A peer-reviewed version of this preprint was published in PeerJ on 22 September 2015.

View the peer-reviewed version (peerj.com/articles/1229), which is the preferred citable publication unless you specifically need to cite this preprint.

Zhai M, Chen F, Zhao Y, Wu Y, Li G, Gao Y, Qi Y. 2015. Novel epitopes identified from efflux pumps of *Mycobacterium tuberculosis* could induce cytotoxic T lymphocyte response. PeerJ 3:e1229
https://doi.org/10.7717/peerj.1229

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

Mingxia Zhai, Fei Chen, Yuanyuan Zhao, Yahong Wu, Guodong Li, Yanfeng Gao, Yuanming Qi

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.

PeerJ PrePrints

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

Ming-xia Zhai, Fei Chen, Yuan-yuan Zhao, Ya-hong Wu, Guo-dong Li, Yan-feng Gao*, Yuan-ming Qi*

School of Life Sciences, Zhengzhou University, Zhengzhou 450001, China

*Correspondence: Prof. Yanfeng Gao or Yuan-ming Qi.

School of Life Sciences, Zhengzhou University, 100 Kexue Road, Zhengzhou 450001, China

Tel.: +86-371-67781107; Fax: +86-371-67783235. E-mail: gaoyf@zzu.edu.cn; or qym@zzu.edu.cn

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015

26

27 **INTRODUCTION**

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

36 As an intracellular pathogen living in macrophages, more and more evidence suggested the
37 important role of cellular immunity in controlling the dissemination of Mtb. It has been
38 proved that, in addition to $CD4^+$ T cells, MHC class I restricted $CD8^+$ T cells also play very
39 important role in immune responses against Mtb. And large amounts of previous results have
40 proved the role of $CD8^+$ T cell-mediated immune responses in Mtb challenged mouse models,
41 nonhuman primates as well as patients (D'Souza et al. 2000; Flynn & Chan 2001; Flynn et al.
42 1992; Lazarevic & Flynn 2002; Sousa et al. 2000).

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

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

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015

51 *smegmatis* increased the minimum inhibitory concentrations of ciprofloxacin (Pasca et al.
52 2004). And Rv2937（drrB）behaved as a functional efflux pump referring to rifampin,
53 tetracycline and erythromycin together with drrA (Choudhuri et al. 2002). Because that DrrA
54 and Rv2688c have homology with some proteins in human, Rv2937, Rv2686c, and Rv2687c
55 were chosen as target antigens to identify HLA-A2 restricted cytotoxic T lymphocyte
56 epitopes.

57 Eight potential peptides derived from Rv2937, Rv2686c, and Rv2687c were predicted by
58 using the online tools SYFPEITHI, BIMAS, and NetCTL. These peptides were synthesized
59 and their ability to induce immune response was tested both in PBMCs of HLA-A2$^+$ donors
60 (*in vitro*) and HLA-A2/K^b transgenic mice (*in vivo*).
61

62 ## MATERIALS AND METHODS

63 ### Prediction and synthesis of candidate peptides

64 By using epitope prediction tools, BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/)
65 (Parker et al. 1994), SYFPEITHI
66 (http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm) (Rammensee et al.
67 1999), and NetCTL (http://www.cbs.dtu.dk/services/NetCTL/) (Larsen et al. 2007), potential
68 HLA-A2-restricted T cell epitopes derived from Rv2937, Rv2686c, and Rv2687c were
69 predicted. Peptides with relative high scores were synthesized by using standard solid phase
70 Fmoc strategy. The peptides were purified by reverse phase-high performance liquid
71 chromatography (RP-HPLC), and then their molecular weights were confirmed by
72 electrospray ionization-mass spectrometry (ESI-MS). The HBV core antigen-derived T helper
73 epitope (sequence128–140: TPPAYRPPNAPIL) was used to enhance the immune activity in
74 the mice vaccination experiment (Milich et al. 1988; Vissers et al. 1999).
75

76 ### Blood samples, animals and cell lines

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

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015
PeerJ PrePrints

81 sample collection from healthy donors and animal experiments were approved by the Ethics
82 Committee of Zhengzhou University (No. 20120312). The human transporter associated with
83 antigen processing (TAP) -deficient T2 cell line was kindly provided by professor Yu-zhang
84 Wu (Third Military Medical University, China), and the cells were cultured in RPMI 1640
85 medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37˚C in an
86 incubator with a humidified atmosphere containing 5% CO_2.
87
88 **Generation of CTLs from HLA-A2 healthy donors**
89 The procedures of generation of CTLs in vitro were performed in accordance with the
90 protocols described by our laboratory (Liu et al. 2012; Shi et al. 2013). Briefly, PBMCs were
91 isolated from six HLA-A2$^+$ PPD$^+$ and an HLA-A2$^+$ PPD$^-$ healthy donors with centrifugation
92 at a Ficoll-Paque density gradient and then cultured in IMDM medium supplemented with
93 10% FBS under the condition of 37˚C, 5% CO_2 (Han et al. 2006). After 24h, PBMCs at the
94 concentration of 1×10^6/ml were stimulated with the candidate peptides (10μg/ml) in the
95 presence of 3μg/ml β2-m for 4h. The next day, human recombinant IL-2 (50 U/ml) and IL-7
96 (50 U/ml) were added. Once a week, these cells were re-stimulated same as the procedures
97 above. Seven days after the third round of stimulation, the cytotoxic assay and ELISPOT
98 assay were performed.
99
100 **Generation of CTLs from HLA-A2.1/K^b transgenic mice**
101 CTLs from HLA-A2.1/K^b transgenic mice were generated as previously described (Liu et
102 al. 2012; Shi et al. 2013). Briefly, HLA-A2.1/K^b transgenic mice were grouped randomly, they
103 were injected subcutaneously at the base of the tail with 100μg each peptide emulsified in
104 incomplete Freund's adjuvant (IFA) in the presence of 140μg of the T helper epitope every
105 five days (Eguchi et al. 2006; Tourdot et al. 2000). On day 11, splenocytes of each mouse
106 were separated and then re-stimulated with the corresponding peptide (10μg/ml) *in vitro* for
107 another five days. Then, the LDH cytotoxicity and ELISPOT assays were employed.
108
109 **ELISPOT assay**
110 ELISPOT assay was performed according to the instruction of the commercial kit (Dakewe,
111 China). Peptide-pulsed T2 cells (stimulator cells, 1×10^5), along with the induced CTLs
112 (effector cells, 1×10^5), were seeded into an anti-human (or anti-mouse) IFN-γ antibody coated
113 96-well plate (Ding et al. 2009). After incubation for 16 h at 37°C, cells were removed and
114 plates were processed. Spots were counted with a computer-assisted spot analyzer (Dakewe,

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015

115 China).

116

117 **Cytotoxicity assay**

118 Cytotoxic activity was tested by the non-radioactive cytotoxicity assay kit (Promega, US)
119 at gradient E:T ratio according to the manufacturer's instruction. T2 cells were loaded with
120 10μg/ml peptide for 1h at 37°C as target cells. The effector cells were co-cultured with target
121 cells (1×10^4/well) at various effector/target ratios for 5h at 37°C under 5% CO_2. The
122 percentage of specific lysis of the target cells was determined according to the following
123 formula. Percentage of specific lysis = [(experimental release − effector spontaneous release −
124 target spontaneous release) / (target maximum release − target spontaneous release)] × 100.

125

126 **Statistical analysis**

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

130

131 **RESULTS**

132 **Peptides selected as potential CTL epitopes**

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

137

138 **IFN-γ release ELISPOT assay *in vitro***

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

145

146 ***In vitro* cytotoxic activity of peptide-specific CTLs**

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

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015

149 they induced could lyse target cells. In the cytotoxicity assay, peptide-pulsed T2 cells were
150 considered as target cells, the effector cells were obtained from CTLs induced from PBMCs
151 of HLA-A2$^+$ PPD$^+$ donor. As shown in Fig. 2, the specific lysis percentages of the CTLs
152 induced by Rv2937-p168, Rv2937-p266, Rv2686c-p151, Rv2686c-p181, and Rv2686c-p184
153 from PBMCs of HLA-A2$^+$ PPD$^+$ healthy donors were increased gradiently from E/T ratio
154 12.5:1 to 50:1. However, after incubating with anti-HLA-A2 monoclonal antibody, the
155 specific lysis rates of the CTLs derived from HLA-A2$^+$ PPD$^+$ were greatly reduced. These
156 results indicated that these peptides could induce HLA-A2-restricted CTL response in PPD$^+$
157 healthy donor.

158

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

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

169 The cytotoxic activity of these splenocytes was also measured by an LDH cytotoxicity
170 assay. Peptide-loaded T2 cells were served as target cells and the effector/target ratios were
171 20:1, 40:1, and 80:1. As shown in Fig. 4, at the E:T ratio of 80:1, the CTLs induced by
172 Rv2937-p168, Rv2937-p266, Rv2686c-p151, and Rv2686c-p181 could significantly kill the
173 target cells. To our surprise, although Rv2686c-p184 could induce the most potent IFN-γ
174 release activity in PBMCs of HLA-A2$^+$ PPD$^+$ donors, it could not induce CTLs with killing
175 effects in HLA-A2.1/K^b transgenic mice.

176

177 **DISCUSSION**

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

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015

183 Accumulating data showed that subunit vaccine containing peptide or protein antigens could
184 exhibit protective activity and very good safety (Ivanyi 2014).
185 HLA-A2 is one of the most common supertypes of human leukocyte antigen particularly in
186 Asian with an estimated frequency of nearly 50% (Mehra et al. 2001), and cell-mediated
187 immunity to Mtb is believed important to control the latent Mtb infection, therefore,
188 identification of HLA-A2 restricted cytotoxic T lymphocyte epitopes derived from efflux
189 pump antigens could be helpful to develop vaccines against drug-resistant TB caused by
190 intrinsic drug efflux. Recently, most researchers who hammer at identification of CTL
191 epitopes of Mtb antigens focused their work on secretary protein, such as ESAT-6(Lalvani et
192 al. 1998) , 19-kDa lipoprotein(Mohagheghpour et al. 1998), Ag85B(Geluk et al. 2000),
193 16-kDa antigen(Caccamo et al. 2002) and MPT51(Suzuki et al. 2004), and so on. We also
194 identified such kind of epitopes from antigen CFP21 and RD region (Chen et al. 2012; Lv et
195 al. 2010). Furthermore, we firstly reported that efflux pumps could also be considered as
196 target antigens for TB immunotherapy and found that Rv1410c could serve as a candidate to
197 the vaccine design against drug-resistant Mtb (Zhu et al. 2011). Then we screened classical
198 efflux pump family members in the genome of TB to find more promising target antigens. As
199 shown in the present study, we found that epitopes, Rv2937-p168, Rv2937-p266,
200 Rv2937-p168, Rv2686c-p181, and Rv2686c-p184 identified from ABC family members,
201 elicited good capacity to induce CTL response in HLA-A2$^+$ PPD$^+$ healthy donors and/or
202 HLA-A2.1/K^b transgenic mice. Work is still need to be done to identify more promising
203 antigens related to drug-resistant Mtb. We are now also working on extrinsic in TB. Hopefully,
204 we can combine all these epitopes derived from secretary and drug-resistant antigens to
205 develop multi-valent subunit vaccines.
206 As we all known, early diagnosis of TB is fundamental for tuberculosis control. In the last
207 decade, ELISPOT assay in the TB diagnosis is considered to have high specifity and
208 sensitivity (Lalvani & Pareek 2010; Milotic et al. 2011). Most of the five candidate peptides
209 showed good activity among the six HLA-A2$^+$ PPD$^+$ healthy donors, but not in HLA-A2$^+$
210 PPD$^-$. Although we do not know whether these PPD$^+$ donors have drug-resistant Mtb infection,
211 the results indicated that these epitopes might also be used as TB diagnosis.
212 In the present study, we used so called 'reversal immunology' strategy to predict antigen
213 epitopes by using on-line tools instead of time-consuming overlapping peptides method,
214 which was very efficient. However, using just one computational algorithm to predict CTL
215 epitopes may lead to large amounts of false positive and false negatives. So we used epitope
216 prediction tool NetCTL combined with the widely used BIMAS and SYFPEITHI databases.

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015

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

222

223 CONCLUSIONS

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

230

231

232 REFERENCES

233 Black GF, Weir RE, Floyd S, Bliss L, Warndorff DK, Crampin AC, Ngwira B, Sichali L,
234 Nazareth B, Blackwell JM, Branson K, Chaguluka SD, Donovan L, Jarman E, King E, Fine
235 PE, and Dockrell HM. 2002. BCG-induced increase in interferon-gamma response to
236 mycobacterial antigens and efficacy of BCG vaccination in Malawi and the UK: two
237 randomised controlled studies. Lancet 359:1393-1401.
238 Caccamo N, Milano S, Di Sano C, Cigna D, Ivanyi J, Krensky AM, Dieli F, and Salerno A.
239 2002. Identification of epitopes of Mycobacterium tuberculosis 16-kDa protein recognized by
240 human leukocyte antigen-A*0201 CD8(+) T lymphocytes. J Infect Dis 186:991-998.
241 Chen F, Zhai MX, Zhu YH, Qi YM, Zhai WJ, and Gao YF. 2012. In vitro and in vivo
242 identification of a novel cytotoxic T lymphocyte epitope from Rv3425 of Mycobacterium
243 tuberculosis. Microbiol Immunol 56:548-553.
244 Choudhuri BS, Bhakta S, Barik R, Basu J, Kundu M, and Chakrabarti P. 2002.
245 Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter
246 encoded by the genes drrA and drrB of Mycobacterium tuberculosis. Biochem J 367:279-285.
247 D'Souza CD, Cooper AM, Frank AA, Ehlers S, Turner J, Bendelac A, and Orme IM. 2000. A
248 novel nonclassic beta2-microglobulin-restricted mechanism influencing early lymphocyte
249 accumulation and subsequent resistance to tuberculosis in the lung. Am J Respir Cell Mol
250 Biol 23:188-193.
251 De Rossi E, Ainsa JA, and Riccardi G. 2006. Role of mycobacterial efflux transporters in drug
252 resistance: an unresolved question. FEMS Microbiol Rev 30:36-52.
253 Ding FX, Wang F, Lu YM, Li K, Wang KH, He XW, and Sun SH. 2009. Multiepitope
254 peptide-loaded virus-like particles as a vaccine against hepatitis B virus-related hepatocellular
255 carcinoma. Hepatology 49:1492-1502.

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015

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.

PeerJ PrePrints

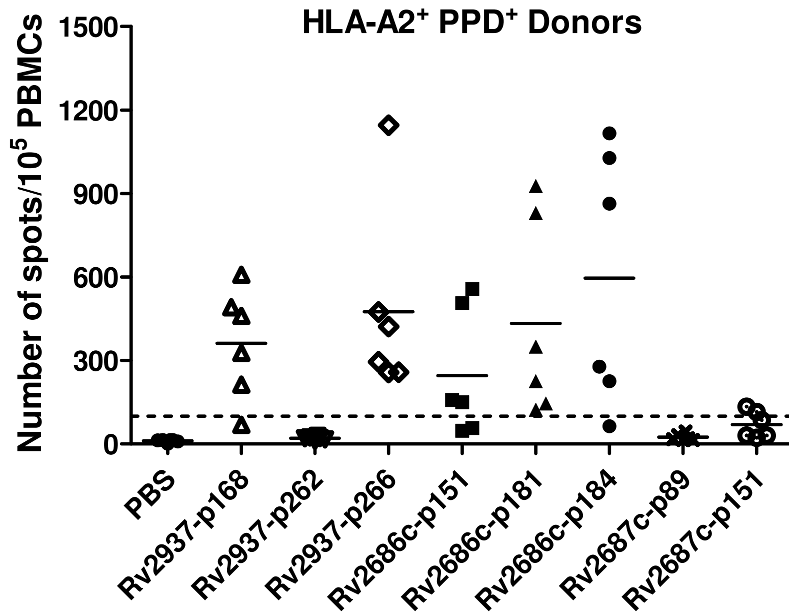
PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015

300 Mohagheghpour 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, Guglierame 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

PeerJ PrePrints

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015

344



345

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

350

351

352

353

354

355

356

357

358

359

360

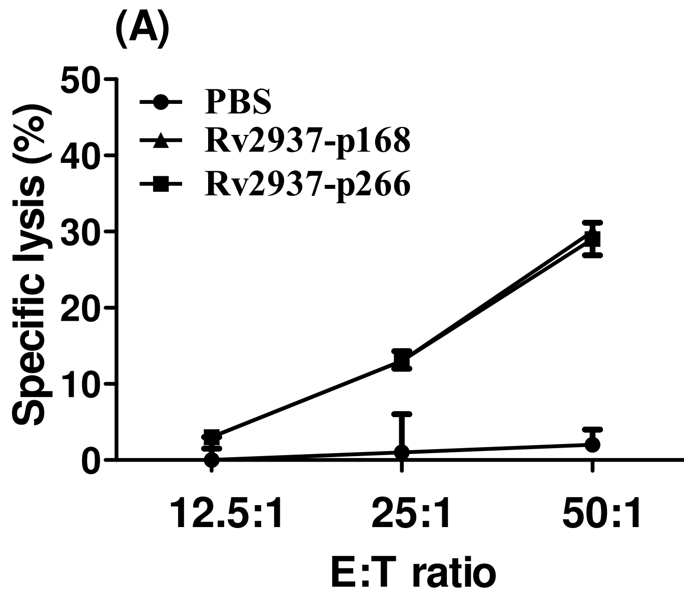
361

362

363

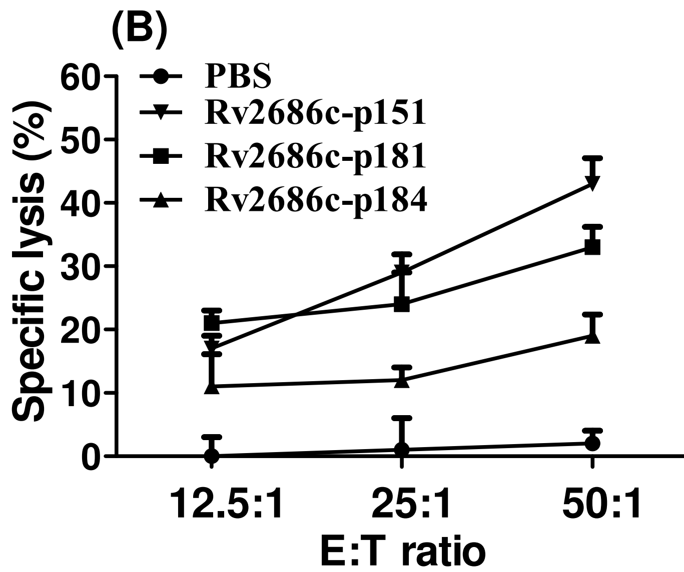
PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015
PeerJ PrePrints

364



365

366

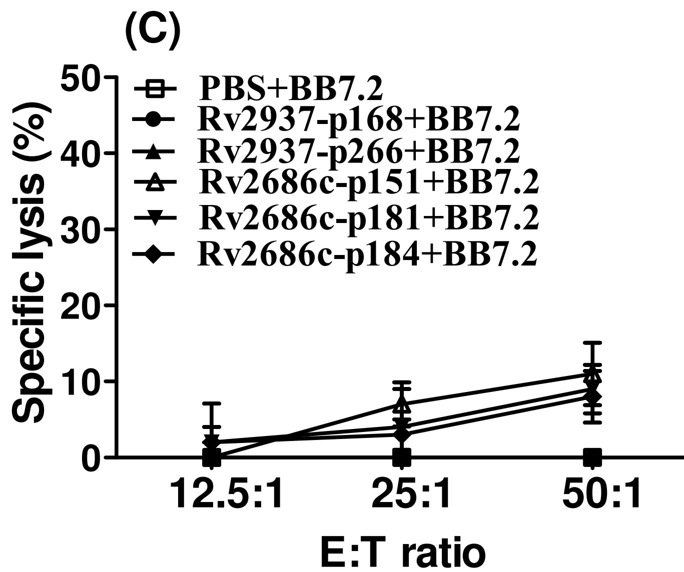


367

368

369

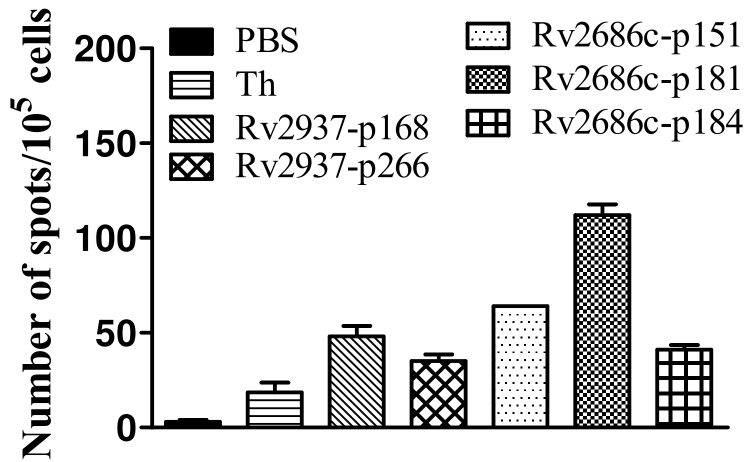
PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015
PeerJ PrePrints



370
371
372 Figure 2. Specific lysis of T2 cells loaded with synthetic peptides by the CTLs generated from
373 PBMCs of HLA-A2+ PPD+ donors. (A-B) The effector cells were obtained from CTLs
374 induced from PBMCs of HLA-A2+ PPD+ donors. (C) The HLA-A2 molecules on the T2 cells
375 surface were blocked by anti-HLA-A2 monoclonal antibody.
376
377
378
379
380
381
382
383
384
385
386
387
388
389

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015
PeerJ PrePrints

390



391

392

393 Figure 3. IFN-γ release ELISPOT assay of the splenocytes from the immunized HLA-A2.1/K^b

394 transgenic mice. HLA-A2.1/K^b transgenic mice were immunized with 100μg each peptide

395 emulsified in incomplete Freund's adjuvant (IFA) in the presence of 140μg of the T helper

396 epitope every five days for three times.

397

398

399

400

401

402

403

404

405

406

407

408

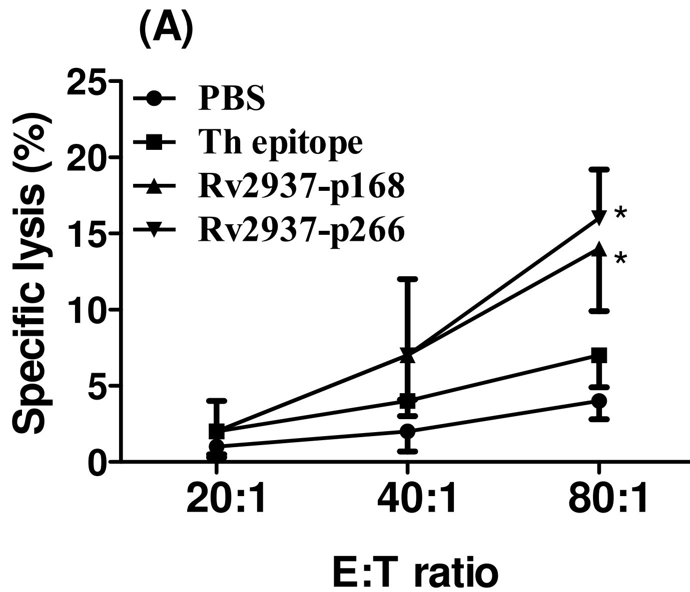
409

410

411

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015
PeerJ PrePrints

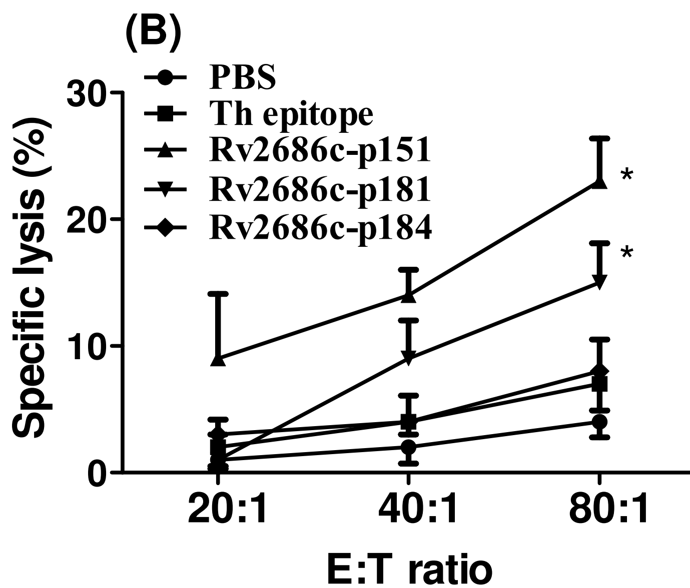
412



413

414

415



416

417 Figure 4. Specific lysis of T2 cells loaded with synthetic peptides by the CTLs generated from

418 the immunized HLA-A2.1/K^b transgenic mice (*n*=5). The data from each peptide immunized

419 group were compared with the Th epitope alone negative control.

420

421

422

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015
PeerJ PrePrints

423

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	YLAAKLTVL	29	1.3019
	151	FLAAVIPLA	22	1.2362

425

PeerJ PrePrints

PeerJ PrePrints | https://dx.doi.org/10.7287/peerj.preprints.987v1 | CC-BY 4.0 Open Access | rec: 16 Apr 2015, publ: 16 Apr 2015