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Novel epitopes identified from efflux pumps of *Mycobacterium tuberculosis* could induce cytotoxic T lymphocyte response

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Overcoming drug-resistance is one of the major challenges to control tuberculosis (TB). The up-regulation of efflux pumps is one common mechanism that leads to drug-resistance. Therefore, immunotherapy targeting these efflux pump antigens could be promising strategy to be combined with current chemotherapy. Considering that CD8+ cytotoxic T lymphocytes (CTLs) induced by antigenic peptides (epitopes) could elicit HLA-restricted anti-TB immune response, efflux pumps from classical ABC family (*Mycobacterium tuberculosis*, Mtb) were chosen as target antigens to identify CTL epitopes. HLA-A2 restricted candidate peptides from Rv2937, Rv2686c and Rv2687c of *Mycobacterium tuberculosis* were predicted, synthesized and tested. Five peptides could induce IFN- γ release and cytotoxic activity in PBMCs from HLA-A2⁺ PPD⁺ donors. Results from HLA-A2/K^b transgenic mice immunization assay suggested that four peptides Rv2937-p168, Rv2937-p266, Rv2686c-p151, and Rv2686c-p181 could induce significant CTL response *in vivo*. These results suggested that these novel epitopes could be used as immunotherapy candidates to TB drug-resistance.

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INTRODUCTION

Tuberculosis is a serious infection disease in developing countries, which is caused by *Mycobacterium tuberculosis* (Dye & Williams 2010). To date, the available strategies against tuberculosis mainly rely on chemotherapeutic regimens. However, with the emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB), it becomes more difficult to fight against tuberculosis. In addition to acquired drug resistance (Ramaswamy & Musser 1998), intrinsic drug resistance has been proved in *Mycobacterium tuberculosis* (Mtb) (De Rossi et al. 2006), in which active drug efflux pump does make contributions to drug resistance (Escribano et al. 2007; Spies et al. 2008).

As an intracellular pathogen living in macrophages, more and more evidence suggested the important role of cellular immunity in controlling the dissemination of Mtb. It has been proved that, in addition to CD4⁺ T cells, MHC class I restricted CD8⁺ T cells also play very important role in immune responses against Mtb. And large amounts of previous results have proved the role of CD8⁺ T cell-mediated immune responses in Mtb challenged mouse models, nonhuman primates as well as patients (D'Souza et al. 2000; Flynn & Chan 2001; Flynn et al. 1992; Lazarevic & Flynn 2002; Sousa et al. 2000).

Although a lot of researchers are trying to develop new vaccines, BCG is the only approved vaccine against tuberculosis, which efficacy in adults has been questioned (Black et al. 2002). Considering all these reasons mentioned above, we believed that it would be very worthy to identify HLA-A2 restricted cytotoxic T lymphocyte epitopes derived from drug efflux pump antigens of Mtb. It would help us to develop effective subunit vaccines against TB, especially XDR and MDR strains.

Classic ABC family is the most well-known efflux pump responsible for intrinsic drug resistance. It was shown that the over-expression of Rv2686c-Rv2687c-Rv2688c in *M.*

smegmatis increased the minimum inhibitory concentrations of ciprofloxacin (Pasca et al. 2004). And Rv2937 (drrB) behaved as a functional efflux pump referring to rifampin, tetracycline and erythromycin together with drrA (Choudhuri et al. 2002). Because that DrrA and Rv2688c have homology with some proteins in human, Rv2937, Rv2686c, and Rv2687c were chosen as target antigens to identify HLA-A2 restricted cytotoxic T lymphocyte epitopes.

Eight potential peptides derived from Rv2937, Rv2686c, and Rv2687c were predicted by using the online tools SYFPEITHI, BIMAS, and NetCTL. These peptides were synthesized and their ability to induce immune response was tested both in PBMCs of HLA-A2⁺ donors (*in vitro*) and HLA-A2/K^b transgenic mice (*in vivo*).

MATERIALS AND METHODS

Prediction and synthesis of candidate peptides

By using epitope prediction tools, BIMAS (http://bimas.dcrf.nih.gov/molbio/hla_bind/) (Parker et al. 1994), SYFPEITHI (<http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm>) (Rammensee et al. 1999), and NetCTL (<http://www.cbs.dtu.dk/services/NetCTL/>) (Larsen et al. 2007), potential HLA-A2-restricted T cell epitopes derived from Rv2937, Rv2686c, and Rv2687c were predicted. Peptides with relative high scores were synthesized by using standard solid phase Fmoc strategy. The peptides were purified by reverse phase-high performance liquid chromatography (RP-HPLC), and then their molecular weights were confirmed by electrospray ionization-mass spectrometry (ESI-MS). The HBV core antigen-derived T helper epitope (sequence 128–140: TPPAYRPPNAPIL) was used to enhance the immune activity in the mice vaccination experiment (Milich et al. 1988; Vissers et al. 1999).

Blood samples, animals and cell lines

Whole blood was prepared from HLA-A2⁺ (PPD⁺ or PPD⁻) healthy donors. The HLA-A2.1/K^b transgenic mice were previously gifted by professor Xue-tao Cao (Second Military Medical University) (Vitiello et al. 1991). All mice at 8 to 12 weeks in the experiments were housed in a specific pathogen-free environment in our laboratory. The

sample collection from healthy donors and animal experiments were approved by the Ethics Committee of Zhengzhou University (No. 20120312). The human transporter associated with antigen processing (TAP) -deficient T2 cell line was kindly provided by professor Yu-zhang Wu (Third Military Medical University, China), and the cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in an incubator with a humidified atmosphere containing 5% CO₂.

Generation of CTLs from HLA-A2 healthy donors

The procedures of generation of CTLs *in vitro* were performed in accordance with the protocols described by our laboratory (Liu et al. 2012; Shi et al. 2013). Briefly, PBMCs were isolated from six HLA-A2⁺ PPD⁺ and an HLA-A2⁺ PPD⁻ healthy donors with centrifugation at a Ficoll-Paque density gradient and then cultured in IMDM medium supplemented with 10% FBS under the condition of 37°C, 5% CO₂ (Han et al. 2006). After 24h, PBMCs at the concentration of 1×10⁶/ml were stimulated with the candidate peptides (10μg/ml) in the presence of 3μg/ml β2-m for 4h. The next day, human recombinant IL-2 (50 U/ml) and IL-7 (50 U/ml) were added. Once a week, these cells were re-stimulated same as the procedures above. Seven days after the third round of stimulation, the cytotoxic assay and ELISPOT assay were performed.

Generation of CTLs from HLA-A2.1/K^b transgenic mice

CTLs from HLA-A2.1/K^b transgenic mice were generated as previously described (Liu et al. 2012; Shi et al. 2013). Briefly, HLA-A2.1/K^b transgenic mice were grouped randomly, they were injected subcutaneously at the base of the tail with 100μg each peptide emulsified in incomplete Freund's adjuvant (IFA) in the presence of 140μg of the T helper epitope every five days (Eguchi et al. 2006; Tourdot et al. 2000). On day 11, splenocytes of each mouse were separated and then re-stimulated with the corresponding peptide (10μg/ml) *in vitro* for another five days. Then, the LDH cytotoxicity and ELISPOT assays were employed.

ELISPOT assay

ELISPOT assay was performed according to the instruction of the commercial kit (Dakewe, China). Peptide-pulsed T2 cells (stimulator cells, 1×10⁵), along with the induced CTLs (effector cells, 1×10⁵), were seeded into an anti-human (or anti-mouse) IFN-γ antibody coated 96-well plate (Ding et al. 2009). After incubation for 16 h at 37°C, cells were removed and plates were processed. Spots were counted with a computer-assisted spot analyzer (Dakewe,

China).

Cytotoxicity assay

Cytotoxic activity was tested by the non-radioactive cytotoxicity assay kit (Promega, US) at gradient E:T ratio according to the manufacturer's instruction. T2 cells were loaded with 10µg/ml peptide for 1h at 37°C as target cells. The effector cells were co-cultured with target cells (1×10^4 /well) at various effector/target ratios for 5h at 37°C under 5% CO₂. The percentage of specific lysis of the target cells was determined according to the following formula. Percentage of specific lysis = [(experimental release – effector spontaneous release – target spontaneous release) / (target maximum release – target spontaneous release)] × 100.

Statistical analysis

All data were presented as means ± S.D. Comparisons between experimental groups and relevant controls were analyzed by Student's t test. $P < 0.05$ was considered as a statistically significant difference.

RESULTS

Peptides selected as potential CTL epitopes

By using the on-line prediction tools, eight potential HLA-A2 restricted T cell epitopes were selected from the three candidate efflux pump antigens, Rv2937, Rv2686c, and Rv2687c. The peptides were synthesized and their molecular weights were confirmed by ESI-MS. (Table 1).

IFN-γ release ELISPOT assay *in vitro*

CTLs were induced from the PBMCs of six HLA-A2⁺ PPD⁺ and an HLA-A2⁺ PPD⁻ healthy donors. IFN-γ release ELISPOT assay was employed to test the capacity of the eight peptides to induce CTL response. As shown in Fig. 1, among the six HLA-A2⁺ PPD⁺ donors, Rv2937-p168, Rv2937-p266, Rv2686c-p151, Rv2686c-p181, and Rv2686c-p184 could induce more frequent and potent CTL response, while all these peptides could only induce very weak response in the HLA-A2⁺ PPD⁻ donor (Fig. S1).

In vitro cytotoxic activity of peptide-specific CTLs

Based on the results of the ELISPOT assay, Rv2937-p168, Rv2937-p266, Rv2686c-p151, Rv2686c-p181, and Rv2686c-p184 were selected to investigate whether the specific T cells

they induced could lyse target cells. In the cytotoxicity assay, peptide-pulsed T2 cells were considered as target cells, the effector cells were obtained from CTLs induced from PBMCs of HLA-A2⁺ PPD⁺ donor. As shown in Fig. 2, the specific lysis percentages of the CTLs induced by Rv2937-p168, Rv2937-p266, Rv2686c-p151, Rv2686c-p181, and Rv2686c-p184 from PBMCs of HLA-A2⁺ PPD⁺ healthy donors were increased gradiently from E/T ratio 12.5:1 to 50:1. However, after incubating with anti-HLA-A2 monoclonal antibody, the specific lysis rates of the CTLs derived from HLA-A2⁺ PPD⁺ were greatly reduced. These results indicated that these peptides could induce HLA-A2-restricted CTL response in PPD⁺ healthy donor.

Cytotoxic T lymphocyte response in HLA-A2.1/K^b transgenic mice

HLA-A2.1/K^b transgenic mice immunization model is a widely used putative model to study the *in vivo* CTL activity of HLA-A2-restricted epitopes. Since Rv2937-p168, Rv2937-p266, Rv2686c-p151, Rv2686c-p181, and Rv2686c-p184 could induce good immune response *in vitro*, we then investigated whether these peptides could stimulate CTL response in HLA-A2.1/K^b transgenic mice. After immunization of the candidate peptides with Th epitope and IFA adjuvant, the splenocytes were isolated and stimulated with the corresponding peptide *in vitro* for another five days. IFN- γ release ELISPOT assay and LDH cytotoxicity assay were performed. As shown in Fig. 3, all of the five peptides showed more potent activity to induce IFN- γ release than the negative control group.

The cytotoxic activity of these splenocytes was also measured by an LDH cytotoxicity assay. Peptide-loaded T2 cells were served as target cells and the effector/target ratios were 20:1, 40:1, and 80:1. As shown in Fig. 4, at the E:T ratio of 80:1, the CTLs induced by Rv2937-p168, Rv2937-p266, Rv2686c-p151, and Rv2686c-p181 could significantly kill the target cells. To our surprise, although Rv2686c-p184 could induce the most potent IFN- γ release activity in PBMCs of HLA-A2⁺ PPD⁺ donors, it could not induce CTLs with killing effects in HLA-A2.1/K^b transgenic mice.

DISCUSSION

Tuberculosis is considered as a major public health concern worldwide because of the high mortality and morbidity associated with it. And as reported, the emergence of XDR-TB and MDR-TB forced patients having fewer options for treatment and risking higher mortalities, especially in HIV/TB co-infected ones (Principi & Esposito 2015). Development of novel therapeutic vaccines provides a promising strategy to combine with chemotherapy.

Accumulating data showed that subunit vaccine containing peptide or protein antigens could exhibit protective activity and very good safety (Ivanyi 2014).

HLA-A2 is one of the most common supertypes of human leukocyte antigen particularly in Asian with an estimated frequency of nearly 50% (Mehra et al. 2001), and cell-mediated immunity to Mtb is believed important to control the latent Mtb infection, therefore, identification of HLA-A2 restricted cytotoxic T lymphocyte epitopes derived from efflux pump antigens could be helpful to develop vaccines against drug-resistant TB caused by intrinsic drug efflux. Recently, most researchers who hammer at identification of CTL epitopes of Mtb antigens focused their work on secretory protein, such as ESAT-6 (Lalvani et al. 1998), 19-kDa lipoprotein (Mohaghehpour et al. 1998), Ag85B (Geluk et al. 2000), 16-kDa antigen (Caccamo et al. 2002) and MPT51 (Suzuki et al. 2004), and so on. We also identified such kind of epitopes from antigen CFP21 and RD region (Chen et al. 2012; Lv et al. 2010). Furthermore, we firstly reported that efflux pumps could also be considered as target antigens for TB immunotherapy and found that Rv1410c could serve as a candidate to the vaccine design against drug-resistant Mtb (Zhu et al. 2011). Then we screened classical efflux pump family members in the genome of TB to find more promising target antigens. As shown in the present study, we found that epitopes, Rv2937-p168, Rv2937-p266, Rv2937-p168, Rv2686c-p181, and Rv2686c-p184 identified from ABC family members, elicited good capacity to induce CTL response in HLA-A2⁺ PPD⁺ healthy donors and/or HLA-A2.1/K^b transgenic mice. Work is still need to be done to identify more promising antigens related to drug-resistant Mtb. We are now also working on extrinsic in TB. Hopefully, we can combine all these epitopes derived from secretory and drug-resistant antigens to develop multi-valent subunit vaccines.

As we all known, early diagnosis of TB is fundamental for tuberculosis control. In the last decade, ELISPOT assay in the TB diagnosis is considered to have high specificity and sensitivity (Lalvani & Pareek 2010; Milotic et al. 2011). Most of the five candidate peptides showed good activity among the six HLA-A2⁺ PPD⁺ healthy donors, but not in HLA-A2⁺ PPD⁻. Although we do not know whether these PPD⁺ donors have drug-resistant Mtb infection, the results indicated that these epitopes might also be used as TB diagnosis.

In the present study, we used so called 'reversal immunology' strategy to predict antigen epitopes by using on-line tools instead of time-consuming overlapping peptides method, which was very efficient. However, using just one computational algorithm to predict CTL epitopes may lead to large amounts of false positive and false negatives. So we used epitope prediction tool NetCTL combined with the widely used BIMAS and SYFPEITHI databases.

NetCTL integrates prediction of binding affinity, transporter of antigenic peptide efficiency and proteasomal cleavage (Larsen et al. 2007), SYFPEITHI is a motif-matrix-based prediction method for MHC binding prediction (Rammensee et al. 1999) and BIMAS is based on peptide/MHC complex half-life (Parker et al. 1994). Our results suggested that this strategy could be very efficient and successful.

CONCLUSIONS

In conclusion, we have identified five HLA-A2 restricted cytotoxic T lymphocyte epitopes derived from drug efflux pump antigens of *Mycobacterium tuberculosis*. The epitopes Rv2937-p168, Rv2937-p266, Rv2686c-p151, Rv2686c-p181, and Rv2686c-p184 showed good capacity to induce CTL response in HLA-A2⁺ PPD⁺ donors. Except Rv2686c-p184, other epitopes could also elicit CTL response when immunized in HLA-A2.1/K^b transgenic mice. These epitopes could serve as candidates for TB diagnosis and immunotherapy.

REFERENCES

- Black GF, Weir RE, Floyd S, Bliss L, Warndorff DK, Crampin AC, Ngwira B, Sichali L, Nazareth B, Blackwell JM, Branson K, Chaguluka SD, Donovan L, Jarman E, King E, Fine PE, and Dockrell HM. 2002. BCG-induced increase in interferon-gamma response to mycobacterial antigens and efficacy of BCG vaccination in Malawi and the UK: two randomised controlled studies. *Lancet* 359:1393-1401.
- Caccamo N, Milano S, Di Sano C, Cigna D, Ivanyi J, Krensky AM, Dieli F, and Salerno A. 2002. Identification of epitopes of *Mycobacterium tuberculosis* 16-kDa protein recognized by human leukocyte antigen-A*0201 CD8(+) T lymphocytes. *J Infect Dis* 186:991-998.
- Chen F, Zhai MX, Zhu YH, Qi YM, Zhai WJ, and Gao YF. 2012. In vitro and in vivo identification of a novel cytotoxic T lymphocyte epitope from Rv3425 of *Mycobacterium tuberculosis*. *Microbiol Immunol* 56:548-553.
- Choudhuri BS, Bhakta S, Barik R, Basu J, Kundu M, and Chakrabarti P. 2002. Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes *drdA* and *drdB* of *Mycobacterium tuberculosis*. *Biochem J* 367:279-285.
- D'Souza CD, Cooper AM, Frank AA, Ehlers S, Turner J, Bendelac A, and Orme IM. 2000. A novel nonclassic beta2-microglobulin-restricted mechanism influencing early lymphocyte accumulation and subsequent resistance to tuberculosis in the lung. *Am J Respir Cell Mol Biol* 23:188-193.
- De Rossi E, Ainsa JA, and Riccardi G. 2006. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS Microbiol Rev* 30:36-52.
- Ding FX, Wang F, Lu YM, Li K, Wang KH, He XW, and Sun SH. 2009. Multiepitope peptide-loaded virus-like particles as a vaccine against hepatitis B virus-related hepatocellular carcinoma. *Hepatology* 49:1492-1502.

256 Dye C, and Williams BG. 2010. The population dynamics and control of tuberculosis. *Science*
257 328:856-861.

258 Eguchi J, Hatano M, Nishimura F, Zhu X, Dusak JE, Sato H, Pollack IF, Storkus WJ, and
259 Okada H. 2006. Identification of interleukin-13 receptor alpha2 peptide analogues capable of
260 inducing improved antiglioma CTL responses. *Cancer Res* 66:5883-5891.

261 Escribano I, Rodriguez JC, Llorca B, Garcia-Pachon E, Ruiz M, and Royo G. 2007.
262 Importance of the efflux pump systems in the resistance of *Mycobacterium tuberculosis* to
263 fluoroquinolones and linezolid. *Chemotherapy* 53:397-401.

264 Flynn JL, and Chan J. 2001. Immunology of tuberculosis. *Annu Rev Immunol* 19:93-129.

265 Flynn JL, Goldstein MM, Triebold KJ, Koller B, and Bloom BR. 1992. Major
266 histocompatibility complex class I-restricted T cells are required for resistance to
267 *Mycobacterium tuberculosis* infection. *Proc Natl Acad Sci U S A* 89:12013-12017.

268 Geluk A, van Meijgaarden KE, Franken KL, Drijfhout JW, D'Souza S, Necker A, Huygen K,
269 and Ottenhoff TH. 2000. Identification of major epitopes of *Mycobacterium tuberculosis*
270 AG85B that are recognized by HLA-A*0201-restricted CD8+ T cells in HLA-transgenic mice
271 and humans. *J Immunol* 165:6463-6471.

272 Han JF, Zhao TT, Liu HL, Lin ZH, Wang HM, Ruan ZH, Zou LY, and Wu YZ. 2006.
273 Identification of a new HLA-A*0201-restricted cytotoxic T lymphocyte epitope from CML28.
274 *Cancer Immunol Immunother* 55:1575-1583.

275 Ivanyi J. 2014. Function and Potentials of *M. tuberculosis* Epitopes. *Front Immunol* 5:107.

276 Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Andersen P, Dockrell H, Pasvol
277 G, and Hill AV. 1998. Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes
278 specific for *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* 95:270-275.

279 Lalvani A, and Pareek M. 2010. Interferon gamma release assays: principles and practice.
280 *Enferm Infecc Microbiol Clin* 28:245-252.

281 Larsen MV, Lundegaard C, Lamberth K, Buus S, Lund O, and Nielsen M. 2007. Large-scale
282 validation of methods for cytotoxic T-lymphocyte epitope prediction. *BMC Bioinformatics*
283 8:424.

284 Lazarevic V, and Flynn J. 2002. CD8+ T cells in tuberculosis. *Am J Respir Crit Care Med*
285 166:1116-1121.

286 Liu W, Zhai M, Wu Z, Qi Y, Wu Y, Dai C, Sun M, Li L, and Gao Y. 2012. Identification of a
287 novel HLA-A2-restricted cytotoxic T lymphocyte epitope from cancer-testis antigen PLAC1
288 in breast cancer. *Amino Acids* 42:2257-2265.

289 Lv H, Gao Y, Wu Y, Zhai M, Li L, Zhu Y, Liu W, Wu Z, Chen F, and Qi Y. 2010.
290 Identification of a novel cytotoxic T lymphocyte epitope from CFP21, a secreted protein of
291 *Mycobacterium tuberculosis*. *Immunol Lett* 133:94-98.

292 Mehra NK, Jaini R, Rajalingam R, Balamurugan A, and Kaur G. 2001. Molecular diversity of
293 HLA-A*02 in Asian Indians: predominance of A*0211. *Tissue Antigens* 57:502-507.

294 Milich DR, Hughes JL, McLachlan A, Thornton GB, and Moriarty A. 1988. Hepatitis B
295 synthetic immunogen comprised of nucleocapsid T-cell sites and an envelope B-cell epitope.
296 *Proc Natl Acad Sci U S A* 85:1610-1614.

297 Milotic DM, Popovic-Grle S, Katalinic-Jankovic V, and Simunovic A. 2011. [Comparison of
298 new and old tests for the diagnosis of latent tuberculosis infection (quantiferon and TST)].
299 *Lijec Vjesn* 133:396-402.

300 Mohaghehpour N, Gammon D, Kawamura LM, van Vollenhoven A, Benike CJ, and
 301 Engleman EG. 1998. CTL response to Mycobacterium tuberculosis: identification of an
 302 immunogenic epitope in the 19-kDa lipoprotein. *J Immunol* 161:2400-2406.
 303 Parker KC, Bednarek MA, and Coligan JE. 1994. Scheme for ranking potential HLA-A2
 304 binding peptides based on independent binding of individual peptide side-chains. *J Immunol*
 305 152:163-175.
 306 Pasca MR, Gugliera P, Arcesi F, Bellinzoni M, De Rossi E, and Riccardi G. 2004.
 307 Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in Mycobacterium
 308 tuberculosis. *Antimicrob Agents Chemother* 48:3175-3178.
 309 Principi N, and Esposito S. 2015. The present and future of tuberculosis vaccinations.
 310 *Tuberculosis (Edinb)* 95:6-13.
 311 Ramaswamy S, and Musser JM. 1998. Molecular genetic basis of antimicrobial agent
 312 resistance in Mycobacterium tuberculosis: 1998 update. *Tuber Lung Dis* 79:3-29.
 313 Rammensee H, Bachmann J, Emmerich NP, Bachor OA, and Stevanovic S. 1999.
 314 SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50:213-219.
 315 Shi RR, Liu J, Zou Z, Qi YM, Zhai MX, Zhai WJ, and Gao YF. 2013. The immunogenicity of
 316 a novel cytotoxic T lymphocyte epitope from tumor antigen PL2L60 could be enhanced by
 317 4-chlorophenylalanine substitution at position 1. *Cancer Immunol Immunother* 62:1723-1732.
 318 Sousa AO, Mazzaccaro RJ, Russell RG, Lee FK, Turner OC, Hong S, Van Kaer L, and Bloom
 319 BR. 2000. Relative contributions of distinct MHC class I-dependent cell populations in
 320 protection to tuberculosis infection in mice. *Proc Natl Acad Sci U S A* 97:4204-4208.
 321 Spies FS, da Silva PE, Ribeiro MO, Rossetti ML, and Zaha A. 2008. Identification of
 322 mutations related to streptomycin resistance in clinical isolates of Mycobacterium
 323 tuberculosis and possible involvement of efflux mechanism. *Antimicrob Agents Chemother*
 324 52:2947-2949.
 325 Suzuki M, Aoshi T, Nagata T, and Koide Y. 2004. Identification of murine H2-Dd- and
 326 H2-Ab-restricted T-cell epitopes on a novel protective antigen, MPT51, of Mycobacterium
 327 tuberculosis. *Infect Immun* 72:3829-3837.
 328 Tourdot S, Scardino A, Saloustrou E, Gross DA, Pascolo S, Cordopatis P, Lemonnier FA, and
 329 Kosmatopoulos K. 2000. A general strategy to enhance immunogenicity of low-affinity
 330 HLA-A2. 1-associated peptides: implication in the identification of cryptic tumor epitopes.
 331 *Eur J Immunol* 30:3411-3421.
 332 Vissers JL, De Vries IJ, Schreurs MW, Engelen LP, Oosterwijk E, Figdor CG, and Adema GJ.
 333 1999. The renal cell carcinoma-associated antigen G250 encodes a human leukocyte antigen
 334 (HLA)-A2.1-restricted epitope recognized by cytotoxic T lymphocytes. *Cancer Res*
 335 59:5554-5559.
 336 Vitiello A, Marchesini D, Furze J, Sherman LA, and Chesnut RW. 1991. Analysis of the
 337 HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice
 338 carrying a chimeric human-mouse class I major histocompatibility complex. *J Exp Med*
 339 173:1007-1015.
 340 Zhu YH, Gao YF, Chen F, Liu W, Zhai MX, Zhai WJ, Qi YM, and Ye Y. 2011. Identification
 341 of novel T cell epitopes from efflux pumps of Mycobacterium tuberculosis. *Immunol Lett*
 342 140:68-73.
 343

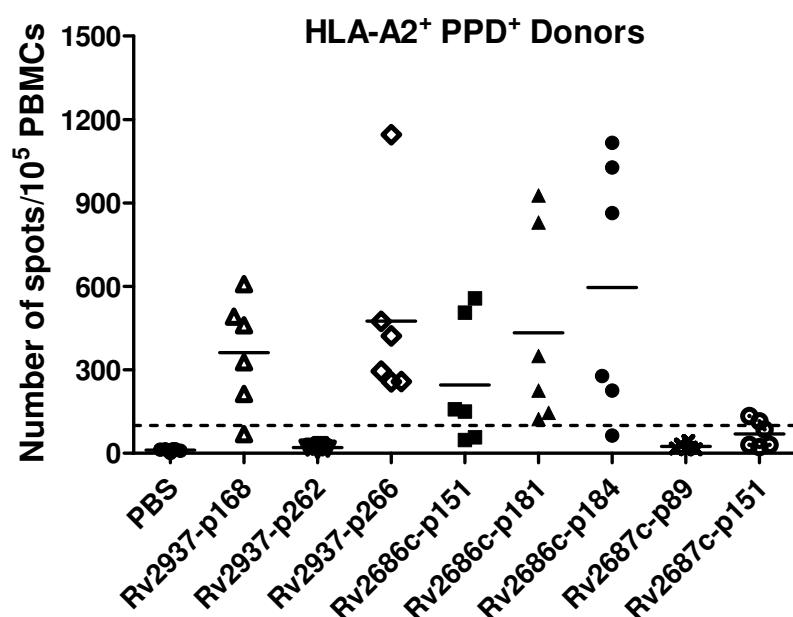
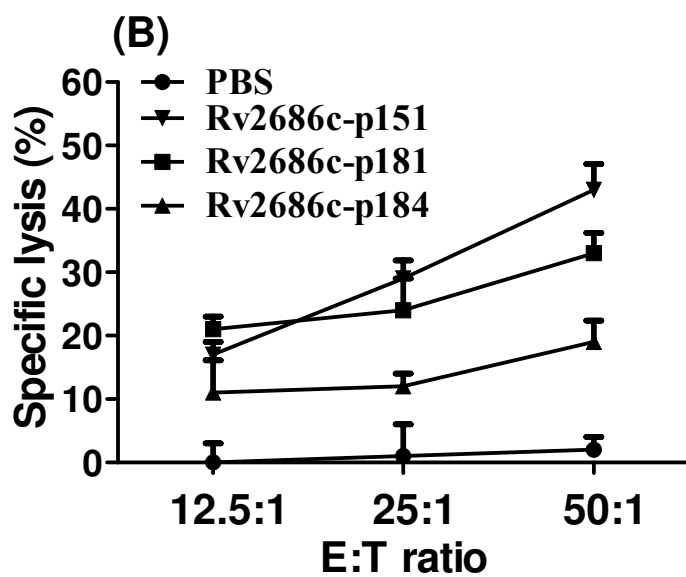
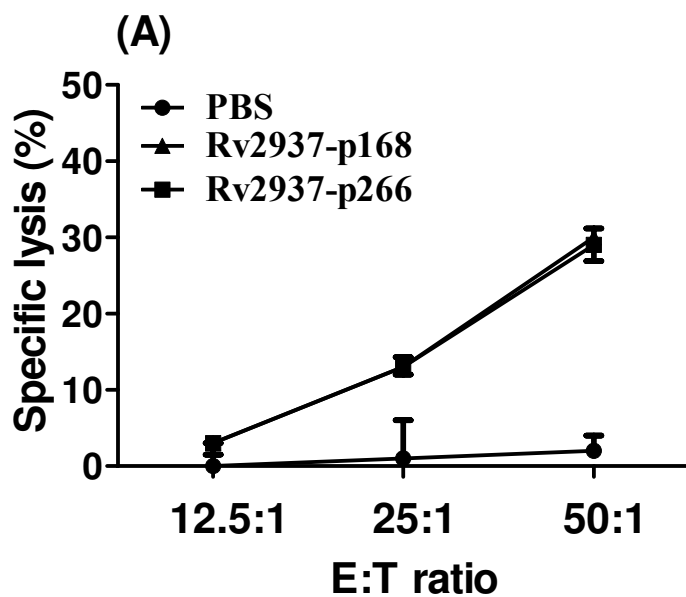


Figure 1. IFN- γ release ELISPOT assay by CTLs induced from PBMCs of HLA-A2⁺ PPD⁺ donors. Each peptide was tested by using the samples from the same group of six donors. PBMCs from healthy donors were separated and stimulated once a week with synthetic peptides and IL-2 for three rounds.



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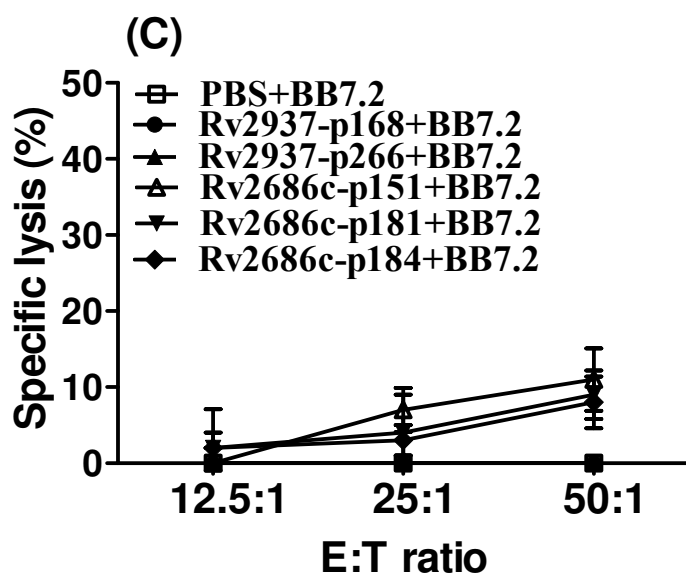
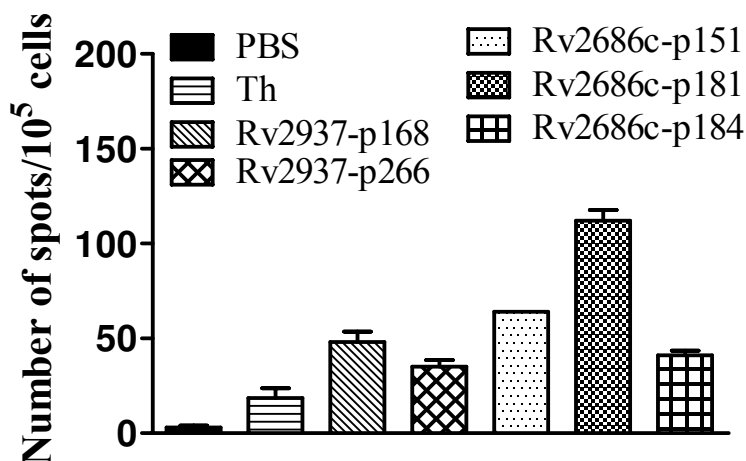


Figure 2. Specific lysis of T2 cells loaded with synthetic peptides by the CTLs generated from PBMCs of HLA-A2⁺ PPD⁺ donors. (A-B) The effector cells were obtained from CTLs induced from PBMCs of HLA-A2⁺ PPD⁺ donors. (C) The HLA-A2 molecules on the T2 cells surface were blocked by anti-HLA-A2 monoclonal antibody.

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Figure 3. IFN- γ release ELISPOT assay of the splenocytes from the immunized HLA-A2.1/K^b transgenic mice. HLA-A2.1/K^b transgenic mice were immunized with 100 μ g each peptide emulsified in incomplete Freund's adjuvant (IFA) in the presence of 140 μ g of the T helper epitope every five days for three times.

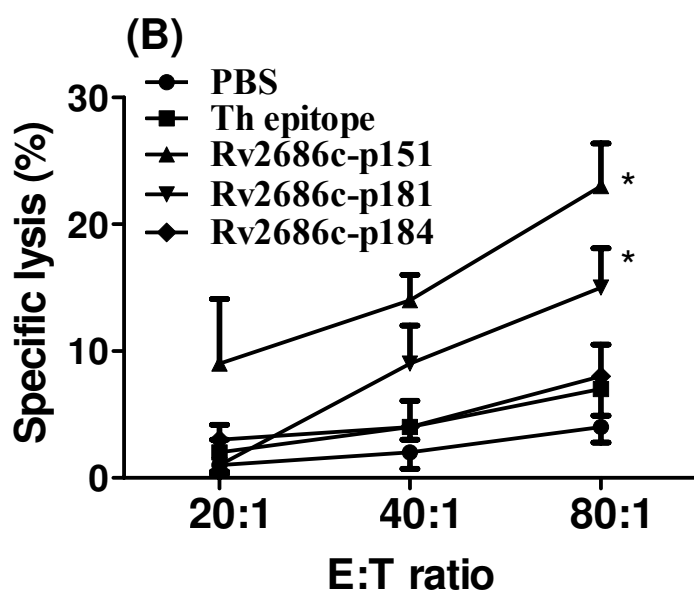
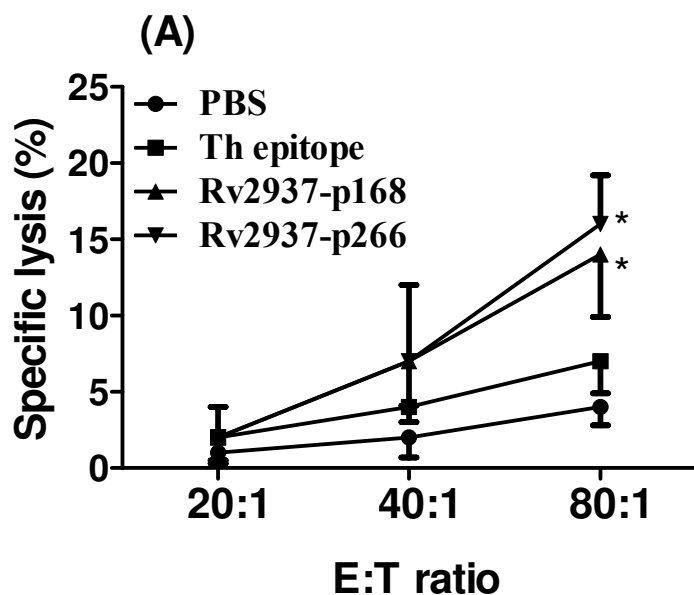


Figure 4. Specific lysis of T2 cells loaded with synthetic peptides by the CTLs generated from the immunized HLA-A2.1/K^b transgenic mice ($n=5$). The data from each peptide immunized group were compared with the Th epitope alone negative control.

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424 Table 1. HLA-A2 CTL epitopes predicted from efflux pumps of *Mycobacterium tuberculosis*

Antigen	Position	Sequence	SYFPEITHI	NetCTL
Rv2937	168	YIVGFCLLV	24	1.2629
	262	VMAPTLTWL	27	1.2098
	266	TLTWLFAFV	22	1.1149
Rv2686c	151	GLVAGLSAV	28	0.9644
	181	ALGMLIAGL	30	0.9898
	184	MLIAGLPCL	29	1.3358
Rv2687c	89	YLA AKLTVL	29	1.3019
	151	FLAAVIPLA	22	1.2362

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