



UNIVERSITI PUTRA MALAYSIA

***CHARACTERIZATION OF M1A2 MONOCLONAL ANTIBODY AND
In Vitro CYTOTOXICITY ASSESSMENT***

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FBSB 2012 56

**CHARACTERIZATION OF M1A2 MONOCLONAL ANTIBODY AND
In Vitro CYTOTOXICITY ASSESSMENT**

By

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**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of Requirements for the Degree of Doctor of Philosophy**

June 2012

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Faculty: Biotechnology and Biomolecular Sciences

Despite all the big achievements in diagnosis and clinical advances, cancer as a life threatening illness, remains a problematic issue. Clinical successes of monoclonal antibodies confirm the capacity of immunotherapeutics to amend the demands in cancer treatment. To find a potential therapeutic and diagnostic product, several hybridoma clones were established earlier by fusion of lymphocytes from BALB/c mice sensitized with MCF7 breast carcinoma cell line and Sp20/0-Ag 14 myeloma cells. M1A2 is one of the stable hybridoma clones producing an IgM monoclonal antibody with κ light chain. In this study, the M1A2 hybridoma clone was recloned by limiting dilution technique and the monoclonal antibody reactivity was screened against MCF7 and HT29 cell lines using cell-ELISA. High producer hybridoma clone was selected and the monoclonal antibody was produced in culture media (*in*

vitro) and in ascitic fluid of peritoneal cavity of pristine primed BALB/c mice. Then the antibody was purified by an affinity chromatography on an F PLC system. The purified monoclonal antibody was characterized by immunological experiments such as immunofluorescence assay and immunohistochemistry. Then the target antigen was identified using protein techniques such as gel electrophoresis, immunoblotting and MALDI TOF/TOF mass spectrometry. Finally, the cytotoxicity potential of antibody was examined by MTT assay and complement-dependent cytotoxicity as well as antibody-dependent cellular cytotoxicity experiments.

The results displayed the reactivity of M1A2 monoclonal antibody against tested human, mouse and rabbit cell lines in cell-ELISA technique. The reactivity results were further confirmed by immunofluorescence assay, which illustrated FITC-labelling in cells' cytoplasm. Immunohistochemical studies also revealed the positive staining of both human normal and cancerous tissues with M1A2 mAb with positive nuclear staining in less-differentiated carcinomas and cytoplasmic in well-differentiated cancerous as well as normal breast, colon and ovary tissues. The flow cytometry analysis also verified the reactivity of M1A2 mAb toward both normal and cancerous cell lines. The HT29 cell line showed the highest percentage of stained cells with $90.07 \pm 1.15\%$ followed by PBMC, HeLa and MCF7 with $88.7 \pm 0.35\%$, $77.3 \pm 2.66\%$ and $52 \pm 0.76\%$, positive staining respectively measured by flow cytometry. Additional experiments were performed to identify M1A2 mAb target protein. A 65 kDa protein band was recognized on PVDF membrane in western blot experiment and a protein with a same molecular weight was immunoprecipitated from HT29 whole cell lysate by the M1A2 mAb. Then the mAb

target protein was identified using MALDI TOF/TOF mass spectrometry and data mining suggested this protein belongs to heat shock protein family named Hsp60. The antibody also displays *in vitro* cytotoxicity toward MCF7 and PBMC with an approximate IC₅₀ value of 403 µg/ mL.

These findings support the introduction of M1A2 mAb as a new monoclonal antibody that recognize Hsp60 protein. With regard to the importance of heat shock proteins especially Hsp60 in new cancer research studies, the M1A2 mAb has the potential to develop as diagnostic and or therapeutic monoclonal antibody in future works.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor of Falsafah

PENCIRIAN M1A2 ANTIBODI MONOKLONAL DAN IN VITRO SITOTOKSITI PENILAIAN

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Walaupun terdapat pencapaian besar dalam diagnosis dan kemajuan klinikal, kanser sebagai penyakit kehidupan yang mengancam, kekal suatu isu yang bermasalah. Kejayaan klinikal antibodi monoklonal mengesahkan keupayaan immunotherapeutics untuk meminda permintaan dalam perawatan kanser. Untuk mencari produk terapeutik dan diagnostik yang berpotensi, beberapa hybridoma klon telah ditubuhkan lebih awal oleh gabungan limfosit dari tikus BALB / c yang sensitif dengan sel payudara karsinoma MCF7 dan sel-sel myeloma Sp20/0-Ag 14. M1A2 adalah salah satu klon hybridoma yang stabil menghasilkan antibodi IgM monoklonal dengan rantai cahaya κ . Dalam kajian ini, klon M1A2 hybridoma telah diklon semula dengan cara teknik cairan terbatas dan kereaktifan antibodi monoklonal telah diuji terhadap sel MCF7 dan HT29 menggunakan sel-ELISA. Klon hybridoma penghasil yang tinggi telah dipilih dan antibodi monoklonal telah dihasilkan dalam media kultur (*in vitro*) dan dalam cecair ascitik kaviti peritoneal

tikus BALB/c. Kemudian antibodi tersebut telah dituliskan melalui kromatografi afiniti pada sistem FPLC. Antibodi monoklonal tulen telah dicirikan melalui eksperimen imunologi seperti asai immunofluorescence dan immunohistokimia. Kemudian antigen sasaran telah dikenal pasti menggunakan teknik protein seperti elektroforesis gel, immunoblotting dan MALDI TOF/TOF spektrometri jisim. Akhirnya potensi sitotoksiti antibodi telah diuji menggunakan asai MTT dan sitotoksiti komplemen-dependent dan juga eksperimen sitotoksiti selular yang bergantung kepada antibodi.

Keputusan yang dipapar menunjukkan kereaktifan tindak balas M1A2 antibodi monoklonal terhadap sel manusia, tikus dan arnab dalam teknik sel-ELISA. Keputusan kereaktifannya telah disahkan oleh asai immunofluorescence yang telah digambarkan oleh penglabelan-FITC dalam sitoplasma sel. Kajian immunohistochemical ini juga mendedahkan pewarnaan positif kedua-dua tisu manusia biasa dan ber kanser dengan M1A2 mAb dengan pewarnaan positif nuklear dalam karsinoma kurang-dibezakan dan sitoplasmik dalam kanser yang baik-dibezakan dan juga tisu payudara, kolon dan ovari normal. Analisis aliran sitometri juga mengesahkan kereaktifan M1A2 mAb terhadap sel normal dan ber kanser. Sel HT29 menunjukkan peratusan yang tertinggi dalam sel-sel berwarna yang diukur menggunakan aliran sitometri iaitu $90.07 \pm 1.15\%$ yang diikuti oleh PBMC, HeLa dan MCF7 dengan masing-masing $88.7 \pm 0.35\%$, $77.3 \pm 2.66\%$ dan $52 \pm 0.76\%$. Eksperimen tambahan telah dijalankan bagi mengenalpasti sasaran protein M1A2 mAb. Sejalur protein 65 kDa telah dikenal pasti di atas membran PVDF dalam eksperimen Western blot dan protein dengan berat molekul yang sama telah diimmunoprecipitate

dari keseluruhan lysate sel HT29 oleh mAb M1A2. Kemudian protein sasaran mAb telah dikenal pasti menggunakan MALDI TOF/TOF spektrometri jisim dan pencarian data mencadangkan protein ini tergolong dalam keluarga protein 'heat shock' yang dinamakan Hsp60. Antibodi ini juga memaparkan sitotoksiti terhadap MCF7 dan PBMC *in vitro* dengan nilai IC_{50} 403 μ g / mL.

Penemuan ini menyokong pengenalan M1A2 mAb sebagai antibodi monoklonal baru yang mengenal pasti Hsp60 protein. Berhubung dengan kepentingan protein 'heat shock' terutama Hsp60 dalam kajian penyelidikan kanser yang baru, mAb M1A2 mempunyai potensi untuk dibangunkan sebagai diagnostik dan atau terapeutik antibodi monoklonal dalam kerja-kerja pada masa hadapan.

ACKNOWLEDGEMENT

*Glory and praise to God, the omnipotent, omniscient and omnipresent,
for all of his helps during my life.*

The author wishes to express her deepest appreciation to all the persons who have inspired, advised, and assisted her during this study. Special recognition and sincere appreciation is extended to Prof Dr Abdul Manaf Ali, the chair of the supervisory committee, for his invaluable guidance and encouragement throughout this work. The help and support of other members of the supervisory committee, Prof. Datin Paduka Dr Khatijah Mohd Yusoff, Assoc. Prof. Dr. Muhajir Hamid and Assoc. Prof. Dr. Noorjahan Banu Mohamed Alitheen is deeply appreciated.

My special thanks are due to Prof. Madya Dr Sabariah Abdul Rahman and Dr Shu-Yuan Liao for their great assistant in IHC experiments, Dr. Fatemeh Jahanshiri, and Assoc. Prof. Dr. Mohd. Puad Abdullah for their excellent technical assistance performing several analyses included in this thesis. I would like to express greatest thanks to Prof. Dr. Rasedee Abdullah and the staffs of the Laboratory of Immunotherapeutics and Vaccines (LIVES) and Animal Cell Culture Lab, Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Science, UPM for the positive and dynamic working environment.

I wish to extend my appreciation to everyone, although not individually named here, who had contributed directly or indirectly to my project and thesis. I am indebted to all my colleagues Dr. Mohd Rosni Sulaiman, Dr. Yeap Swee Keong, Dr. Rammanan,

Dr Afshin Ebrahimpour, Ira, Zahra, Morvarid, Tam, Ruzila, Cheewun, Joo EE Beh and Mehdi for sharing their knowledge and experience with me. Finally, I would like to thank University Putra Malaysia and the Ministry of Science, Technology, and Innovation of Malaysia for providing research grant 54953 for supporting this study. Many thanks for giving me this opportunity.

Last, but certainly not least, I extend my never-ending gratitude to my beloved family for their unconditional love, support, and encouragement in my entire life.



I certify that a Thesis Examination Committee has met on 29 June 2012 to conduct the final examination of Fatemeh Bashokouh on her thesis entitled "Characterization of M1A2 Monoclonal Antibody and *In Vitro* Cytotoxicity Assessment" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has been previously, and is not concurrently, submitted for any other degree at University Putra Malaysia or at any other institution.

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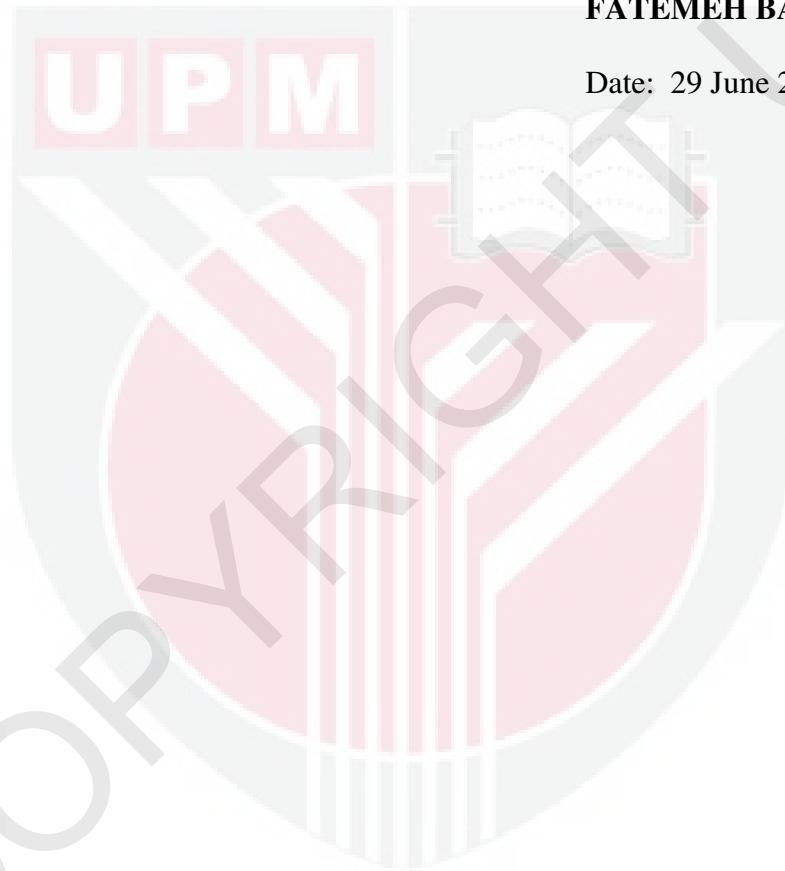


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