

PRR adjuvants restrain high stability peptides presentation on APCs

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Abstract

Adjuvants can affect APCs function and boost adaptive immune responses post-vaccination. However, whether they modulate the specificity of immune responses, particularly immunodominant epitope responses, and the mechanisms of regulating antigen processing and presentation remain poorly defined. Here, using overlapping synthetic peptides, we screened the dominant epitopes of Th1 responses in mice post-vaccination with different adjuvants and found that the adjuvants altered the antigen-specific CD4⁺ T-cell immunodominant epitope hierarchy. MHC-II immunopeptidomes demonstrated that the peptide repertoires presented by APCs were significantly altered by the adjuvants. Unexpectedly, no novel peptide presentation was detected after adjuvant treatment, whereas peptides with high binding stability for MHC-II presented in the control group were missing after adjuvant stimulation, particularly in the MPLA- and CpG-stimulated groups. The low-stability peptide present in the adjuvant groups effectively elicited robust T-cell responses and formed immune memory. Collectively, our results suggest that adjuvants (MPLA and CpG) inhibit high-stability peptide presentation instead of revealing cryptic epitopes, which may alter the specificity of CD4⁺ T-cell-dominant epitope responses. The capacity of adjuvants to modify peptide-MHC (pMHC) stability and antigen-specific T-cell immunodominant epitope responses has fundamental implications for the selection of suitable adjuvants in the vaccine design process and epitope vaccine development.

eLife Assessment

This **important** study provides interesting insights into the mechanisms of action of adjuvants. It shows that adjuvants, MPLA and CpG especially, modulate the peptide repertoires presented on the surface of antigen presenting cells, and surprisingly, adjuvant favored the presentation of low-stability peptides rather than high-stability peptides by antigen presenting cells. As a result, the low stability peptide presented in adjuvant groups elicits T cell response effectively. Evidence in support of these conclusions is **solid**, and this paper would be of interest to vaccinologists and immunologists.

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Introduction

Adjuvants are an important composition of vaccines that enhance or prolong the immune responses to co-administered antigens post-vaccination. The most commonly used adjuvants stimulate antibody responses by relying on B-cells.^(1, 2) B-cell priming and memory forming are dependent on the cognate help of antigen-specific T-cells. T-cells also have direct effector functions against pathogenic infections. Thus, antigen-specific T-cells play a vital role in shaping the immune response during vaccination. However, the mechanisms by which adjuvants regulate the specificity of antigen-specific T-cell responses remain poorly understood.

Antigen-specific T-cell responses mainly focus on several peptides derived from antigen amino acids called immunodominant epitopes.⁽³⁾ Although specific T-cell responses are provoked by the same antigen, their response epitopes may differ, leading to diverse effects. Peptides presented by antigen-presenting cells (APCs) provide the first major signal for T-cells priming and determining their specificity. Although adjuvants can activate APCs and increase the expression of co-stimulatory molecules of APCs,⁽⁴⁾ the effect of adjuvants on the peptide repertoires presented by APCs or modulation of epitope-specific T-cell responses remains unknown.

Helicobacter pylori infects more than half the global population and causes chronic gastritis, peptic ulcers, gastric mucosa-associated lymphoid tissue lymphoma (MALT), and gastric cancer.^(5, 6) Studies in human and mouse models showed that the Th1 response provides more important protection against *H. pylori* than a humoral immune response. Unfortunately, no effective vaccines have been developed to drive T-cell responses.⁽⁷⁾ *H. pylori* provides an ideal experimental model to determine the mechanisms by which adjuvants regulate T-cell responses.

Studies on widely used adjuvants have found that some pattern recognition receptor (PRR) ligand adjuvants can induce a strong T-cell response.^(8, 9) PRR ligand adjuvants that target PRRs on the surface of innate immune cells, such as macrophages and dendritic cells, promote cytokine secretion and upregulate co-stimulatory molecule expression.⁽¹⁰⁾ Typical PRR adjuvants include MPLA, CpG, and MDP, which induce effective T-cell responses and have been successfully used in many vaccines such as HPV, HSV, and COVID-19 vaccines.^(11–13) However, their impact on antigen processing and presentation on APCs and immunodominant epitope responses is unknown.

In this study, using MPLA, CpG, and MDP adjuvants and *H. pylori* antigens, we demonstrated that immunodominant epitopes recognized by antigen-specific T-cells were altered by adjuvants. Furthermore, we showed that the adjuvants MPLA and CpG modulate the peptide repertoires present on the APCs surface. Surprisingly, instead of revealing cryptic epitopes or presenting high-stability peptides, peptides with high binding stability for MHC-II were restrained, and low-stability peptides were presented by APCs after adjuvant treatment. The low-stability peptide presented in the adjuvant groups effectively elicits a T-cell response. Thus, altering pMHC stability in APCs provides a fundamentally new mechanism for PRR adjuvants to regulate adaptive immunity. The implications of these observations are discussed.

Results

Immunodominant T-cell epitope hierarchy varies in mice vaccinated with different adjuvants

To determine the influence of adjuvants on the specificity of immune responses, BALB/c mice were vaccinated with antigen UreB, an effective antigen for the *H. pylori* vaccine, combined with the adjuvants CpG, MDP, and MPLA. Then, antigen-specific T-cells from immunized mice were expanded and their interferon- γ (IFN- γ) responses to 93 UreB overlapping 18mer peptides were screened using flow cytometry. T-cells from the CpG group exhibited a dominant response to 18mer peptides U313–330 and U403–426, while T-cells from the MDP group exhibited a dominant response to U409–426 and U481–498. T-cells from the MPLA group primarily recognized U313–U330 and U505–522, whereas U481–U504 was the dominant region in T-cells from the no-adjuvant group (**Figure 1** [↗](#)). These data indicate that immunodominant epitopes responding to antigen-specific T-cells varied in different adjuvant vaccination groups.

Profiling MHC-II peptides in adjuvant-treated APCs

Considering that T-cell repertoires are the same in naïve mice, we speculate that dominant epitope variations in different adjuvant vaccination groups result from alterations in APCs peptide presentation. To investigate the MHC-II–peptide repertoire presented by APCs, antigens from *H. pylori* ultrasonic supernatant were used to pulse H-2^d A20 cells combined with the adjuvants CpG, MDP, and MPLA. MHC-II–peptide complexes were immunoprecipitated. Bound peptides were eluted and identified by liquid chromatography–tandem MS (LC–MS/MS; **Figure 2A** [↗](#)). The whole proteomes of protein-pulsed A20 cells were also analyzed by LC–MS/MS to examine the effect of adjuvants on extracellular antigen phagocytosis and antigen processing- and presentation-associated protein expression.

A total of 4074 peptides were identified: 3408, 3257, 3227, and 3330 peptides in the PBS, MDP, MPLA, and CpG groups, respectively. As expected, the number and length distributions of the peptides were not influenced by the adjuvants (**Figures 2B** [↗](#) and **2C** [↗](#)). Most identified peptides were 16 mer. Next, the binding motifs of all identified peptides were analyzed (**Figure 2D** [↗](#)). No obvious differences were observed following the adjuvant treatment. The binding motifs of the peptides per individual MHC-II allele, H2-IA and H2-IE, were also analyzed (**Figure S1** [↗](#)). No major differences were detected. The amino acids observed at the main anchor locations were consistent with the expected binding motifs. The binding affinity data predicted using NetMHCIIpan showed that most MS-detected peptides bind to MHC-II alleles (%rank <10) and are generally assigned to the H2-IA allele (**Figure 2E** [↗](#); **Figure S1** [↗](#)). These results indicate that adjuvants did not affect MHC-II binding characteristics.

We also selected another H-2^d cell J774A.1, a macrophage cell line, for immunopeptidome analysis in this study. Briefly, 5×10^8 J774A.1 cells were used for immunopeptidomics. Moreover, fewer than 350 peptides were observed at a peptide spectrum match (PSM) level of < 1.0% false discovery rate (FDR). However, more than 5500 peptides were detected in 10^8 A20 cells at FDR < 1.0% (**Figure S2A** [↗](#)). CD86 and MHC-II molecule expression on J774A.1 cells was substantially lower than that on A20 cells (**Figure S2B** [↗](#)). Low MHC-II expression on J774A.1 cells could be the reason for the lack of peptides identified by LC–MS/MS. Thus, A20 cells instead of J774A.1 cells were used for the subsequent experiments.

Adjuvants affected the presentation of exogenous peptides exclusively

Next, we examined the exogenous peptides across the *H. pylori* genome from the MHC-II immunopeptidome. Surprisingly, *H. pylori* contained more than 3,000 proteins, but less than 30 proteins were detected in the MHC-II immunopeptidomes (**Figure 3A** [↗](#)). To test whether the MHC-

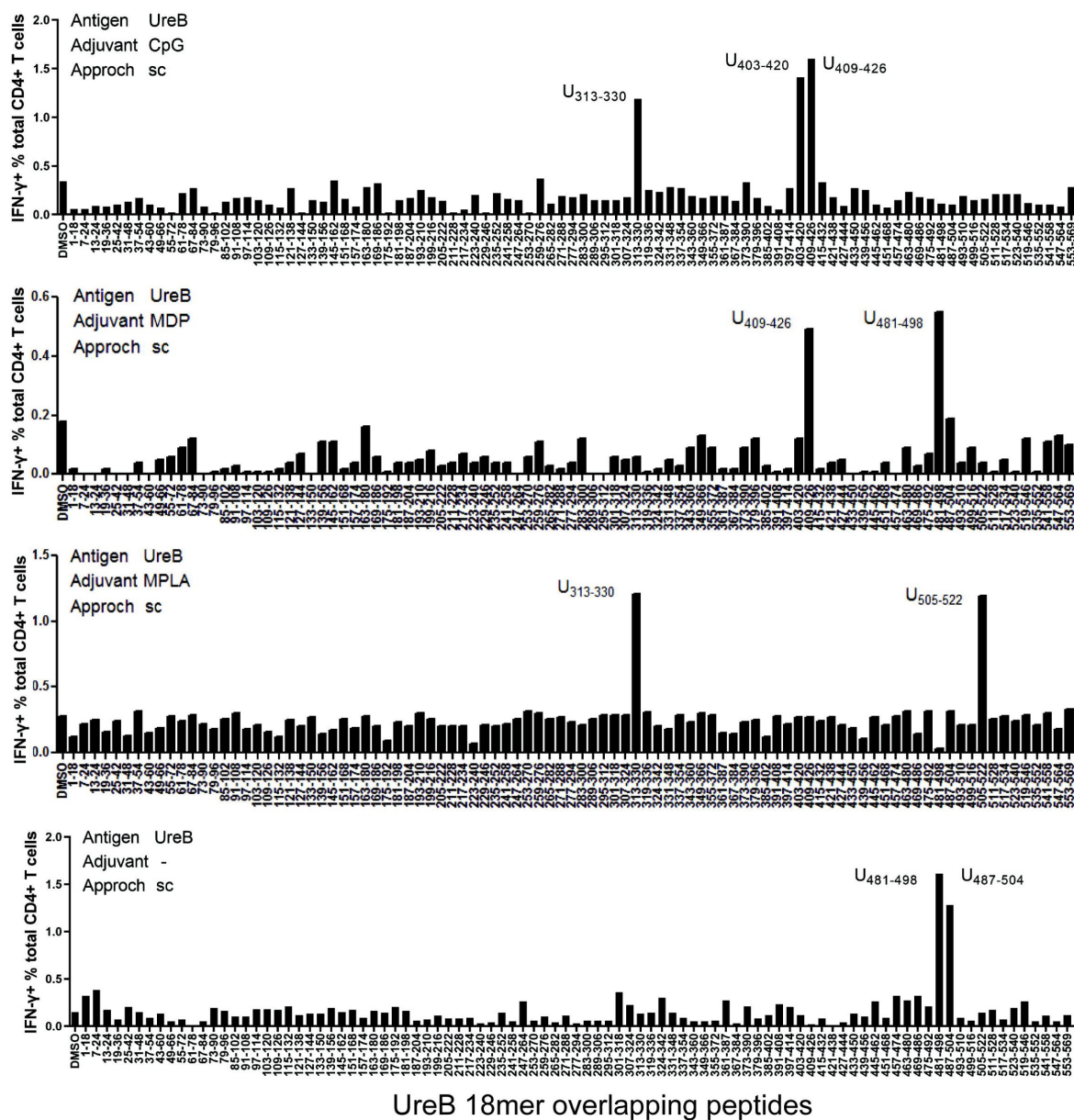


Fig. 1.

Immunodominant T-cell epitopes in different adjuvants vaccination mice.

Spleens were collected from mice on day 10 post-vaccination with the antigen UreB incorporated with adjuvants CpG, MDP, and MPLA; cultured *in vitro*; and stimulated with a panel of overlapping UreB 18 mer peptides to assess the responsiveness of CD4⁺ T-cells for interferon-γ (IFN-γ) using ICS. The percentages of CD4⁺ T-cells secreting IFN-γ against each peptide were determined using flow cytometry. Locations of the dominant peptides in different groups are indicated.

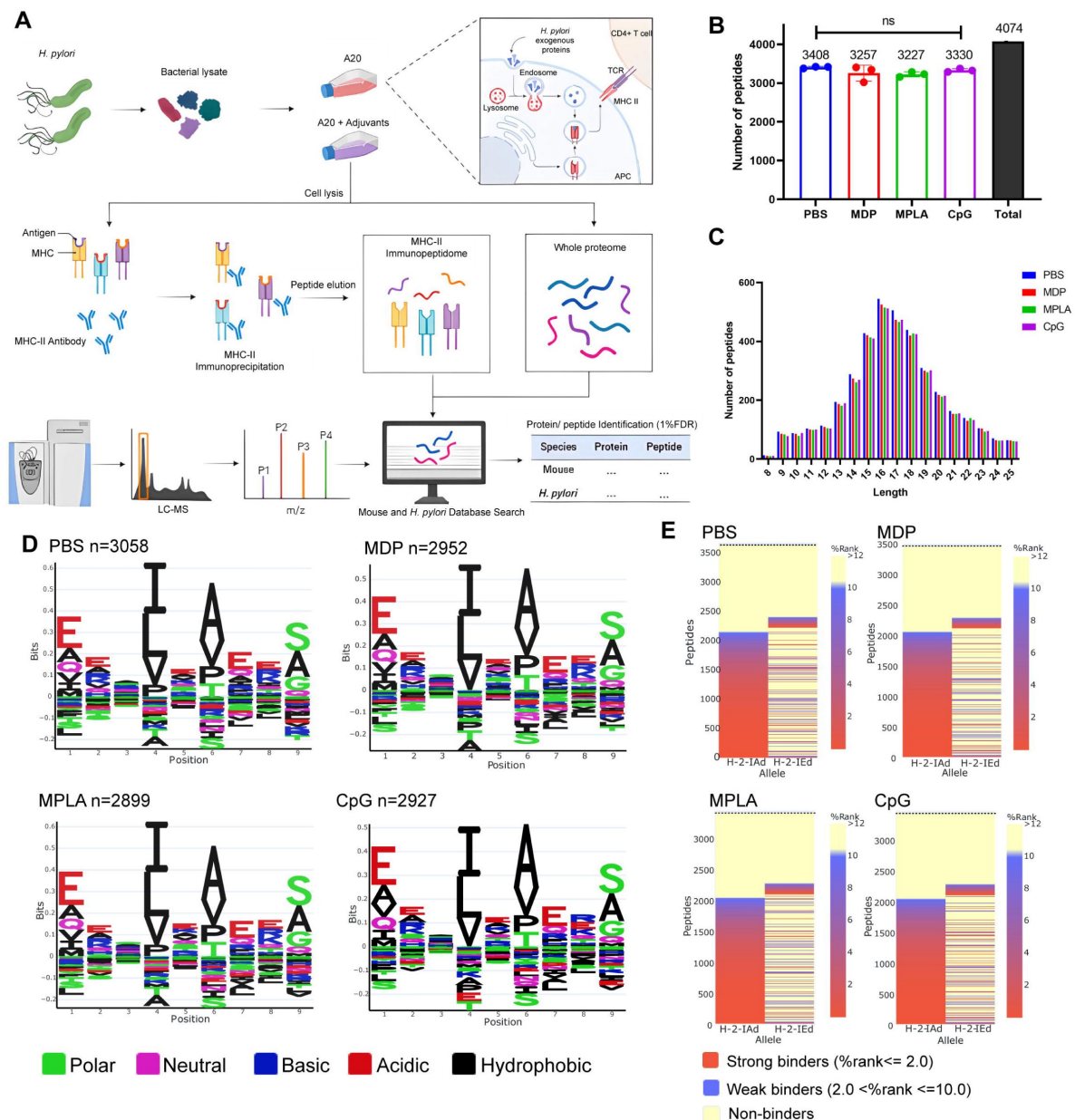


Fig. 2.

MHC-II peptidome and proteome measurements in adjuvant-treated antigen-presenting cells (APCs).

A20 cells were treated with CpG ODN, MDP, or MPLA incorporated with *Helicobacter pylori* antigens for 12 h. Most cells (10^8) were lysed for immunopeptidomics and the remaining cells (10^7) were used for proteomics. **(A)** Experimental flow chart. **(B)** Number of MHC peptides identified in the different adjuvant-treated groups ($n=3$). The numbers indicate mean values. **(C)** Length distribution of MHC peptides in different adjuvant-treated groups. **(D)** Sequence motifs of the MHC peptides identified in the adjuvant-treated groups. **(E)** Binding heatmaps of all eluted MHC peptides between 9–22 mer in adjuvant-treated groups were predicted and assigned to alleles using NetMHCIIpan. ns: not significantly different ($p>0.05$).

IP (MHC-immunoprecipitation) proteins had higher expression in bacteria, we ranked the individual *H. pylori* proteins according to their abundance. The abundance of MHC-IP-identified proteins in the entire *H. pylori* proteome MS data was analyzed, and we found that the abundance of these proteins was similar to that of other bacterial proteins (**Figure 3B** [↗](#)). We then verified whether MHC-IP proteins harbor more peptides compatible with MHC-II by comparing the ratio between the number of peptides predicted to bind MHC-II alleles and the total number of 13- to 17-mer. MHC-IP proteins contained more presentable peptides than most other bacterial proteins (**Figure 3C** [↗](#)).

By analyzing the host and exogenous MHC-II peptides present in different adjuvant groups, we found that 82.4% host MHC-II peptides were present in all the groups; however, only 34.4% exogenous MHC-II peptides were conserved after adjuvant treatment (**Figure 3D** [↗](#); **Figure S3** [↗](#)). These data indicate that adjuvants affect exogenous peptide presentation. Many exogenous peptides are missing after adjuvant MPLA or CpG treatment. However, no changes were detected in the number of peptides in the host (**Figure 3E** [↗](#)). These data indicate that the repertoires of exogenous peptides presented by APCs are affected exclusively by adjuvants, and a smaller number of peptides are present.

Adjuvants may affect antigen processing but not phagocytosis

To test whether the changes in exogenous peptide presentation among adjuvant groups could be explained by differences in antigen phagocytosis, we examined whole-proteome data. We observed a strong correlation between the bacterial protein abundances in different adjuvant-treated groups (**Figure 4A** [↗](#)). We then compared the abundance of bacterial proteins in the proteome and MHC-II immunopeptidomes and found that the bacterial protein abundance in the immunopeptidome changed significantly among the adjuvant-treated groups, and no peptides of several bacterial antigens were detected in some groups. However, only several proteins showed altered abundance in the proteome (**Figure 4B** [↗](#)). These results suggest that the changes in exogenous peptide presentation among the adjuvant groups cannot be explained by antigen phagocytosis.

To further investigate how adjuvants affect bacterial antigen processing and presentation, we ranked individual bacterial proteins and MHC-II peptides according to their abundance and compared them with those from the host. The overall abundance of bacterial proteins in the APCs proteome was low (**Figure S4A** [↗](#)). Individual bacterial protein expression was below that of most host proteins in both the PBS- and adjuvant-treated groups (**Figure 5A** [↗](#)). In contrast to the low abundance of bacterial proteins, the intensities of their MHC-II peptides were similar to those of peptides from the host in the PBS-treated group according to the immunopeptidome data (**Figure 5B** [↗](#)), indicating that bacterial peptides are preferentially present. However, in the MPLA- and CpG-treated groups, the intensities of bacterial MHC-II peptides in the immunopeptidome were much lower than those in the hosts (**Figure 5B** [↗](#)). These results indicate that adjuvants MPLA and CpG restrain bacterial peptide presentation.

We then investigated whole-proteome data to determine the evidence of adjuvant modification of antigen presentation. We focused on the proteins involved in antigen processing, peptidase function, ubiquitination pathway, and IFN signaling. The ubiquitination pathway and IFN signaling play crucial roles in the modification of antigen presentation and immune responses. Through KEGG enrichment analysis, we found that many proteins involved in antigen processing, peptidase function, ubiquitination pathways, and IFN signaling were altered after adjuvant treatment, particularly in the MPLA- and CpG-treated groups (**Figure 5C** [↗](#); **Figure S4B** [↗](#)). The expression of each protein is shown in **Figure S4C** [↗](#) and Supplementary Table 2. These data suggest that MPLA and CpG adjuvants may affect the antigen processing of APCs, resulting in fewer peptide presentations.

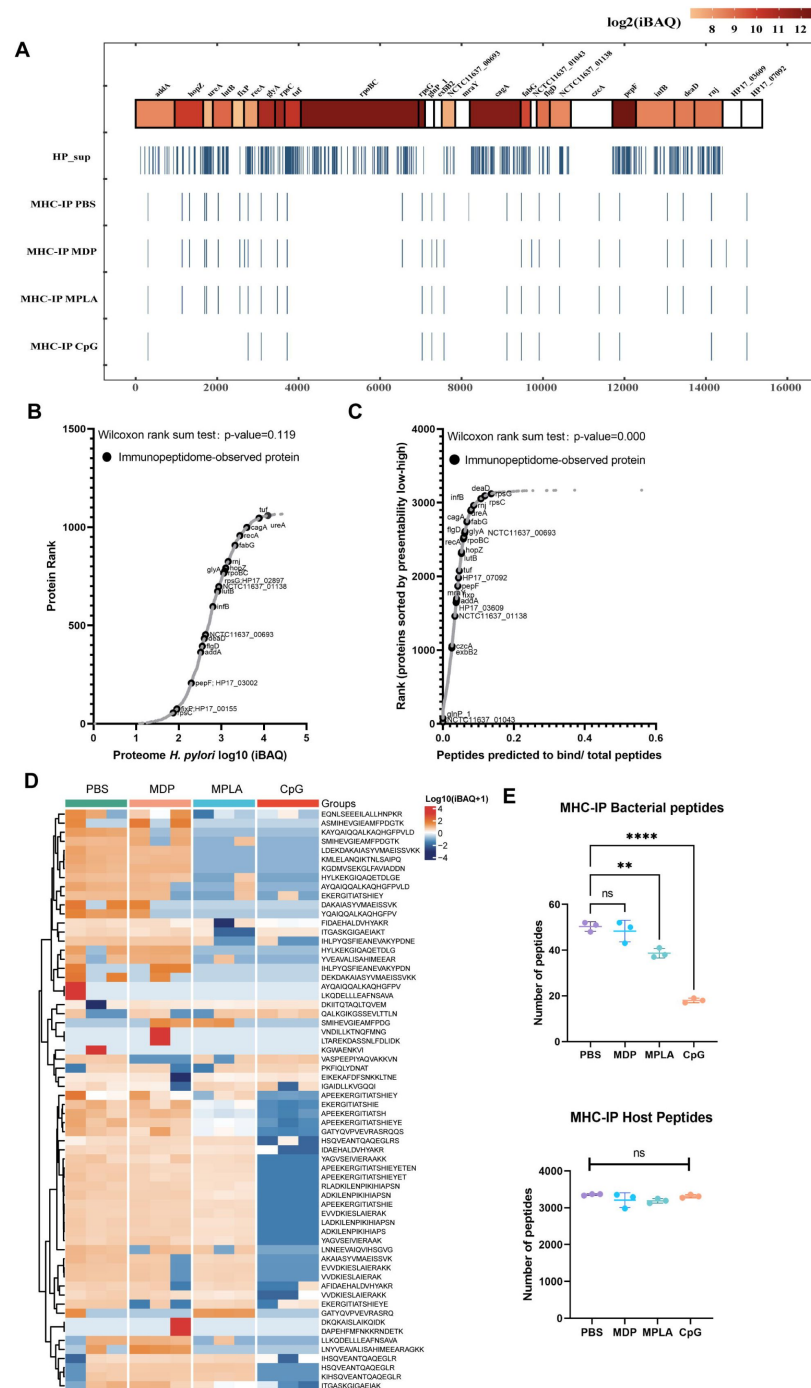


Fig. 3.

Profiling exogenous MHC-II peptides in adjuvant-treated antigen-presenting cells (APCs).

(A) Peptide locations across the *Helicobacter pylori* genome from MHC-II immunopeptidomes. (B) Rank plot of each protein abundance detected in the whole-proteome of bacterial ultrasonic supernatant antigens. Proteins identified in immunopeptidomes are annotated with their respective gene names. (C) MHC-II presentation potential of bacterial proteins. All reported *H. pylori* proteins were ranked according to the ratio between the number of peptides predicted to be presented by MHC-II alleles (rank≤2) and the total number of 13- to 17-mer. Proteins identified in immunopeptidomes are annotated with their respective gene names. (D) Heatmap of exogenous MHC peptides from different adjuvant groups. The identified sequences are shown. (E) Numbers of MHC peptides derived from bacteria and host were compared among different adjuvant groups. n=3. **p<0.01, ****p<0.0001.

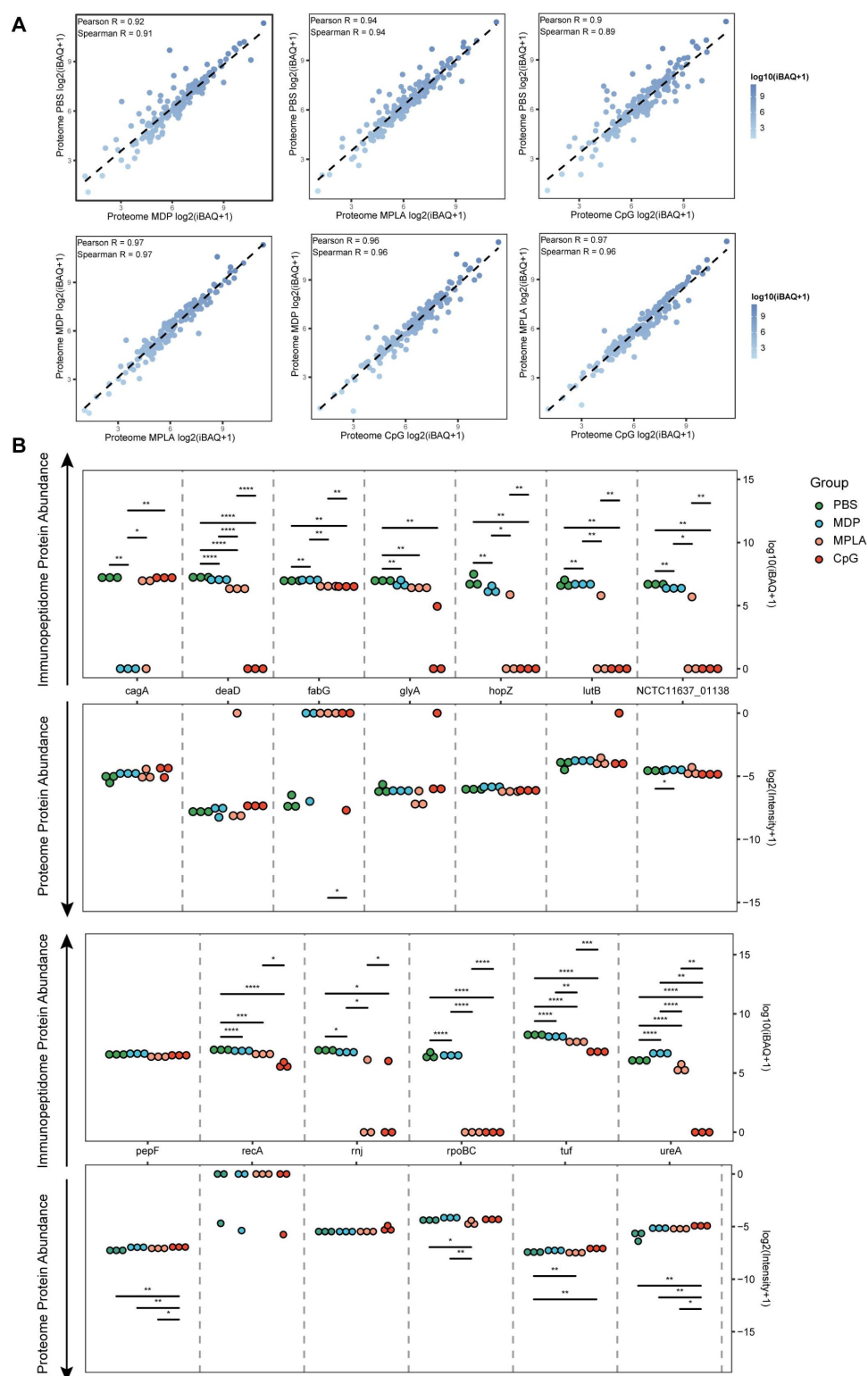


Fig. 4.

Antigen phagocytosis of antigen-presenting cells (APCs) treated with different adjuvants.

(A) Comparison of bacterial protein abundance in APCs 12 h post-adjuvant stimulation from whole proteomes. **(B)** Abundances of bacterial proteins from immunopeptidome and proteome were compared among adjuvant groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

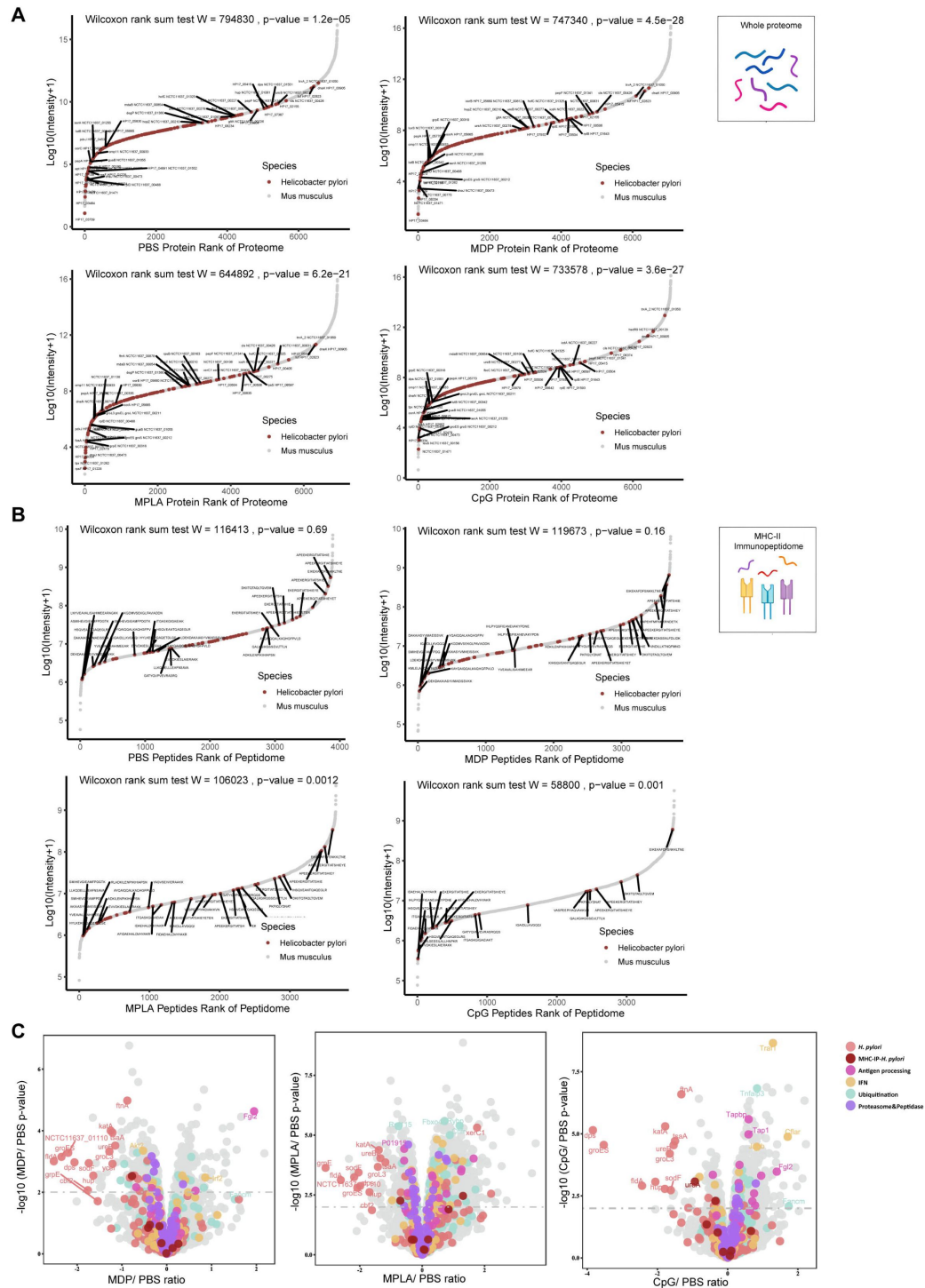


Fig. 5.

The effects of adjuvants on antigen presentation.

(A) Rank plot of host and bacterial protein abundances in the whole-proteome and (B) MHC-peptide abundances from immunopeptidomes of different adjuvant groups. Bacterial proteins are marked with red, and some of them are annotated with their respective gene names. Bacterial MHC peptides are annotated with their respective amino acid sequence. (C) Volcano plots comparing protein levels between PBS- and adjuvant-treated groups in the whole-proteome. Proteins involved in antigen processing, ubiquitination, proteasome & peptidase, and interferon (IFN) pathways are colored accordingly. Above the dashed line ($p < 0.01$) means significant.

High-stability epitopes were deficient in MPLA- and CpG-treated groups

We confirmed that the number of peptides from the exogenous antigens was significantly reduced in the MPLA- and CpG-treated groups. To further characterize the adjuvant effects on peptide presentation, the MHC-binding stability of the peptides present in the adjuvant-treated groups and that of the peptide-deficient post-adjuvant stimulation were analyzed using the IEDB website. Compared to that in the PBS-treated group, the IC⁵⁰ of the peptides binding to H2-IA and H2-IE alleles in the CpG- or MPLA-treated groups were much higher than those of the deficient peptides in the corresponding groups, which indicated that the peptides presented in the MPLA- and CpG-treated groups have lower binding stability for MHC-II (**Figure 6A** [↗](#)). Similar results were obtained at cutoffs of the predicted percentile rank <2 (Strong Binders) or <10 (Weak Binders) (**Figure S5** [↗](#)). Furthermore, we found that the peptides present in both adjuvant- and PBS-treated control groups were mainly derived from proteins *tuf*, *recA*, etc., and the deficient peptides in the MPLA- or CpG-treated group were mainly derived from proteins such as *ureA* and *hopZ* (**Figure 6B** [↗](#)). To validate the amino acid sequences and binding stability of MS-detected peptides, 10 peptides derived from the top four presented and deficient proteins shown in **Figure 6B** [↗](#), were synthesized (**Figure 6C** [↗](#)). The tandem mass spectra of the synthetic peptides and experimental spectra were compared, and strong correlations between fragment ions and retention times were observed (**Figure 6D** [↗](#)). We then performed an MHC-II competition-binding assay to detect the binding stability of the 10 synthetic peptides in the presence of one competing MHC-II ligand. We confirmed that the peptides missing in the MPLA-/CpG-treated groups had better binding stability for MHC-II molecules than the peptides presented in the adjuvant-treated groups (**Figure 6E** [↗](#)). These data indicate that epitopes with high binding stability were deficient after MPLA and CpG treatment.

Low-stability peptides presented in adjuvant-treated groups effectively induce robust T-cell responses

To evaluate whether the low-stability peptides presented in the adjuvant-treated groups could induce T-cell responses, mice were immunized with a pool of 10 synthetic peptides. Cell responses to individual peptides were detected using IFN- γ ELISpot assay on days 10 (effective phase) and 28 (memory phase) post-immunization (**Figure 7A** [↗](#)). We found positive responses to the low-stability peptides *recA* #23 (AFIDAEHALDVHYAKR) and NCTC11637-00693 #38 (IHSQVEANTQAQEGRL) as well as highly stable peptides *ureA* #2 (ASMIHEVGIEAMFPDGTK), *ureA* #3 (YVEAVALISAHIMEEAR), and *hopZ* #53 (KMLELANQIKTNLSAIPQ) on day 10 (**Figure 7B** [↗](#)). On day 28, the response to the low-stability peptide *recA* #23 (AFIDAEHALDVHYAKR) was not weaker than that of the other peptides (**Figure 7C** [↗](#)). Peptides with low MHC-II stability skew T-cell repertoires toward high-affinity clonotypes that have excellent responses against pathogen infection.^{(14 [↗](#), 15 [↗](#))} To assess the functional avidities of T-cell responses induced by the low-stability peptide presented in adjuvant-treated groups, peptide-specific CD4⁺ T-cells were expanded *in vitro*. Moreover, the magnitude of IFN- γ responses was detected by ICS using flow cytometry on stimulation with a set of titrated peptides. We found that the low-stability peptide *recA* #23 induced more robust CD4⁺ T-cell responses at lower peptide concentrations (**Figure 7D** [↗](#)). These data suggest that the low-stability peptide presented in the adjuvant-treated groups could induce robust CD4⁺ T-cell responses effectively.

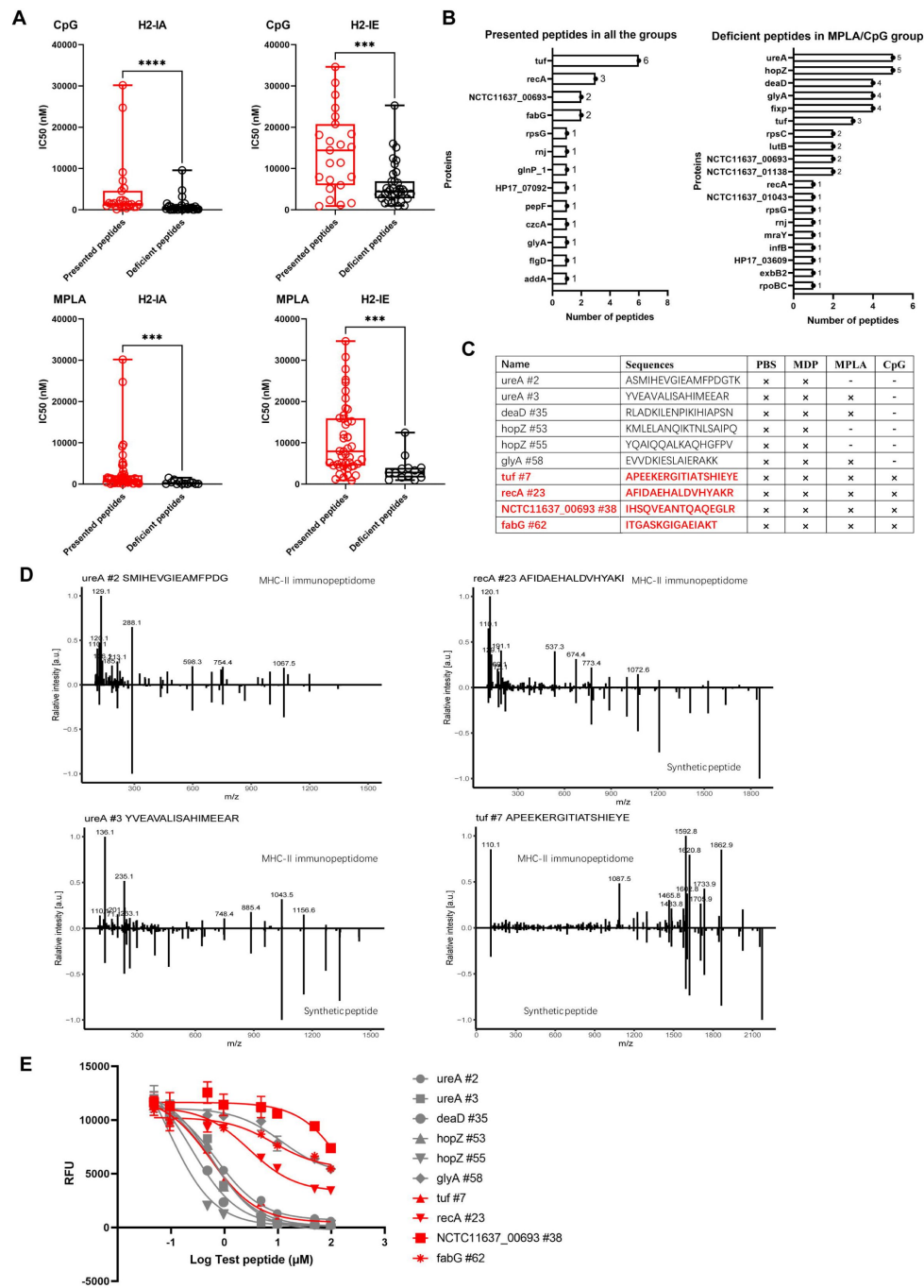


Fig. 6.

Binding affinity of MS-detected peptides was determined.

(A) IC_{50} of the presented and deficient peptides post-adjuvant stimulation from immunopeptidome binding to H2-IA and H2-IE were predicted by the NN align method using the IEDB website. The data of peptides from adjuvants MPLA and CpG are shown. High IC_{50} means low binding stability. (B) Distribution of proteins corresponding to bacterial MHC peptides from immunopeptidome. The numbers of peptides identified by MS for each protein are indicated. (C) Information of 10 synthetic peptides from Top4 presented and deficient proteins. x: Presence of peptides in the corresponding group. -: Peptides missing in the corresponding group. (D) Mirror plots with fragment ion mass spectra to confirm the sequences of MHC peptides from immunopeptidome. Positive y-axis, MHC-II IP sequences; negative y-axis, synthetic peptides. (E) Competitive binding curve of synthetic peptides for MHC-II H2-IA allele. The binding curves of peptides presented in adjuvant groups are marked with red.

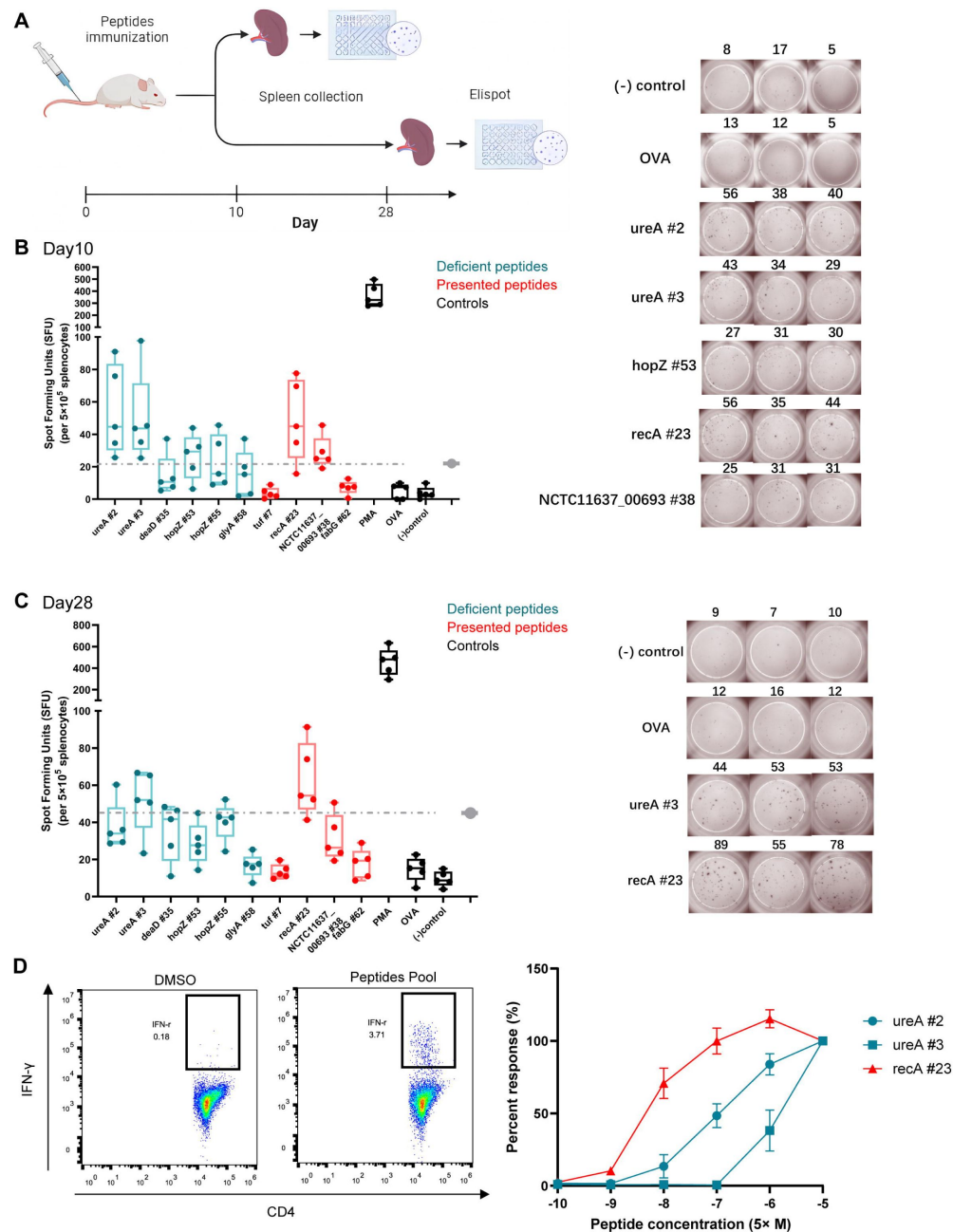


Fig. 7.

T-cell responses induced by MS-detected peptides with different binding stability were analyzed.

Five BALB/c mice were immunized with a pool of 10 synthetic peptides. On days 10 and 28 post-immunization, the splenocytes were isolated and stimulated with individual peptides for interferon- γ (IFN- γ) Elispot assay. **(A)** Experimental flow chart. **(B)** and **(C)** Elispot results on days 10 and 28. OVA peptide and non-stimulated wells were used as negative controls. PMA stimulation was used as the positive control. Responses to the peptides in the adjuvant groups are marked in red. The dashed line represents the $3 \times$ median of the OVA peptide used as the threshold for a positive response. Boxes show quartiles, bars indicate medians, and whiskers show distributions. Elispot images of positive responses from one immunized mouse are shown. Numbers indicate spot counts. **(D)** Epitope-specific CD4 $^{+}$ T-cells from immunized mice spleens were expanded *in vitro* and the IFN- γ producing CD4 $^{+}$ T-cells were assessed using the peptides pool (left). The present peptide recA #23 and deficient peptides ureA #2 and ureA #3 after adjuvant treatment were titrated to restimulate the expanded cells (right). The IFN- γ responses of CD4 $^{+}$ T-cells were detected by FACS. The responses induced by the indicated peptides at 50 μM were considered 100%. All other responses were evaluated based on their relative strengths.

Discussion

We demonstrated that adjuvants alter the specificity of the dominant Th1 epitope responses post-vaccination. Using an LC-MS/MS-based MHC-II immunopeptidome, the APCs-presented peptides of *H. pylori* antigens under the stimulation of different adjuvants were analyzed. The peptide motifs binding to MHC-II were generally consistent following stimulation with adjuvants. However, the exogenous peptide repertoires of APCs changed. Low- and high-stability peptides were presented by APCs in the control group; however, fewer peptides were detected in the adjuvant groups, and peptides with high binding stability for MHC-II presented in the control group were missing after adjuvant stimulation, particularly in the CpG group. The low-stability peptide presented in the adjuvant groups showed good immunoreactivity, effectively inducing effector and memory immune responses. Thus, our data suggested that adjuvants can restrain pMHC-II stability in APCs to regulate dominant epitope-specific T-cell responses.

The protective effects of vaccines depend on the immune responses induced post-vaccination. For prophylactic vaccines, adaptive immune responses are important and restricted to a few epitopes. However, not all epitope-specific responses were protective as some epitope-induced immune responses exacerbate inflammation and autoimmune diseases.^(16–18) Some epitopes induce Treg expansion and relieve inflammation.⁽¹⁹⁾ Thus, the alteration of dominant epitope responses directly affects vaccine effectiveness. Maeda *et al.* characterized the modulation of adjuvants LT and LTB on antibody responses to a co-administered antigen, EDIII. They showed that LT- and LTB-adjuvanted specific antibodies displayed distinct linear epitope-binding patterns and recognized fewer peptides than the non-adjuvanted or alum-adjuvanted groups.⁽²⁰⁾ However, Borriello *et al.* showed that mannans formulated with alum as an adjuvant broadened the epitope responses of anti-spike neutralizing antibodies.⁽²¹⁾ Chung *et al.* reported that ISCOMATRIX™ adjuvant promotes HA1 antibodies against large conformational epitopes spanning the receptor-binding domain, while antibodies targeting the C-terminus of the HA1 domain are generated in the unadjuvanted group. However, the number of recognized epitopes did not differ between groups.⁽²²⁾ Although the capacity of adjuvants to modulate the epitope specificity of antibodies has been described, the effects of adjuvants on T-cell epitope specificity remain unclear.

Lo-Man R *et al.* reported that *Salmonella enterica* expressing the MalE protein induced new CD4⁺ T-cell responses to peptides silent in the purified Ag with adjuvant CFA administration group.⁽²³⁾ Andersen *et al.* showed that an adenovirus vector expressing Ag85B/ESAT-6 fusion proteins induced a CD8⁺ T-cell response against the ESAT-6 epitope, while a CD4⁺ T-cell response to an epitope located in Ag85B was detected in the liposomal adjuvant group.⁽²⁴⁾ These studies suggest that specific T-cell responses are variable and antigen delivery formulations can affect CD4⁺ T-cell response specificity. In this study, using a series of synthesized overlapping peptides, we demonstrated that adjuvants modulate the hierarchy of epitope-specific Th1 responses post-vaccination.

Successful epitope-specific T-cell priming requires T-cell receptor (TCR) recognition by the pMHC on the APC surfaces. Adjuvants activate APCs and enhance co-stimulatory signals.^(25, 26) However, whether adjuvants can regulate the pMHC presentation on the APC surface lacks direct evidence. In this study, using LC-MS/MS-based immunopeptidomes, we confirmed that adjuvants affected the peptide repertoires presented by MHC-II molecules on APCs, and fewer peptides were presented by APCs after MPLA and CpG stimulation. A narrow peptide repertoire increases T-cell priming possibility and improves T-cell response specificity.⁽²⁷⁾ Thus, we speculate that the adjuvants MPLA and CpG may enhance vaccine-induced immune responses by restricting the diverse epitope presentation of APCs rather than inducing cryptic epitope presentation. Furthermore, we found that peptides with high binding stability for MHC-II were restrained, and

low-stability peptides were present on APCs after MPLA and CpG adjuvant treatment. Peptides with low MHC-II stability skew antigen-specific T-cell repertoires toward high TCR affinity clonotypes.⁽¹⁴⁾ Our data explain the findings of Baumgartner *et al.* that MPLA and CpG adjuvants induce high TCR affinity antigen-specific T-cell clonotype responses.⁽²⁸⁾ T-cell clonotypes with high TCR affinity have excellent abilities against pathogenic infections and tumors.^(15, 29, 30) In this study, we also observed robust T-cell responses induced by the peptide with low stability through the titration assay (**Figure 7D**), although no single T-cell clonotype was used. Unfortunately, only one peptide, recA #23, with low binding stability and induced significant Th1 responses, was identified in this study. To further confirm that low-stability peptides can induce stronger and higher TCR-affinity antigen-specific T-cell clonotype responses than high-stability peptides, further studies should monitor more peptides with different stabilities.

In this study, we found that the peptide repertoires presented by APCs were significantly affected by the adjuvants CpG and MPLA, but not MDP. All three adjuvants belong to the PRR ligand adjuvant family. CpG and MPLA bind to TLRs and MDP is recognized by NOD2. Although the receptors are different, many common molecules are involved both in TLR and NLD pathway activation. Unfortunately, we did not demonstrate why the MDP had different impacts on peptide presentation compared with other adjuvants. Further investigation is required to clarify the mechanism by which MPLA, CpG, and MDP adjuvants modulate the presentation of peptides with different stabilities.

APC subtypes differ in the expression of endolysosomal proteases and DO/DM molecules^(31–33) and exhibit different abilities to process Ag and present peptides.^(34, 35) Kanellopoulos *et al.* reported that DCs focused HEL-specific CD4⁺ T-cell responses against an I-E^d-restricted peptide HEL103-117, while B-cells presented an additional I-A^d-restricted peptide HEL7-31.⁽³⁶⁾ Schnurr *et al.* showed that CD1c⁺ blood DCs and MoDCs presented peptides of the antigen NY-ESO-1 on both MHC-I and MHC-II, while pDCs were limited to MHC-II presentation.⁽³⁷⁾ In this study, the antigen UreB adjuvanted with different adjuvants induced antigen-specific CD4⁺ T-cell responses *in vivo* post-vaccination, whereas no UreB peptides presented by A20 cells were detected *in vitro* using LC-MS/MS, possibly due to the differences in APC types involved, with many APC subtypes involved *in vivo*, particularly DCs, but only the B-cell line (A20) used *in vitro*. Another reason may be that the UreB peptide abundance was below the detection limit of LC-MS/MS *in vitro*.

A high abundance of pathogenic proteins is often preferred as candidate antigens in vaccine design. However, the amount of pathogen antigens were not consistent with that of antigens phagocytosed and presented by APCs. Weingarten-Gabbay *et al.* found that nucleocapsid protein (N) was the most abundant viral protein in SARS-CoV-2 infected A549 and HEK293T cells, but only one HLA-I peptide from N was present.⁽³⁸⁾ Thus, antigens with high expression may be less presented than expected and induce poor T-cell responses. We found that proteins harboring more peptides compatible with the MHC-binding motifs were more likely to be present (**Figure 3C**). Screening dominant epitopes from all pathogen antigens is important for vaccine design. This is an advantage of immunopeptidomes based on LC-MS/MS. Several epitopes with good immunogenicity detected in this study may be used as candidates for epitope vaccine development.

Taken together, our results reveal that adjuvants influence the pMHC stability on APCs and change the epitope response specificity. In the process of adjuvant selection for vaccines, the adjuvant effect on immune response strength as well as response specificity, particularly for PRR ligand adjuvants, which mainly induce Th1- or CTL-biased responses, should be considered.

Limitations of the study

First, the peptides screened *in vitro* using cell lines did not reflect the *in vivo* situation. The epitope presentation of APCs can be affected by the APC subtypes and inflammatory cytokines at the inoculation site(39), which are difficult to simulate and reproduce *in vitro*. Second, peptide abundance below the limit of detection may be missed when using LC–MS/MS-based assays, which may cause false negatives. The possibility that these peptides induce immune responses cannot be ruled out. Third, human MHC molecules are more complex than mouse MHC molecules. Thus, whether the conclusions of this study can be generalized to the human population requires further investigation.

Materials and Methods

Synthetic peptides, antibodies, and other reagents

The 18mer peptides overlapping by 12 amino acids derived from antigen UreB were synthesized and purified (purity >90%) by ChinaPeptides (Shanghai, China). All peptides were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO, USA) and stored at –80 °C. Information on the antibodies and other reagents used is given in Supplementary Table 1.

Cell culture

Mouse A20 (H-2^d, B-cell lymphoma cell line) and J774A.1 (H-2^d, monocyte/macrophage cell line) cells were obtained from the American Tissue Culture Collection (ATCC, VA, USA). A20 cells were cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). J774A.1 cells were maintained in DMED (Gibco) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. All cell lines were maintained at 37 °C in a humidified incubator with 5% CO₂.

Mice immunization and specific T-cell bulk culture

This study was approved by the Animal Ethics Review Committee of the Eighth Affiliated Hospital of Sun Yat-Sen University.

Six- to eight-week-old SPF female BALB/c mice were immunized subcutaneously with 100 µg recombinant *H. pylori* UreB (rUreB, purity >95%) emulsified in adjuvants CpG (20 µg/ mouse), MDP (30 µg/ mouse), and MPLA (10 µg/ mouse). Mice immunized with the same antigen without adjuvants were used as controls. Ten days later, the mice were euthanized with Carbon Dioxide (CO₂) and their spleens were harvested. Antigen-specific T-cells were expanded *in vitro* as described previously.(40) Briefly, the lymphocytes from spleens were isolated using a Ficoll–Hypaque (Dakewe, Shanghai, China) gradient, pulsed with 0.5 µM rUreB protein in the presence of 5 U/mL rmIL-2 (PeproTech, NJ, USA), and *in vitro* cultured in “RF-10” medium consisting of RPMI 1640 (Gibco, CA, USA) supplemented with 10% fetal calf serum (Gibco), 1% L-glutamine, 1×2-mercaptoethanol (Gibco), and 100 U/mL penicillin/ streptomycin. On day 5, the live cells were collected using a Ficoll–Hypaque gradient and cultured in a complete medium containing 20 U/mL rmIL-2. Half the medium was replaced when required.

Flow cytometry

Bulk cultured T-cells were stimulated with 5 µM peptides for 5 h in the presence of brefeldin A (BioLegend). The cells were collected and stained with specific antibodies against surface markers. Intracellular cytokine staining was performed after fixation. Cells were acquired using an

LSRFortessa Flow Cytometer (BD Biosciences) or a Navios Flow Cytometer (Beckman Coulter, FL, USA). The data were analyzed using FlowJo software (Tree Star, CA, USA).

***H. pylori* lysate preparation**

Helicobacter pylori strain NCTC 11637 (ATCC) was cultured on brain–heart infusion plates with 10% rabbit blood (Sbjbio, Nanjing, China) at 37 °C under microaerophilic conditions. Then, *H. pylori* was amplified in Brucella broth with 5% FBS (BI) at 37 °C under microaerophilic conditions with gentle shaking for 24 h. The bacteria were collected, washed, and re-suspended in PBS (Gibco) for lysis using an ultrasonic dismembrator (Biosafes, Nanjing, China). The lysates were centrifuged at 4 °C and 12000×*g* for 20 min. The supernatant was stored at –80 °C for subsequent experiments.

MHC-II complex immunoprecipitation

A20 cells were cultured in T⁷⁵ cell culture flasks (Corning, NY, USA). The expanded cells were pulsed with *H. pylori* lysates in combination with adjuvants MPLA, MDP, or CpG for 12 h, collected, washed twice with sterile PBS, and divided into two fractions. One fraction, approximately 10⁷ cells, was used for whole-proteome analysis. The remaining 10⁸ cells were lysed in cold lysis buffer (1.0% w/v CHAPS, Protease Inhibitor tablet, and PMSF) for MHC-II complex immunoprecipitation. The cell lysates were centrifuged at 18,000×*g* for 20 min. The supernatant (containing the MHC–peptide complexes) was transferred into a new 1.5 mL microcentrifuge tube (Corning) containing a mixture of Sepharose CNBr-activated beads (Cytivia, Utah, USA) and 2 mg Anti-Mouse H2-IA^d/IE^d (M5/114) antibody (BioXcell, NH, USA). The immune complexes were captured on the beads by incubating on a rotor at 4 °C for 18 h. Sequentially, the immune complexes were transferred to a polypropylene column (Bio-Rad Laboratories, Hercules, CA, USA) and washed with 10 mL buffer A (150 mM NaCl, 20 mM Tris, pH 8.0), 10 mL buffer B (400 mM NaCl, 20 mM Tris, pH 8.0), 10 mL buffer A, and 10 mL buffer C (Tris 20 mM, pH 8.0). The MHC-II–peptide complexes were eluted with 300 µL 10% glacial acetic acid (Macklin, Shanghai, China) three times. The eluate was stored at –80 °C until mass spectrometry analysis was performed.

MHC-II peptidome LC–MS/MS data generation

MHC peptides were eluted and desalted from the beads as described previously.⁽⁴¹⁾ The lyophilized peptides were re-suspended in ddH₂O containing 0.1% formic acid and 2 µL aliquots were loaded to a nanoViper C18 (Acclaim PepMap 100, 75 µm×2 cm) trap column. Online chromatographic separation was performed using an Easy nLC 1200 system (Thermo Fisher Scientific, MA, USA). The trapping and desalting procedures were performed with 20 µL 100% solvent A (0.1% formic acid). Then, an elution gradient of 5–38% solvent B (80% acetonitrile, 0.1% formic acid) in 60 min was used on an analytical column (Acclaim PepMap RSLC, 75 µm × 25 cm C18-2 µm 100Å). Data-dependent acquisition (DDA) mass spectrometry was used to acquire tandem MS data on a Q Exactive mass spectrometer (Thermo Fisher) fitted with a Nano Flex ion source using an ion spray voltage of 1.9 kV and an interface heater temperature of 275 °C. For a full mass spectrometry survey scan, the target value was 3×10⁶ and the scan ranged from 350 to 2,000 *m/z* at a resolution of 70,000 and a maximum injection time of 100 ms. For the MS2 scan, only spectra with a charge state of 2–5 were selected for fragmentation by high-energy collision dissociation with a normalized collision energy of 28. The MS2 spectra were acquired in the ion trap in rapid mode with an AGC target of 8,000 and a maximum injection time of 50 ms. The dynamic exclusion was 25 s.

Whole-proteome LC–MS/MS data generation

Protein aliquots were mixed with 200 µL 8 M urea in Nanosep Centrifugal Devices (PALL) and centrifuged at 12,000×*g* at 20 °C for 20 min. All the centrifugation steps were performed under the same conditions, allowing for maximal concentration. Then, 200 µL 8 M urea solution with 10 mM DTT was added, and the reduction reaction was performed for 2 h at 37 °C. The solution was removed by centrifugation and 200 µL 8 M urea solution with 50 mM iodoacetamide (IAA) was

added. The samples were incubated in the dark for 15 min at room temperature. The ultra-fraction tube was washed with 200 μ L 8 M urea three times and 200 μ L 25 mM ammonium bicarbonate three times by centrifugation at 12,000 $\times g$ for 20 min at room temperature. Then, 100 μ L 25 mM ammonium bicarbonate containing 0.01 μ g/ μ L trypsin was added to each filter tube and incubated at 37 °C for 12 h. The filter tubes were washed twice with 100 μ L 25 mM ammonium bicarbonate by centrifugation at 12,000 $\times g$ for 10 min. The flow-through fractions were collected and lyophilized.

The lyophilized peptides were re-suspended in ddH₂O containing 0.1% formic acid, and 2 μ L aliquots were loaded to a nanoViper C18 (Acclaim PepMap 100, 75 μ m $\times$ 2 cm) trap column. Online chromatographic separation was performed using an Easy nLC 1200 system (Thermo Fisher). The trapping and desalting procedures were carried out with 20 μ L 100% solvent A (0.1% formic acid). Then, an elution gradient of 5–38% solvent B (80% acetonitrile, 0.1% formic acid) in 60 min was used on an analytical column (Acclaim PepMap RSLC, 75 μ m $\times$ 25 cm C18-2 μ m 100 Å). A TimsTof Pro2 mass spectrometer (Bruker, USA) fitted with a Bruker captive spray ion source was operated in DIA-PASEF mode with a scan range of 100–1700 m/z and 10 PASEF ramps. The TIMS settings were 100 ms ramp and accumulation time (100% duty cycle) and 9.42 Hz ramp rate; this resulted in 1.8 s cycle time and setting at a 5000 absolute intensity threshold. The collision energy remained at the default with a base of 1.60/K0[V s/cm²] set at 59 eV and a base of 0.60/K0[V s/cm²] set at 20 eV. Active exclusion was enabled with a 0.4 min release. TIMS ranges were initially set from one range of 0.60–1.60/K0[V s/cm²], as seen in most published studies.

Peptide identification verification

The screened peptides were verified using synthetic peptides. Peptides were synthesized by ChinaPeptides (Shanghai, China) at a purity > 90% and dissolved to 10mM with DMSO. For LC–MS/MS measurements, the peptides were pooled and further diluted with 0.1% FA/3% ACN to load 120 fmol/mL on the column. LC–MS/MS measurements were performed as previously described. The experimental and synthetic sequences were confirmed by plotting fragment ion mass spectra.

MHC-II-peptide binding assay *in vitro*

A competition assay based on the binding of a high-affinity biotin-labeled control peptide (biotin–(Ahx)–(Ahx)–YAHAAHAAHAAHAAHAA) to MHC-II molecules was used to test peptide binding to MHC-II molecules. The assays were performed as previously described.⁽⁴²⁾ Briefly, test peptides were diluted to a series of concentrations and co-incubated with 0.1 μ M biotin-labeled peptide and 50 nM purified MHC-II proteins (ProImmune, Oxford, UK) at 37 °C for 24 h in the presence of octyl- β -D-glucopyranoside (Sigma-Aldrich). The binding reaction was neutralized and the products were transferred to MHC Class II (I-A/I-E) monoclonal antibody (M5/114.15.2) (Thermo Fisher)-coated ELISA plates (Corning), which were incubated at 4 °C overnight. Finally, diluted europium–streptavidin (Abcam, MA, USA) was added to each well of the ELISA plate, and the fluorescence was read using a time-resolved fluorescent plate reader (Thermo Fisher Scientific, MA, USA) with europium settings. Each peptide was tested at eight concentrations in three independent experiments.

Peptide immunization and ELISpot assay

BALB/c mice were immunized subcutaneously with a pool of synthetic peptides (50 μ g for each peptide) emulsified in Complete Freund's Adjuvant (Sigma-Aldrich). At 10 and 28 days post-vaccination, the mice were euthanized, and the spleens were removed for ELISpot assays.

Splenocytes (500,000 cells/well) were treated with red blood cell lysis buffer and stimulated with 5 μ M peptides in triplicate in ELISpot plates (Dakewe Biotech, Shenzhen, China) for 18 h. IFN- γ secretion was detected using capture and detection antibodies and imaged using an ELISpot &

FluoroSpot Reader (Mabtech, Stockholm, Sweden). OVA peptide^{323–339} (ISQAVHAAHAEINEAGR) and non-stimulated wells were used as negative controls. PMA (BioGems, NJ, USA) was used as a positive control. A 3-fold increase over baseline was used as the threshold for positive responses.

Peptide–MHC-II binding motif prediction

The peptide–MHC-II binding motifs, alignment, and peptide clustering were predicted using the MhcVizPipe (MVP) software tool (<https://github.com/CaronLab/MhcVizPipe>). The MHC-II alleles I-A^d or I-E^d were selected while maintaining all standard parameters.

MHC-II binding prediction

The online tools NetMHCIIpan 4.1, SMM alignment, and NN_ alignment in IEDB (<https://www.iedb.org/>) were used to predict the binding of peptides to MHC-II alleles. To test whether MS-detected proteins harbored more peptides compatible with MHC-II binding motifs, all *H. pylori* strain NCTC 11637 protein sequences were retrieved from the UniProt database, and the ratio between the number of peptides predicted to bind MHC-II alleles and the total number of 13- to 17-mer at a cutoff of predicted percentile rank values (%) <2 were compared.

Statistical analysis

Data are shown as mean ±SD. One-way analysis of variance (ANOVA) was performed to compare statistical significance among three or more groups. Student's *t*-test was used to compare the differences between the two groups; however, when the variances differed, the Mann–Whitney U test was used. The chi-square test was used to analyze the differences in the constituent ratios between the two groups. Pearson's correlation was used to test the correlation between two continuous variables, and Spearman's correlation was used for categorical variables. SPSS statistical software (version 25; SPSS Inc., IL, USA) and GraphPad Prism (version 9.0; GraphPad Software, CA, USA) were used for the statistical analyses. *p*<0.05 was considered statistically significant.

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Author contributions

Bin Li: Funding acquisition; Project administration; Investigation; Formal analysis; draft manuscript; Writing—review and editing.

Jin Zhang: Investigation; Data analysis; Methodology; Writing—review and editing.

Taojun He: Data curation; Investigation; Methodology.

Hanmei Yuan: Methodology.

Hui Wu: Methodology.

Peng Wang: Project administration; Supervision; Validation; Resources; Writing—review and editing.

Chao Wu: Conceptualization; Funding acquisition; Project administration; Supervision; Validation; Resources; Writing—review and editing.

Competing interests

The authors declare no competing interests.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The original mass spectra and peptide spectrum matches used in this study have been deposited in the public proteomics repository iProX (<https://www.iprox.cn/page/home.html>) under accession number IPX0007611000. Data are also available from the corresponding authors on reasonable request.

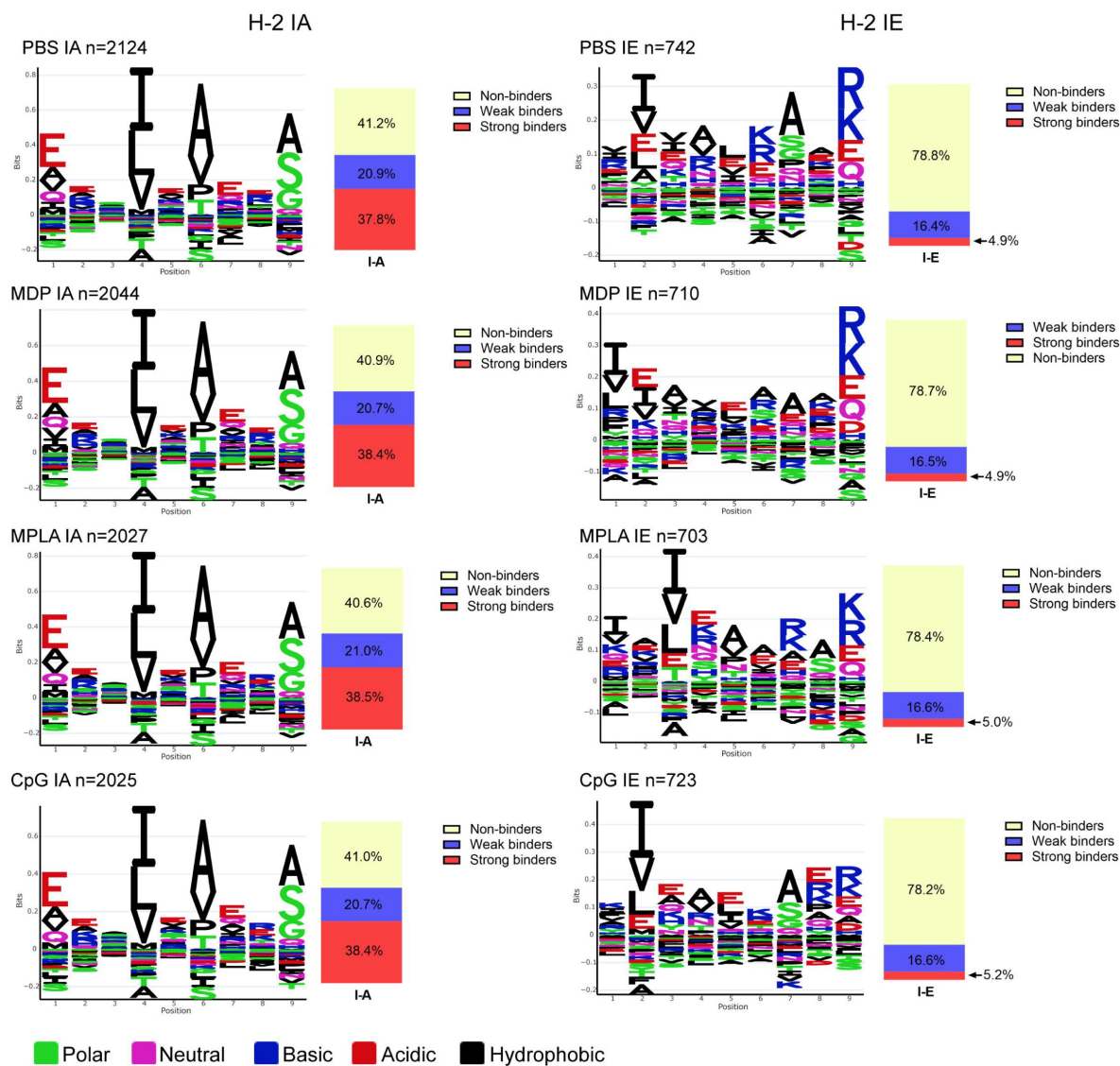


Figure S1.

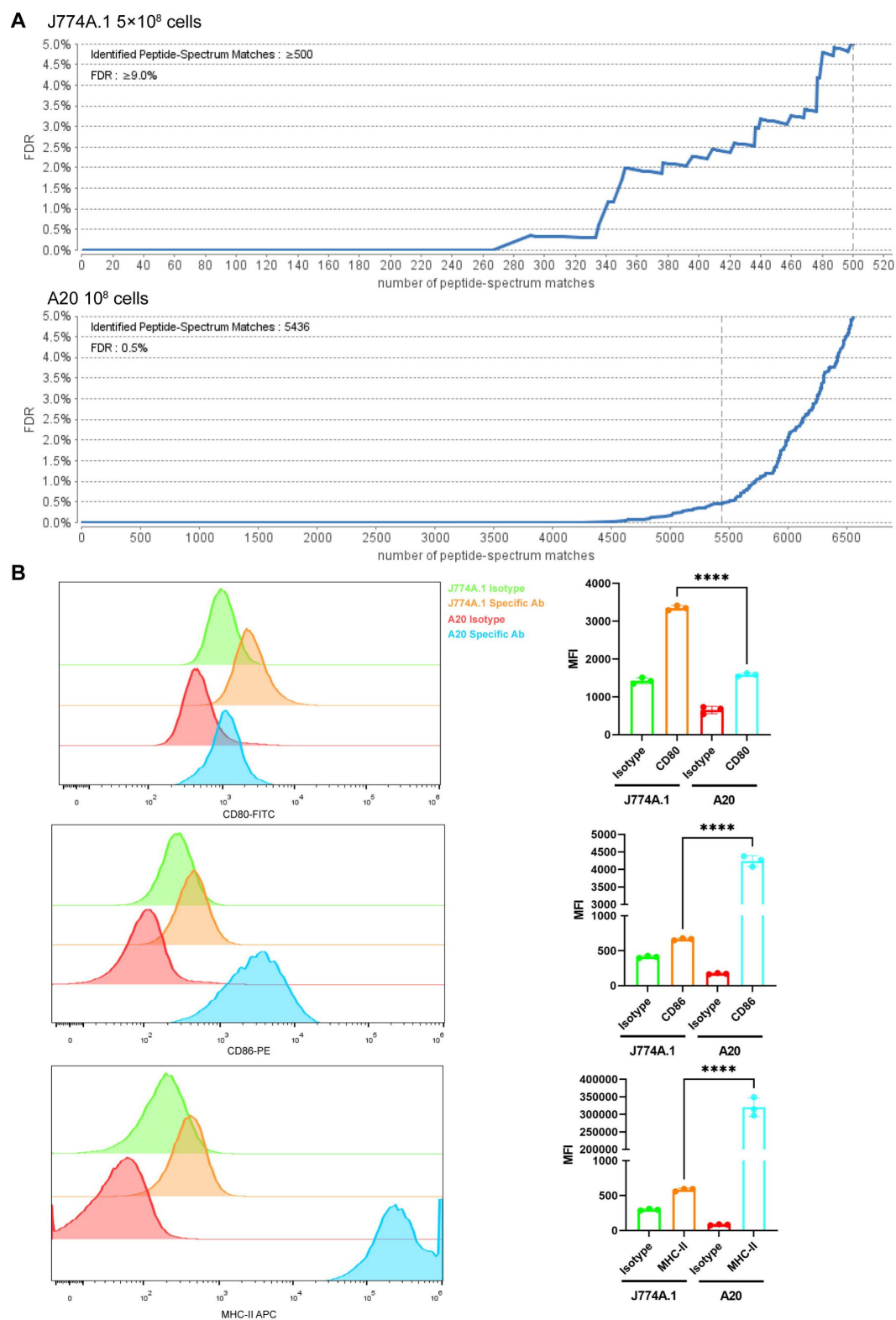


Figure S2.

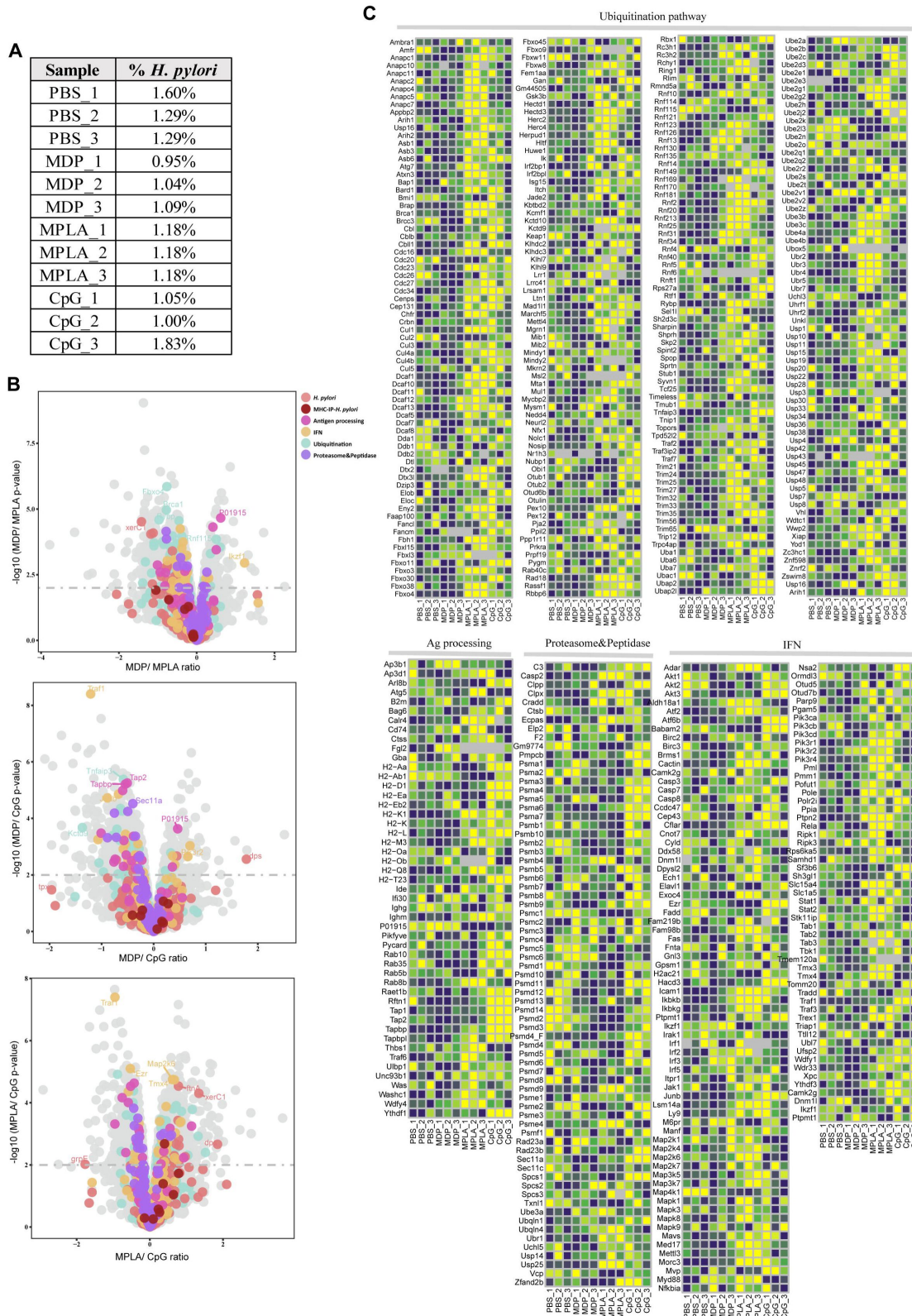


Figure S4.

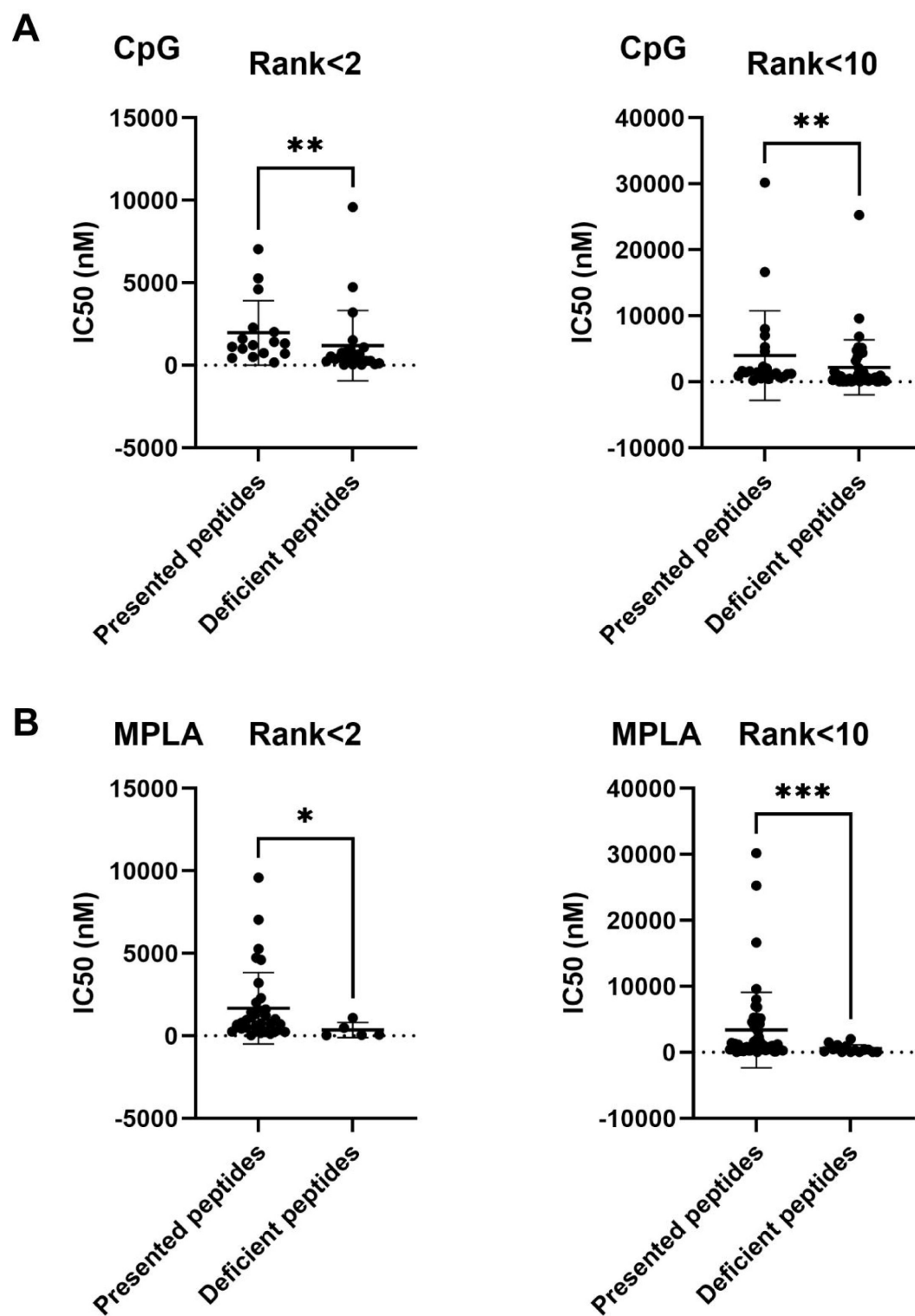


Figure S5.

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Reviewer #1 (Public Review):

Summary:

Li et al investigated how adjuvants such as MPLA and CpG influence antigen presentation at the level of the Antigen presenting cell and MHCII : peptide interaction. They found that use of MPLA or CpG influences the exogenous peptide repertoire presented by MHC II molecules. Additionally, their observations included the finding that peptides with low-stability peptide:MHC interactions yielded more robust CD4+ T cell responses in mice. These phenomena were illustrated specifically for 2 pattern recognition receptor activating adjuvants. This work represents a step forward for how adjuvants program CD4+ Th

responses and provide further evidence regarding expected mechanisms of PRR adjuvants in enhancing CD4⁺ T cell responses in the setting of vaccination.

Strengths:

The authors use a variety of systems to analyze this question. Initial observations were collected in an *H. pylori* model of vaccination with a demonstration of immunodominance differences simply by adjuvant type, followed by analysis of MHC:peptide as well as proteomic analysis with comparison by adjuvant group. Their analysis returns to peptide immunization and analysis of strength of relative CD4⁺ T cell responses, through calculation of IC₅₀ values and strength of binding. This is a comprehensive work. The logical sequence of experiments makes sense and follows an unexpected observation through to trying to understand that process further with peptide immunization and its impact on Th responses. This work will premise further studies into the mechanisms of adjuvants on T cells

Weaknesses:

While MDP has a different manner of interaction as an adjuvant compared to CpG and MPLA, it is unclear why MDP has a different impact on peptide presentation and it should be further investigated, or at minimum highlighted in the discussion as an area that requires further investigation.

It is alluded by the authors that TLR activating adjuvants mediate selective, low affinity, exogenous peptide binding onto MHC class II molecules. However, this was not demonstrated to be related specifically to TLR binding. Wonder if some work with TLR deficient mice (TLR 4KO for example) could evaluate this phenomenon more specifically

Lastly, it is unclear if the peptide immunization experiment reveals a clear pattern related to high and low stability peptides among the peptides analyzed.

<https://doi.org/10.7554/eLife.99173.2.sa2>

Reviewer #2 (Public Review):

Adjuvants boost antigen-specific immune responses to vaccines. However, whether adjuvants modulate the epitope immunodominance and the mechanisms involved in adjuvant's effect on antigen processing and presentation are not fully characterized. In this manuscript, Li et al report that immunodominant epitopes recognized by antigen-specific T cells are altered by adjuvants.

Using MPLA, CpG, and MDP adjuvants and *H. pylori* antigens, the authors screened the dominant epitopes of Th1 responses in mice post-vaccination with different adjuvants and found that adjuvants altered antigen-specific CD4⁺ T cell immunodominant epitope hierarchy. They show that adjuvants, MPLA and CpG especially, modulate the peptide repertoires presented on the surface of APCs. Surprisingly, adjuvant favored the presentation of low-stability peptides rather than high-stability peptides by APCs. As a result, the low stability peptide presented in adjuvant groups elicits T cell response effectively.

<https://doi.org/10.7554/eLife.99173.2.sa1>

Author response:

Reviewer #1 (Public Review):

Summary:

Li et al investigated how adjuvants such as MPLA and CpG influence antigen presentation at the level of the Antigen-presenting cell and MHCII : peptide interaction. They found that the use of MPLA or CpG influences the exogenous peptide repertoire presented by MHC II molecules. Additionally, their observations included the finding that peptides with low-stability peptide:MHC interactions yielded more robust CD4+ T cell responses in mice. These phenomena were illustrated specifically for 2 pattern recognition receptor activating adjuvants. This work represents a step forward for how adjuvants program CD4+ Th responses and provides further evidence regarding the expected mechanisms of PRR adjuvants in enhancing CD4+ T cell responses in the setting of vaccination.

Strengths:

The authors use a variety of systems to analyze this question. Initial observations were collected in an H pylori model of vaccination with a demonstration of immunodominance differences simply by adjuvant type, followed by analysis of MHC:peptide as well as proteomic analysis with comparison by adjuvant group. Their analysis returns to peptide immunization and analysis of strength of relative CD4+ T cell responses, through calculation of IC:50 values and strength of binding. This is a comprehensive work. The logical sequence of experiments makes sense and follows an unexpected observation through to trying to understand that process further with peptide immunization and its impact on Th responses. This work will premise further studies into the mechanisms of adjuvants on T cells.

Weaknesses:

Comment 1. While MDP has a different manner of interaction as an adjuvant compared to CpG and MPLA, it is unclear why MDP has a different impact on peptide presentation and it should be further investigated, or at minimum highlighted in the discussion as an area that requires further investigation.

Thank you for the suggestion. We investigated the reasons for the different effects of MDP on peptide presentation compared with those of CpG and MPLA. We found that the expression of some proteins involved in antigen processing and presentation, such as CTSS, H2-DM, Ifi30, and CD74, was substantially lower in the MDP-treated group than in the CpG- and MPLA-treated groups. To further confirm whether these proteins play a key role during adjuvant modification of peptide presentation, we knocked down them using shRNA and then performed immunopeptidomics. The original mass spectra and peptide spectrum matches have been deposited in the public proteomics repository iProX (<https://www.iprox.cn/page/home.html>) under accession number IPX0007611000. Unfortunately, the expected results for peptide presentation repertoires were not observed. Thus, we hypothesized that the different effects of MDP on peptide presentation might not result from differences in protein expression. We cannot exclude the possibility that some other proteins that may be important in this process were overlooked. We are still working on the mechanisms and do not have an exact conclusion. Thus, we did not present related data in this manuscript.

The related statements were added in the Discussion section on page 13, lines 292–299: “In this study, we found that the peptide repertoires presented by APCs were significantly affected by the adjuvants CpG and MPLA, but not MDP. All three adjuvants belong to the PRR ligand adjuvant family. CpG and MPLA bind to TLRs and MDP is recognized by NOD2. Although the receptors are different, many common molecules are involved both in TLR and NLD pathway activation. Unfortunately, we did not demonstrate why the MDP had different impacts on peptide presentation compared with other adjuvants. Further investigation is required to clarify the mechanism by which MPLA, CpG, and MDP adjuvants modulate the presentation of peptides with different stabilities.”

Comment 2. It is alluded by the authors that TLR activating adjuvants mediate selective, low affinity, exogenous peptide binding onto MHC class II molecules. However, this was not demonstrated to be related specifically to TLR binding. I wonder if some work with TLR deficient mice (TLR 4KO for example) could evaluate this phenomenon more specifically.

Thank you for the suggestion. This is an important point that was overlooked in this study. Based on published research on the mechanisms of PRR adjuvants, CpG and MPLA, we believe that the effect of CpG and MPLA on APCs-selective epitope presentation needs to be bound to the corresponding receptor, although we did not give a definitive conclusion in the manuscript.

To confirm the TLR-activating adjuvants affecting peptides presented on MHC molecules specifically through TLR binding, we have used CRISPR-cas9 to knock out TLR4 and TLR9 of A20 cells and repeated the experiments, as suggested. We chose TLR4- and TLR9- knockout A20 cell lines instead of TLR-deficient mice because a large number of APCs are required for immunopeptidomics. Moreover, the data observed in this study were based on the A20 cell line. However, these experiments are time-consuming. Unfortunately, we were unable to provide timely data. In addition, we believe that elucidating the downstream molecular mechanisms of TLR activation is necessary, as mentioned in comment 1. All these data will be combined and reported in our upcoming publications.

Comment 3. It is unclear to me if this observation is H pylori model/antigen-specific. It may have been nice to characterize the phenomenon with a different set of antigens as supplemental. Lastly, it is unclear if the peptide immunization experiment reveals a clear pattern related to high and low-stability peptides among the peptides analyzed.

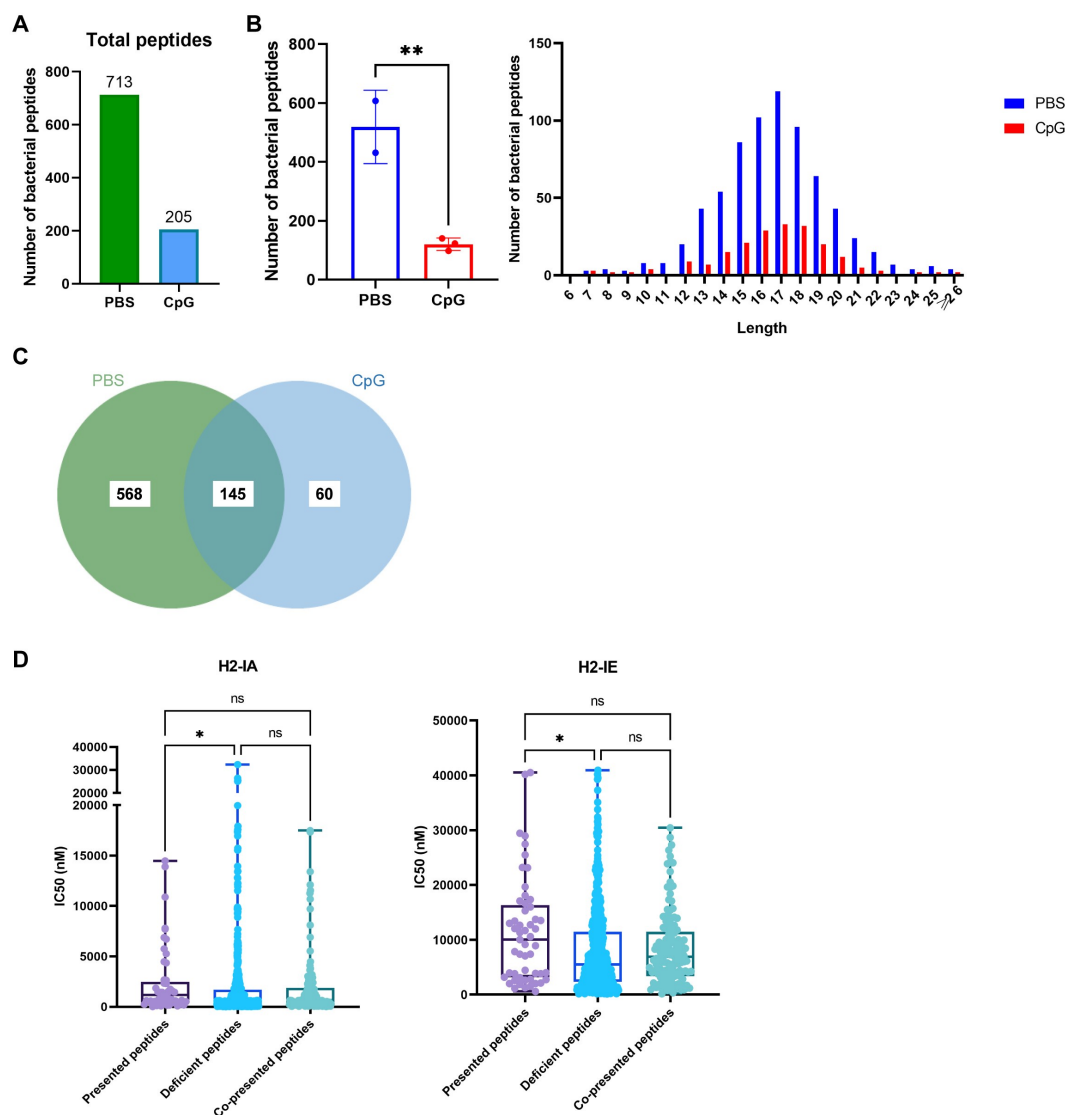
Q1: It is unclear to me if this observation is H. pylori model/antigen-specific. It may have been nice to characterize the phenomenon with a different set of antigens as supplemental.

Thank you for the comment. To confirm the effect of the adjuvant on the exogenous peptide repertoire presented by MHC II molecules, a set of antigens from another bacterium, *Pseudomonas aeruginosa*, was used, and the experiments were repeated. The A20 cells were treated with CpG and pulsed with *Pseudomonas aeruginosa* antigens. Twelve hours later, MHC-II-peptide complexes were immunoprecipitated, and immunopeptidomics were performed. The data are shown below (Author response image 1). Information on the MHC-peptides from *Pseudomonas aeruginosa* is given in the Supplementary Table named “Table S3 Response to comment3”. A total of 713 and 205 bacterial peptides were identified in the PBS and CpG groups (Author response image 1A). The number of exogenous peptides in the CpG-treated group was significantly lower than that in the PBS-treated control group (Author response image 1B). A total of 568 bacterial peptides were presented only in the PBS group; 60 bacterial peptides were presented in the CpG-treated group, and 145 bacterial peptides were presented in both groups (Author response image 1C). We then analyzed the MHC-binding stability of the peptides present in the adjuvant-treated group and that of the peptide-deficient after adjuvant stimulation using the IEDB website. We found that the IC₅₀ of the peptides in the adjuvant-treated group were much higher than those of the deficient peptides, which indicated that the peptides presented in the CpG-treated groups have lower binding stability for MHC-II (Author response image 1D). These results indicate that CpG adjuvant affects the presentation of exogenous peptides with high binding stability, which is consistent with the data reported in our manuscript. Using another set of antigens, we confirmed that our observations were not *H. pylori* model- or antigen-specific.

Author response image 1.

MHC-II peptidome measurements in adjuvant-treated APCs pulsed with *Pseudomonas aeruginosa* antigens.

(A) Total number of bacterial peptides identified in the PBS- and CpG-treated groups. (B) The number and length distribution of bacterial peptides in different groups were compared. (C) Venn diagrams showing the distribution of bacterial peptides in different groups. (D) IC₅₀ of the presented, deficient, and co-presented peptides post-adjuvant stimulation from immunopeptidome binding to H2-IA and H2-IE were predicted using the IEDB website. High IC₅₀ means low binding stability. * $p < 0.05$, ** $p < 0.01$.



Q2: Lastly, it is unclear if the peptide immunization experiment reveals a clear pattern related to high and low-stability peptides among the peptides analyzed.

In this study, we used a peptide immunization experiment to evaluate the responses induced by the screened peptides with different stabilities. In addition to this method, tetramer staining and ELISA have been used to assess epitope-specific T-cell proliferation and cytokine secretion. Among these, tetramer staining is often used in studies involving model antigens.

However, as many peptides were screened in our study, synthesizing a sufficient number of tetramers was difficult. However, we believe that the experimental data obtained in this study support the conclusion. Nevertheless, we agree that more methods applied will make the pattern more clearly.

Reviewer #2 (Public Review):

Adjuvants boost antigen-specific immune responses to vaccines. However, whether adjuvants modulate the epitope immunodominance and the mechanisms involved in adjuvant's effect on antigen processing and presentation are not fully characterized. In this manuscript, Li et al report that immunodominant epitopes recognized by antigen-specific T cells are altered by adjuvants.

Using MPLA, CpG, and MDP adjuvants and H. pylori antigens, the authors screened the dominant epitopes of Th1 responses in mice post-vaccination with different adjuvants and found that adjuvants altered antigen-specific CD4+ T cell immunodominant epitope hierarchy. They show that adjuvants, MPLA and CpG especially, modulate the peptide repertoires presented on the surface of APCs. Surprisingly, adjuvant favored the presentation of low-stability peptides rather than high-stability peptides by APCs. As a result, the low stability peptide presented in adjuvant groups elicits T cell response effectively.

Thanks a lot for your comments.

Reviewer #1 (Recommendations For The Authors):

Recommendation 1. Figure 6: The peptides considered low affinity- it would be helpful to specify from which adjuvant they were collected from. When they are pooled it is unclear if we are analyzing peptides collected from adjuvanting with any of the three adjuvants studied.

Thank you for the suggestion. The related description in Figure 6 has been modified in the revised manuscript. Data for the peptides identified from the adjuvants MPLA- and CpG-treated groups are shown separately.

Recommendation 2. It is unclear to me why the A20 cell line is less preferred to the J774 line for the immunopeptidome analysis - can the authors expand on this?

We apologize for not clearly explaining this in the original manuscript. In fact, the A20 cell line is better than J774A.1 cell line for immunopeptidomics experiments. Compared to J774A.1 cells, more MHC-II peptides were obtained from a smaller number of A20 cells using immunopeptidomics. At the beginning of this study, we chose the J774A.1 cell line as it is a macrophage cell line. J774A.1 cells (up to 5×10^8) were pulsed with the antigens, and MHC-II-peptide complexes were eluted from the cell surface for immunopeptidomics. Unfortunately, only a few hundred peptides from the host were detected and no exogenous peptides were detected. Next, we tested the A20 cell line. In total, 108 A20 cells were used in this study. More than 3500 host peptides and approximately 50 exogenous peptides have been identified. These data indicate that the A20 cell line was better.

To investigate the reasons for this, we detected MHC-II expression on cell surfaces using FACS. Our purpose was to elute peptides from MHC-peptide complexes present on the cell surface. Low MHC expression resulted in the elution of a few peptides. We found the MFI of MHC-II molecules on J774A.1 cell is about 500; however, the MFI of MHC-II molecules on A20 cells is more than 300,000. These data indicate that MHC-II expression on A20 cells was much higher than that on J774A.1 cells. J774A.1 cell is a macrophage cell line. Macrophages have excellent

antigen phagocytic capabilities; however, their ability to present antigens is relatively weak. MHC molecules on the macrophage cell surface can be upregulated in the stimulation of some cytokines, for example, IFN- γ . In this study, we used adjuvants as stimulators and did not want to use additional cytokine stimulators. Thus, J774A.1 cells were not used in the present study.

The related statements are reflected on page 6 lines 120–128 “We also selected another H-2d cell J774A.1, a macrophage cell line, for immunopeptidome analysis in this study. Briefly, 5×10^8 J774A.1 cells were used for immunopeptidomics. Moreover, fewer than 350 peptides were observed at a peptide spectrum match (PSM) level of $< 1.0\%$ false discovery rate (FDR). However, more than 5500 peptides were detected in 108 A20 cells at FDR $< 1.0\%$ (Figure S2A). CD86 and MHC-II molecule expression on J774A.1 cells was substantially lower than that on A20 cells (Figure S2B). Low MHC-II expression on J774A.1 cells could be the reason for the lack of peptides identified by LC-MS/MS. Thus, A20 cells instead of J774A.1 cells were used for the subsequent experiments.”

Recommendation 3. Lines 172-177, can more details be provided about the whole proteome analysis? The plots are shown for relative representation of protein expression to PBS, but it is unclear to me what examples of these proteins are (IFN pathway, Ubiquitination pathway). Could these be confirmed by protein expression analyses in supplemental?

Thank you for the suggestion. In this study, we conducted whole proteome analysis to investigate changes in protein expression across different pathways in the adjuvant groups. Through KEGG enrichment analysis, we compared the differential expression of MHC presentation pathway proteins (such as H2-M, Ifi30, CD74, CTSS, proteasome, and peptidase subunits) between the PBS- and adjuvant-treated groups using our proteome data. In addition, we focused on IFN and ubiquitination pathways that play crucial roles in antigen presentation modification and immune response. The proteins and their relative expression in these pathways are shown in Figure S4B. Details regarding the protein names and expressions are provided in Supplemental Table S2 of the revised manuscript.

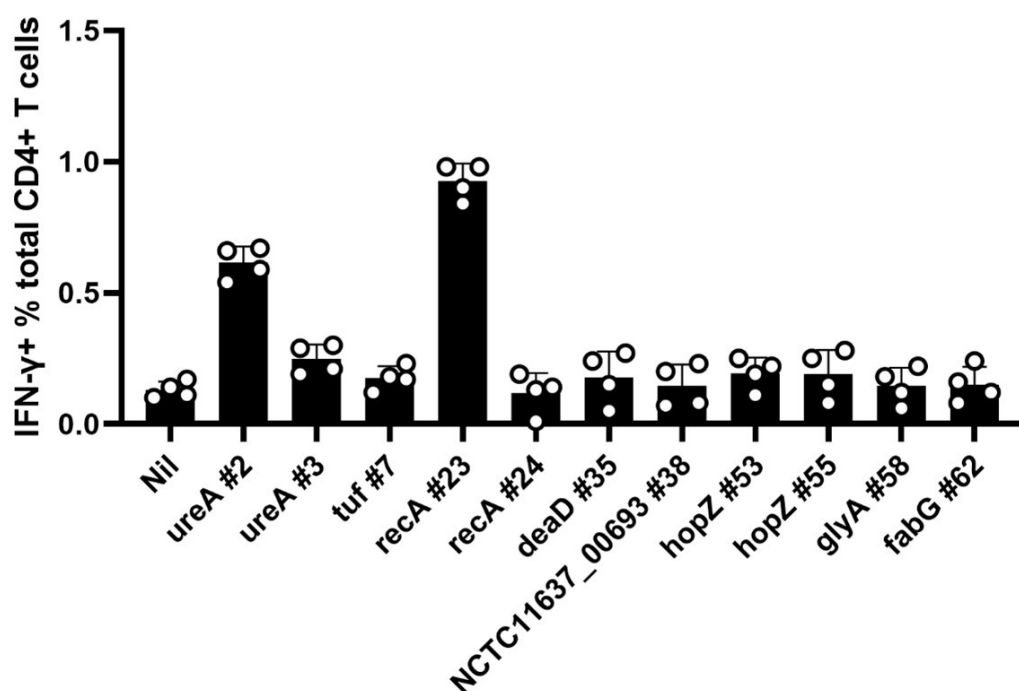
The original statements in the results “Then, we analyzed the whole proteome data to determine whether the proteins involved in antigen presentation and processing were altered. We found that proteins involved in antigen processing, peptidase function, ubiquitination pathway, and interferon (IFN) signaling were altered post adjuvants treatment, especially in MPLA and CpG groups (Figure 5C; Figure S4B and S4C). These data suggest that adjuvants MPLA and CpG may affect the antigen processing of APCs, resulting in fewer peptides presentation.” This has been revised on page 8 lines 172–182 as “We then investigated whole-proteome data to determine the evidence of adjuvant modification of antigen presentation. We focused on the proteins involved in antigen processing, peptidase function, ubiquitination pathway, and IFN signaling. The ubiquitination pathway and IFN signaling play crucial roles in the modification of antigen presentation and immune responses. Through KEGG enrichment analysis, we found that many proteins involved in antigen processing, peptidase function, ubiquitination pathways, and IFN signaling were altered after adjuvant treatment, particularly in the MPLA- and CpG-treated groups (Figure 5C; Figure S4B). The expression of each protein is shown in Figure S4C and Supplementary Table 2. These data suggest that MPLA and CpG adjuvants may affect the antigen processing of APCs, resulting in fewer peptide presentations.”

Recommendation 4. Lines 212-218: I think there needs to be more discussion of interpretation here. Only one of the low-stability peptides required low concentrations for CD4+ T cell responses in vitro. What about the other peptides in the analysis? Perhaps if the data is taken together there is not a clear pattern?

Thank you for the comment. In this study, epitope-specific CD4⁺ T-cells were expanded in vitro from the spleens of peptide-pool-immunized mice. T-cell responses to individual peptides were detected using ICS and FACS. Only one peptide, recA #23, with low binding stability, and one high-stability peptide, ureA #2, induced effective T-cell responses. Peptide ureA #3 with high stability induces low Th1 responses. The other peptides cannot induce CD4⁺ T-cell secreting IFN- γ (Data are shown in Author response image 2). Thus, we compared the strength of IFN- γ responses induced by these three peptides at a set of low concentrations. Data for other peptides without any response could not be taken together.

Author response image 2.

The expanded CD4⁺T cells from peptides immunized mice were screened for their response to the peptides in an ICS assay.



In this study, we used a peptide pool containing four low-stability peptides to vaccinate mice; however, only one peptide induced an effective CD4⁺ T-cell response. We speculate that the possible reasons are as follows. First, the number of peptides used for vaccination is too small. Only four low-stability peptides were synthesized and used to immunize mice. Three of these could not induce an effective T-cell response, possibly because of their low immunogenicity. If more peptides are synthesized and used, more peptides that induce T-cell responses may be observed. Second, epitope-specific T-cell responses are variable. Responses to the subdominant peptides can be inhibited by the dominant peptide. The subdominant peptide can become dominant by changing the peptide dose or in the absence of the dominant peptide. Thus, we believe that responses to the other three peptides may be detected if mice are immunized with a peptide pool that does not contain a response epitope.

The corresponding statements have been added to the Discussion section on page 13 lines 287–291 as “Unfortunately, only one peptide, recA #23, with low binding stability and induced significant Th1 responses, was identified in this study. To further confirm that low-stability peptides can induce stronger and higher TCR-affinity antigen-specific T-cell clonotype

responses than high-stability peptides, further studies should monitor more peptides with different stabilities.”

Recommendation 5. There are some areas where additional editing to text would be beneficial due to grammar (eg lines 122-126; line 116, etc).

The manuscript has been edited by a professional language editing company.

Reviewer #2 (Recommendations For The Authors):

Recommendation 1. It is interesting that there was no difference in IFN γ responses induced by different adjuvants.

Thank you for the comment. Possible reasons for the lack of difference in IFN- γ responses could be as follows. First, all adjuvants used in this study have been confirmed to effectively induce Th1 responses. Second, in this study, IFN- γ responses were examined using expanded antigen-specific T cells in vitro. The in vitro cell expansion efficiency may have affected these results.

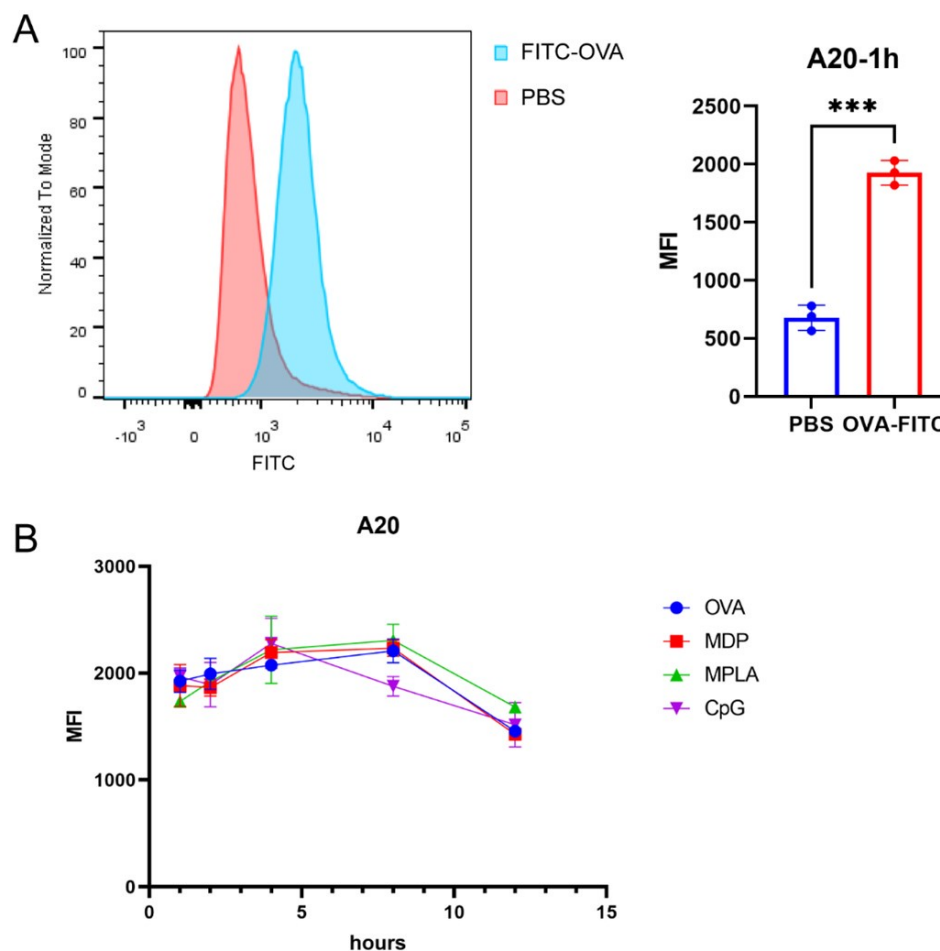
Recommendation 2. The data to support the claim that changes in exogenous peptide presentation among adjuvant groups were not due to differences in antigen phagocytosis is insufficient.

Thank you for the comment. In this study, proteomics of A20 cells pulsed with antigens in different adjuvant-treated groups were used to determine exogenous antigens phagocytosed by cells. In addition, we used fluorescein isothiocyanate (FITC)-labeled OVA to pulse APCs and detected antigen phagocytosis by APCs after treatment with different adjuvants. The MFI of FITC was detected by FACS at different time points. The data are shown below (Author response image 3). No obvious differences in FITC MFI were detected after adjuvant stimulation, indicating that antigen phagocytosis among the adjuvant groups was almost the same.

A20 cells, used as APCs, are the B-cell line. Antigen recognition and phagocytosis by B-cells depends on the B-cell receptor (BCR) on the cell surface. The ability of BCRs to bind to different antigens varies, leading to significant differences in the phagocytosis of different antigens by B-cells. Therefore, detecting the phagocytosis of a single antigen may not reflect the overall phagocytic state of the B-cells. Thus, in this study, we used proteomics to detect exogenous proteins in B-cells pulsed with *H. pylori* antigens, which contain thousands of components, to evaluate their overall phagocytic capacity. Only the proteomic data are presented in our manuscript.

Author response image 3.

Antigen phagocytosis of A20 cells were measured using FITC-labeled OVA. (A) A20 cells were pulsed with FITC-labeled OVA. MFI of FITC was measured after 1 h. (B) MFI of FITC was examined post the stimulation of adjuvants at different time points.



Recommendation 3. It is not clear how MPLA, CpG, and MDP adjuvants modulate the presentation of low vs high stability peptides.

Thank you for pointing this out. We acknowledge that we did not clarify the mechanisms by which adjuvants affect the stability of the peptide presentations of APCs.

We performed experiments to detect the expression of proteins involved in antigen processing and presentation in the different adjuvant-treated groups. Furthermore, shRNAs were used to knock down the expression of key molecules. Immunopeptidomics was used to detect peptide presentation. Unfortunately, the expected results for peptide presentation repertoires were not observed. We are still working on the mechanisms.

Please also see our response to comment 1 of reviewer 1

The related statements were added in the Discussion section on page 13, lines 292–299: “In this study, we found that the peptide repertoires presented by APCs were significantly affected by the adjuvants CpG and MPLA, but not MDP. All three adjuvants belong to the PRR ligand adjuvant family. CpG and MPLA bind to TLRs and MDP is recognized by NOD2. Although the receptors are different, many common molecules are involved both in TLR and NLD pathway activation. Unfortunately, we did not demonstrate why the MDP had different impacts on peptide presentation compared with other adjuvants. Further investigation is

required to clarify the mechanism by which MPLA, CpG, and MDP adjuvants modulate the presentation of peptides with different stabilities.”

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