) which was absent in case of MS3. However, in the cell lysates, MR4 infection induced a signal for pro-IL-1 $\beta$  in WT and *Il1 $\alpha$* <sup>-/-</sup> macrophages (Fig. 4B). These data thus suggested that this strong response induced by MR4 was not a real IL-1 $\beta$  signal and it could be due to the artificial band. To further validate the identity of this signal, we used a host cell free system in which we inoculated mammalian cell medium IMDM with the same number of bacteria as in the BMDMs infection experiments. We also included the laboratory strains of *S. aureus* including 65.8T, MW2 and Newman. After 6 hours, we measured the culture supernatant for mouse IL-1 $\beta$  and TNF- $\alpha$  using standard ELISA procedure. It turned out that all different strains of *S. aureus* exhibited strong signal for both IL-1 $\beta$  and TNF- $\alpha$  in ELISA (Fig. 4C, 4D). Furthermore, we also found the dose dependency of the cross reactive signals in case of MR4 after serially diluting it in cell free system. This kind of dose dependence]y was observed both for mouse IL-1 $\beta$  and TNF- $\alpha$  (Fig 4E & 4F). In contrast, the culture supernatant did not show any cross reactivity with human IL-1 $\beta$  and TNF- $\alpha$  ELISA antibodies (data not shown). Interestingly, a previous work from Eun-Jin Choi et al showed that *S. aureus* induced high level of TNF- $\alpha$  from murine primary cells [22]. And they noticed a sharp dropping of TNF- $\alpha$  after application of the drug Honokiol [22]. This might be indicating a killing of *S. aureus* instead of inhibiting host signals by Honokiol, as no clear dose dependency was observed and the TNF- $\alpha$  signal from this assay can also be a result of cross reactivity if the antibodies used in that study was the same as ours [22]. Similarly, it has also been reported that incubation of macrophages with high MOI of *S. aureus* for 12 or 24 hours led to significant release of IL-1 $\beta$  without caspase-1 activation [23]. In consistent with our findings, in this study the IL-1 $\beta$  production was detected via ELISA, which could be the discrepant signals we report in the current work [23]. Altogether, our data provide a clear evidence about the cross reactivity of *S. aureus* with mouse cytokine assay antibodies including but may not limited to IL-1 $\beta$  and TNF- $\alpha$ , all experimental data related with these assays for *S. aureus* infection must be carefully controlled.



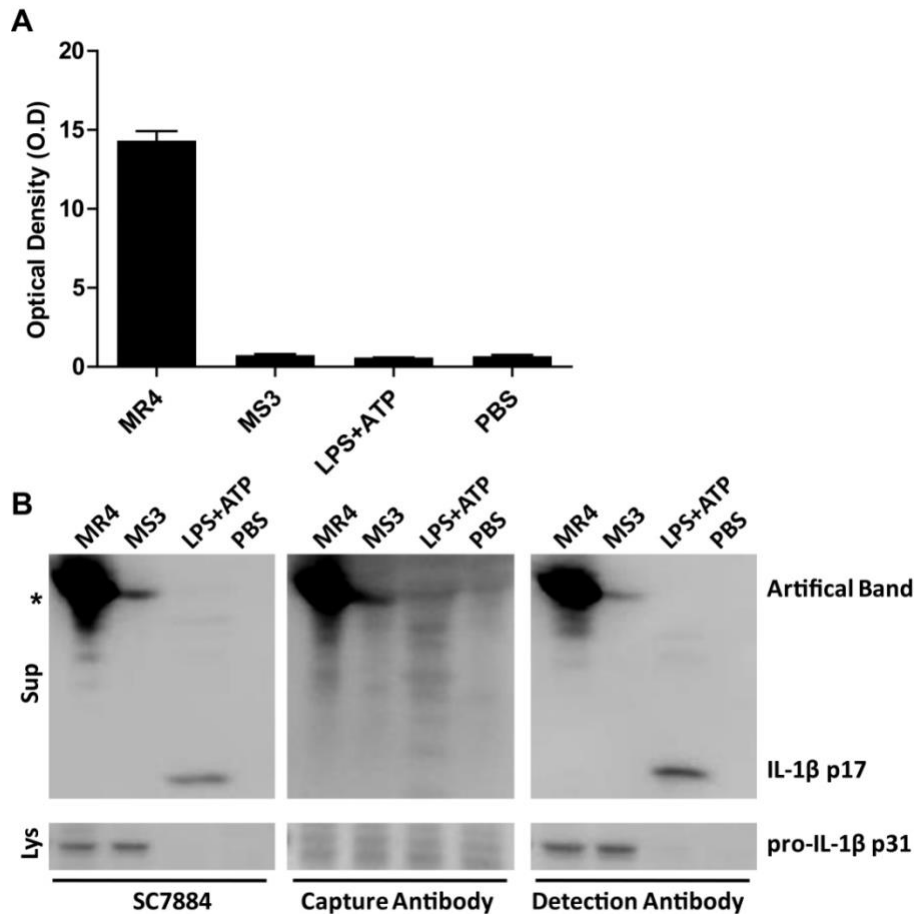
**Fig 4: *Il1 $\beta$ <sup>-/-</sup>* BMDMs failed to abolish the IL-1 $\beta$  signal generated from MR4 infection.** (A), BMDMs from WT, *Il1 $\alpha$ <sup>-/-</sup>* and *Il1 $\beta$ <sup>-/-</sup>* mice were stimulated with MR4 at MOI=1, PBS and LPS+ATP served as controls. Cell culture supernatant was quantified for (A) IL-1 $\beta$  induction via ELISA. (B), BMDMs from WT, *Il1 $\alpha$ <sup>-/-</sup>* and *Il1 $\beta$ <sup>-/-</sup>* mice were stimulated with MR4, MS3 at MOI=1, PBS and LPS+ATP served as controls. Cell culture supernatant (Sup) was analyzed for artificial band (band followed by star) and cleavage of IL-1 $\beta$ . Cell lysate (Lys) was analyzed for pro-IL-1 $\beta$  and  $\beta$ -actin respectively through immunoblotting. (C-D), IMDMs were inoculated with MR4, MS3, 65.8T, MW2, Newman strains, PBS and Brain Heart Infusion (BHI) broth served as controls with the same number as with BMDMs infection from figures (1-3) was analyzed for (C) mouse



IL-1 $\beta$  and (D) mouse TNF- $\alpha$  via ELISA. (E-F) IMDM were inoculated with MR4 with indicated MOIs after incubation (2h) and the supernatant was analyzed for (E) mouse IL-1 $\beta$  and (F) mouse TNF- $\alpha$  via ELISA.

### **3.5 MR4 is cross reactive with multiple IL-1 $\beta$ antibodies of different origins**

In order to rule out the potential involvement of other antibodies from different manufacturers, we screened the supernatants of murine primary cells infected with MR4, MS3, PBS and LPS+ATP as a control after replacing the capture antibody of ELISA system (eBioscience) with conventional Rabbit anti-mouse IL-1 $\beta$  (Santa Cruz Biotechnology, Inc. CA, USA) antibody. Interestingly, we found the same exaggerated cross reactive signals from MR4 as obtained earlier with the capture antibody of eBioscience antibody (Fig. 5A). However, this system could not detect IL-1 $\beta$  standard, so we presented the data in the form of optical density (OD). These data suggest that the cross reactive molecule of MR4 also have the ability to interact with antibodies from other manufacturers in addition to eBioscience ELISA antibodies. This was further validated after immunoblotting of the supernatants of MR4 or MS3 infected BMDMs with capture and detection (eBioscience) antibodies in addition to conventional Rabbit anti-mouse IL-1 $\beta$  (Santa Cruz Biotechnology, Inc. CA, USA) antibody on separate blots via western blotting. Surprisingly, we found the same artificial band by MR4 after interaction with capture and detection antibodies of eBioscience (Fig. 5B). These findings clearly indicate that the potential cross reactive molecule from MR4 has ability to produce artificial signals with other assay systems.



**Fig 5: MR4 also has ability to cross react with IL-1 $\beta$  antibodies of manufacturers other than eBioscience ELISA kit.** (A), BMDMs from WT mouse were stimulated with MR4 or MS3 at MOI=1, PBS and LPS+ATP served as controls. The supernatant was incubated with SC7884 antibody as capture antibody followed by detection antibody and HRP(A), Optical Density (OD) obtained at 450nm is presented. (B), Cell culture supernatant (Sup) was analyzed for artificial band (band followed by star) and cleavage of IL-1 $\beta$ . Cell lysate (Lys) was analyzed for pro-IL-1 $\beta$  through immunoblotting with Rabbit anti-mouse IL-1 $\beta$  (SC7884, Santa Cruz, USA), ELISA capture and detection antibodies (eBioscience) respectively.

## Conclusion

In conclusion, the present study described that *S. aureus* have some unknown epitopes which have the ability of cross reacting with mouse cytokines like IL-1 $\beta$  and TNF- $\alpha$ . So, all reported

data for *S. aureus* induced murine IL-1 $\beta$  or TNF- $\alpha$  secretion monitored via ELISA as indication of host immune responses should be revalidated with alternative approaches.

### **Author's contributions**

N.J., G.X. and G.M. designed research; N.J., G.X., A.L., Y.X. performed the experiments; Y.I., H.X., H.L., G.S. contributed reagents; N.J., G.X. and G.M. wrote the manuscript.

### **Competing Interests**

The authors have no financial conflicts of interest.

### **Acknowledgements**

We thank Dr. Warren Strober for sharing NLRP3 deficient mice, Dr. Vishva M. Dixit for providing ASC deficient mice, Dr. Baolin Sun and Dr. Feng Shao for providing MW2 and Newman strains respectively.

This work was supported by grants from Natural Science Foundation of China (31370892, 31300712, 91429307, 31570895), National Key Basic Research Programs (2014CB541905, 2015CB554302) and National Major Projects for Science and Technology (2014ZX0801011B-001).

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