



รายงานวิจัยฉบับสมบูรณ์

โครงการ บทบาทของสัญญาณ Notch ในการควบคุมการทำงานของมาโครฟาจและการตายแบบอะพอพโตซิสที่ชักนำโดยการติดเชื้อ mycobacteria (The role of Notch signaling in regulating macrophage effector functions and apoptosis induced by mycobacterial infection)

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ชักนำโดยการติดเชื้อ mycobacteria" (The role of Notch signaling in regulating macrophage effector

functions and apoptosis induced by mycobacterial infection)

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สารบัญ

	หน้าที่
บทคัดย่อภาษาไทย	4
บทคัดย่อภาษาอังกฤษ	5
Executive Summary	6
เนื้อหางานวิจัย	10
Introduction	10
Materials and Methods	11
Results	14
Discussion	24
เอกสารอ้างอิง	27
กิตติกรรมประกาศ	29
Output ที่ได้จากโครงการ	30
ภาคผนวก	31

บทคัดย่อ

สัญญาณ Notch เป็นวิถีสัญญาณที่มีการอนุรักษ์ไว้สูง ซึ่งมีหน้าที่ในการควบคุมการเพิ่มจำนวนของเซลล์ การตัดสินใจชะตาของเซลล์และการตายแบบ apoptosis ของเซลล์หลากหลายชนิด ในระหว่างพัฒนาการของระบบสร้างเลือด สัญญาณ Notch ควบคุมการตัดสินใจ lineage ในหลายระดับ รวมทั้งพัฒนาการของเซลล์ไมอีลอยด์และลิมโฟไซต์ แมคโครฟาจมีการแสดงออกของ Notch receptor และ ligand ในระหว่างระยะพัฒนาการต่างๆ ซึ่งหน้าที่ของวิถีสัญญาณ Notch ในแมคโครฟาจที่มีพัฒนาการสมบูรณ์แล้ว ยังไม่มีการศึกษาในรายละเอียด งานวิจัยนี้จึงมีจุดประสงค์ที่จะศึกษาความเกี่ยวข้องของสัญญาณ Notch ในการควบคุมการทำงานของแมคโครฟาจและความเกี่ยวข้องในระหว่างที่มีการติดเชื้อไมโคแบคทีเรียในแมคโครฟาจ แมคโครฟาจปฐมภูมิที่พัฒนาการมาจากไขกระดูกและในเซลล์ไลน์ RAW264.7 ที่ไม่ได้รับการกระตุ้นมีการแสดงออกของ Notch1, 2 และ 4 เมื่อทำการกระตุ้นแมคโครฟาจโดยใช้ lipopolysaccharide พร้อมด้วย interferon-gamma พบว่ามีการเพิ่มการแสดงออกของเฉพาะ Notch1, 2 อีกทั้งสามารถตรวจหาโปรตีน Notch1 ที่ถูกตัด และการแสดงออกของ *Hes1* และ *Deltex* ซึ่งเป็นเงินเป้าหมายของสัญญาณ Notch ก็มีการเหนี่ยวนำขึ้น แสดงว่าเกิดการกระตุ้นวิถีสัญญาณ Notch เมื่อนำอะกอนิสต์แต่ละชนิดของ Toll-like receptor (TLR) (TLR2, TLR3, TLR4, TLR9) มากระตุ้นแมคโครฟาจ พบว่ามีการแสดงออกเพิ่มของ Notch1 เพื่อศึกษาหน้าที่ของสัญญาณ Notch ระหว่างการหน้าที่ของแมคโครฟาจ จึงใช้วิธีทางเภสัชวิทยาและพันธุศาสตร์ เมื่อทำการกีดการตัดด้วย Notch ด้วยเอนไซม์แกมมาซีรีเทส โดยใช้ยาเอนไซม์แกมมาซีรีเทส (GSI) พบว่ามีการลดลงของการผลิตไนโตรออกไซด์ $\text{TNF}\alpha$ และการแสดงออกของ IL-6 mRNA ในขณะที่มีการเพิ่มขึ้นของการแสดงออกของ IL-10 mRNA และ MHC Class II ในทางตรงกันข้าม เมื่อทำการแสดงออกเกินของ Notch ที่มีแอคติวิตี พบว่ามีการเพิ่มขึ้นของไนโตรออกไซด์ และ $\text{TNF}\alpha$ ที่ผลิต เพื่อทดสอบความเกี่ยวข้องของหน้าที่ของสัญญาณ Notch ในระหว่างที่มีการติดเชื้อในแมคโครฟาจ จึงได้ทำการติดเชื้อแมคโครฟาจด้วย *Mycobacterium bovis* สายพันธุ์กลายที่ไม่ก่อให้เกิดโรค BCG หลังจากติดเชื้อพบว่าการเพิ่มขึ้นอย่างต่อเนื่องของการแสดงออกของ Notch1 ในแมคโครฟาจปฐมภูมิ เมื่อทำการกีดวิถีสัญญาณ Notch โดยใช้ GSI ส่งผลให้มีการลดลงของแบคทีเรีย CFU และการอยู่รอดของแมคโครฟาจ ซึ่งมีความสัมพันธ์กับการแสดงออกของเงินด้านการตายแบบ apoptosis *Mcl1* อีกทั้งพบว่ามีมีการแสดงออกเกินของ Notch แมคโครฟาจที่ติดเชื้อมีการตายลดลง ดังนั้น งานวิจัยนี้จึงเป็นงานวิจัยแรกที่แสดงให้เห็นถึงหน้าที่ที่ซับซ้อนของวิถีสัญญาณ Notch ในการควบคุมการทำงานของแมคโครฟาจ และการตายแบบ apoptosis ที่เหนี่ยวนำด้วยการติดเชื้อไมโคแบคทีเรีย ซึ่งจากผลที่ได้นี้ วิถีสัญญาณ Notch อาจจะเป็นเป้าหมายใหม่ในการนำไปใช้บำบัด โดยการเปลี่ยนแปลงการทำงานของแมคโครฟาจให้เหมาะสมและพัฒนาวัคซีนที่มีประสิทธิภาพได้ต่อไป

Abstract

Notch signaling is a well conserved signaling pathway which is involved in regulating cell proliferation, cell fate decision and apoptosis in various cell types. During hematopoiesis, Notch signaling regulates multiple lineage choices, including development of myeloid and lymphoid cells. Macrophages express Notch receptors and ligands at various stages during development. The functions Notch signaling plays in developmentally matured macrophages are largely unexplored. This study aimed at elucidating the involvement of Notch signaling in regulating effector functions of macrophages and its relevance during infection of macrophages with mycobacteria. Notch1, 2 and 4, were expressed in resting primary bone marrow derived macrophages and RAW264.7 macrophage like cell line. Upon activation with lipopolysaccharide together with interferon-gamma, only Notch1 and 2 were upregulated. In addition, cleaved Notch1 was detected and expression of the target genes of Notch signaling, *Hes1* and *Deltaex*, were induced, suggesting that Notch signaling is activated. When each Toll-like receptor (TLR) agonist (TLR2, TLR3, TLR4, TLR9) was used to stimulate macrophages, Notch1 was upregulated in all cases. To address the roles Notch signaling plays during effector phase of macrophages, pharmacological and genetic approaches were used. Suppression of Notch processing by gamma-secretase using gamma-secretase inhibitor (GSI) resulted in decreased nitric oxide, TNF α production and IL-6 mRNA expression, while increased IL-10 mRNA expression and MHC class II expression were detected. In contrast, overexpression of activated form of Notch resulted in increased nitric oxide and TNF α production. To test the roles of Notch signaling in relevance to infection, macrophages were infected with attenuated strains of *Mycobacterium bovis*, BCG. Upon infection, sustaining upregulation of Notch1 was detected in primary macrophages. Inhibition of Notch signaling using GSI resulted in significant decrease in bacterial CFU and viability of macrophages, correlating with decreased expression of anti-apoptotic gene, *Mcl1*. In addition, overexpression of Notch protected macrophages from undergoing apoptosis as a result of BCG infection. Taken together, this study reveals for the first time the complicated roles Notch signaling plays in regulating effector functions and apoptosis induced by mycobacterium infection. Thus, Notch signaling pathway may provide a novel therapeutic target in manipulating macrophage functions and development of more effective vaccines.

หน้าสรุปโครงการ (Executive Summary)
ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่

1. ชื่อโครงการ

(ภาษาไทย) บทบาทของสัญญาณ Notch ในการควบคุมการทำงานของมาโครฟาจและการตายแบบอะพอพโทซิสที่ชักนำโดยการติดเชื้อ mycobacteria

(ภาษาอังกฤษ) The roles of Notch signaling in regulating macrophage effector functions and apoptosis induced by mycobacterial infection

2. ความสำคัญของปัญหา

วิถีสัญญาณ Notch ทำหน้าที่สำคัญระหว่างพัฒนาการของตัวอ่อนและอวัยวะหลากหลายประเภท รวมถึงควบคุมกระบวนการพัฒนาของเซลล์ในระบบภูมิคุ้มกันด้วย โดยวิถีสัญญาณ Notch ทำหน้าที่ควบคุมการแปรสภาพ (differentiation) ของเซลล์ในระบบภูมิคุ้มกันในหลายระดับ ทั้งใน lymphoid และ myeloid lineage อีกทั้งยังมีหน้าที่ควบคุมการเจริญและการตายของเซลล์อีกด้วย ความผิดปกติของวิถีสัญญาณ Notch ที่ทำให้มีการแสดงออกเกินในบางกรณีมีความสัมพันธ์โดยตรงกับการกลายเป็นมะเร็งของเซลล์ในหลายอวัยวะ รวมถึงเซลล์เม็ดเลือดขาวด้วย นอกจากนี้หน้าที่สำคัญในการควบคุมพัฒนาการของระบบภูมิคุ้มกันนี้แล้ว เซลล์เม็ดเลือดขาวที่พัฒนาสมบูรณ์แล้ว ยังมีการแสดงออกของจีนต่างๆ อันเป็นองค์ประกอบของวิถีสัญญาณ Notch โดยใช้เป็นวิถีสัญญาณในการควบคุมการทำงานให้เหมาะสมอีกด้วย เช่น T ลิมโฟไซต์ที่พัฒนาสมบูรณ์แล้วใช้วิถีสัญญาณ Notch เป็นปัจจัยสำคัญในการควบคุมการแปรสภาพของเซลล์ในการตอบสนองของระบบภูมิคุ้มกัน เป็นต้น ดังนั้น จึงได้มีการสันนิษฐานว่าวิถีสัญญาณ Notch อาจทำหน้าที่สำคัญในการควบคุมการทำงานของเซลล์เม็ดเลือดขาวชนิดอื่นๆ หลังจากที่มันพัฒนาการที่สมบูรณ์แล้ว

แมคโครฟาจเป็นเซลล์ที่มีหน้าที่สำคัญในการตอบสนองของภูมิคุ้มกัน โดยเป็นเซลล์ด่านแรกในการรับรู้และป้องกันการเข้าสู่ร่างกายของจุลชีพและแอนติเจนต่างๆ อีกทั้งยังเป็นเซลล์ที่ทำหน้าที่เป็นตัวเชื่อมระหว่างการตอบสนองของระบบภูมิคุ้มกันแบบ innate และแบบ adaptive โดยทำหน้าที่เป็นเซลล์ที่นำเสนอแอนติเจนต่อ T ลิมโฟไซต์ แมคโครฟาจรับรู้การเข้าสู่ร่างกายของจุลชีพโดยใช้กลุ่มโปรตีนรีเซปเตอร์ที่จำเพาะต่อลักษณะเด่นของจุลชีพแต่ละชนิด เช่น รีเซปเตอร์ในกลุ่ม Toll-like receptor (TLR) ซึ่งมีความจำเพาะต่อองค์ประกอบของผนังเซลล์ของแบคทีเรีย หรือ ลักษณะจำเพาะของดีเอ็นเอของไวรัส เป็นต้น เมื่อถูกกระตุ้นผ่านรีเซปเตอร์แล้ว แมคโครฟาจจะเริ่มทำงานทันทีโดยหลั่งสารไซโตไคน์เพื่อชักนำให้เกิดอาการอักเสบ อีกทั้งยังกลืนกินและฆ่าจุลชีพได้ การตายของแมคโครฟาจหลังการติดเชื้อแบคทีเรียหรือไวรัสถือเป็นกลไกหนึ่งที่ระบบภูมิคุ้มกันใช้ในการตอบสนองต่อการติดเชื้อ โดย dendritic cell จะกลืนกินชิ้นส่วนของแมคโครฟาจที่ตายและมีส่วนประกอบของแบคทีเรียอยู่ภายใน จากนั้นจะย่อยแอนติเจนของแบคทีเรียเพื่อนำเสนอแอนติเจนต่อ T ลิมโฟไซต์ต่อไป ดังนั้น การตายของแมคโครฟาจหลังจากการติดเชื้อจึงมีความสำคัญในการที่จะเหนี่ยวนำการตอบสนองแบบจำเพาะให้มีประสิทธิภาพมากขึ้น แบคทีเรียบางชนิดมีกลไกในการหลบหลีกกระบวนการนี้ เช่น *Mycobacterium tuberculosis* ที่ทำก่อให้เกิดโรควัณโรคบางสายพันธุ์มีกลไกในการยับยั้งการตายของแมคโครฟาจได้ โดยกลไกในการยับยั้งการตายนี้ยังไม่เป็นที่ทราบแน่ชัด

ปัจจุบันมีรายงานว่าวิถีสัญญาณ Notch มีหน้าที่ควบคุมการแปรสภาพของแมคโครฟาจจากเซลล์ไมอีลอยด์โปรเจเนเตอร์ และมีรายงานว่าหลังจากที่โมโนไซต์แปรสภาพเป็นแมคโครฟาจแล้วยังคงมีการแสดงออกของจีน *Notch* ต่อไป ซึ่งในปัจจุบันยังไม่มียานวิจัยใดที่ศึกษาเกี่ยวกับหน้าที่ของวิถีสัญญาณ Notch ในแมคโครฟาจที่ได้รับการกระตุ้นโดยแบคทีเรีย ดังนั้น โครงการวิจัยนี้จึงมีจุดประสงค์ที่จะศึกษาว่าวิถีสัญญาณ Notch ทำหน้าที่สำคัญในการควบคุมการทำงานของแมคโครฟาจหรือไม่ และมีส่วนเกี่ยวข้องกับกระบวนการตายของแมคโครฟาจหลังการติดเชื้อแบคทีเรีย โดยจะใช้เชื้อ *mycobacteria* ซึ่งเป็นเชื้ออันเป็นสาเหตุของโรคติดต่อที่เป็นปัญหาทั่วโลกและในประเทศไทยในปัจจุบัน เป็นแบบจำลอง องค์ความรู้ใหม่ที่ได้จากโครงการวิจัยนี้สามารถใช้เป็นข้อมูลพื้นฐานในการพัฒนาวิธีการควบคุมการตอบสนองของระบบภูมิคุ้มกันของแมคโครฟาจผ่านวิถีสัญญาณ Notch ซึ่งจะเป็นประโยชน์ต่อการพัฒนาวัคซีนที่มีประสิทธิภาพสำหรับโรคที่เกิดจากการติดเชื้อ *mycobacteria* ต่อไปในอนาคต

3. วัตถุประสงค์

- 8.1 ศึกษาการแสดงลักษณะของจีน *Notch* ในเซลล์แมคโครฟาจที่ถูกกระตุ้นโดย lipopolysaccharide (LPS) และ peptidoglycan ของแบคทีเรีย
- 8.2 ศึกษาผลการยับยั้งการส่งสัญญาณของ Notch ที่มีต่อการทำงานของเซลล์แมคโครฟาจ
- 8.3 ศึกษาผลกระทบของการแสดงออกเกินของจีน *Notch* ในเซลล์แมคโครฟาจ
- 8.4 ศึกษาความสัมพันธ์ของการแสดงลักษณะของ Notch กับการตายของแมคโครฟาจที่เกิดจากการติดเชื้อ *Mycobacterium bovis* bacillus Calmette-Guerin (BCG)

4. ระเบียบวิธีวิจัย

- 4.1 แยกแมคโครฟาจปฐมภูมิของหนูทดลองจากเซลล์ไขกระดูก
- 4.2 วิเคราะห์การแสดงลักษณะของจีน *Notch 1* และ *Notch 2* ในเซลล์แมคโครฟาจปฐมภูมิของหนูและเซลล์แมคโครฟาจ RAW264.7 ที่ได้รับการกระตุ้นโดย LPS และ peptidoglycan ของแบคทีเรีย
- 4.3 ยับยั้งการส่งสัญญาณของ Notch โดยใช้สารยับยั้งเอนไซม์ γ -secretase ในเซลล์แมคโครฟาจและวิเคราะห์การเปลี่ยนแปลงของแมคโครฟาจเมื่อถูกกระตุ้นโดย LPS โดยจะศึกษาความสามารถในการผลิตสารไซโตไคน์ ความสามารถในการกลืนกินและฆ่าแบคทีเรีย
- 4.4 ทำการสร้างพลาสมิดเวกเตอร์ที่มีชิ้นดีเอ็นเอซึ่งประมวลรหัสของ *Notch*
- 4.5 นำรีคอมบิแนนท์พลาสมิดจากข้อที่ 4.4 เข้าสู่เซลล์ RAW264.7 และคัดเลือกทรานสเฟคแทนท์ที่มีรีคอมบิแนนท์ พลาสมิด
- 4.6 วิเคราะห์การตอบสนองของทรานสเฟคแทนท์ต่อการกระตุ้นโดย LPS ตามรายละเอียดในข้อที่ 4.3
- 4.7 ศึกษาการแสดงลักษณะของจีน *Notch* ในระหว่างการติดเชื้อ *Mycobacterium bovis* bacillus Calmette-Guerin และผลกระทบจากการยับยั้งวิถีสัญญาณ Notch ต่อการตายของแมคโครฟาจ จากการติดเชื้อ *Mycobacterium bovis* bacillus Calmette-Guerin

5. แผนการดำเนินงานตลอดโครงการ

กิจกรรม	ปีที่ 1 ช่วงที่ 1		ปีที่ 1 ช่วงที่ 2			ปีที่ 2 ช่วงที่ 1			ปีที่ 2 ช่วงที่ 2		
	3	6	3	6	3	3	6	3	3	6	6
1. รวบรวมข้อมูลและเตรียมอุปกรณ์	↕										
2. แยกเซลล์แมคโครฟาจปฐมภูมิของหนูทดลองจากเซลล์ไขกระดูก	↕										
3. วิเคราะห์การแสดงลักษณะของจีน <i>Notch 1</i> และ <i>Notch 2</i> ในเซลล์แมคโครฟาจปฐมภูมิของหนูและเซลล์แมคโครฟาจ RAW264.7 ที่ได้รับการกระตุ้นโดย LPS และ peptidoglycan ของแบคทีเรีย			↔								
4. ยับยั้งการส่งสัญญาณของ Notch โดยใช้สารยับยั้งเอ็นไทม์ γ -secretase ในเซลล์แมคโครฟาจและวิเคราะห์การเปลี่ยนแปลงของแมคโครฟาจเมื่อถูกกระตุ้นโดย LPS โดยจะศึกษาความสามารถในการผลิตสารไซโตไคน์ ความสามารถในการกลืนกินและฆ่าแบคทีเรีย				↔			↔				
5. ทำการสร้างพลาสมิดเวคเตอร์ที่มีเอ็นไทม์ซึ่งประมวลรหัสของ <i>Notch</i>				↔							
6. นำรีคอมบิแนนท์พลาสมิดจากข้อที่ 9.4 เข้าสู่เซลล์ RAW264.7 และคัดเลือกทรานสเฟคแทนต์ที่มีรีคอมบิแนนท์พลาสมิด				↔							
7. วิเคราะห์การตอบสนองของทรานสเฟคแทนต์ต่อการกระตุ้นโดย LPS ตามรายละเอียดในข้อที่ 4							↔			↔	
8. ศึกษาการแสดงลักษณะของจีน <i>Notch</i> ในระหว่างการติดเชื้อ <i>Mycobacterium bovis</i> bacillus Calmette-Guerin และผลกระทบจากการยับยั้งวิถีสัญญาณ Notch ต่อการตายของแมคโครฟาจ จากการศึกษาเชื้อ <i>Mycobacterium bovis</i> bacillus Calmette-Guerin										↕	

Introduction

Macrophages play a pivotal role during innate and acquired immune responses. They mount first line defenses during infections, mediate cellular immune response and serve to direct acquire immune response. In contrast, macrophages can also cause damage to host by mediating chronic inflammation leading to tissue destructions and serving as a hiding niche for intracellular pathogens such as intracellular bacteria and viruses [1]. Given multifaceted roles of macrophages in mounting effective immune responses and pathological conditions, signalings governing activation and functions of these cells have been extensively studied. The recognition of pathogen associated molecular patterns through pattern recognition receptors such as Toll-like receptors and c-type lectins by macrophages signals through diverse signaling cascades including NF- κ B and MAPK pathways, which are translated into expression of immune effector functions [2].

Notch signaling is well conserved signaling pathway involved in controlling proliferation, differentiation and cell death of various cell types. In mammals, there are 4 distinct Notch receptors, i.e. Notch1-4, while 5 different Notch ligands are identified. The signal cascade is initiated by ligand-receptor interaction on cell surface, resulting in processing of Notch receptors by 2 enzymes, i.e. TACE and γ -secretase. Upon being cleaved, intracellular domain of Notch receptors is released and translocates to nucleus where it forms a transcriptional activation complex with DNA-binding protein CSL and other transcriptional coactivators such as MAML, PCAF and p300. Consequently, target genes of Notch signaling are expressed by the Notch transcriptional complex. Currently, many genes have been identified as targets of Notch signaling, including transcriptional repressor *Hes* family gene, *Deltex*, *CD25*, *Bcl-2* and *c-myc* [3].

During hematopoiesis, Notch signaling is involved in regulating multiple lineage choices. In myeloid lineage cells, Notch signaling plays essential roles in development of monocyte/macrophage and dendritic cells. In addition, Notch receptors and ligands are expressed in fully functional resting macrophages post development, implying that it may serve to regulate functions of macrophages [4, 5]. In fact, Notch ligands expressed on antigen presenting cells plays an important role in steering differentiation of helper T cells during immune response [6]. Despite the expression pattern of Notch receptor in macrophages, the roles this signaling pathway play during effector phase in these cells are largely unexplored. In this study, we first examined expression of Notch receptors in resting and stimulated bone marrow derived murine macrophages and macrophage cell line. Using pharmacological and genetic approaches, we uncovered that Notch signaling regulates multiple effector functions of macrophages. The involvement of Notch signaling was further studied in setting of bacterial infection. Notch signaling was found to inhibit apoptosis of infected macrophages by maintaining the expression of anti-apoptotic gene *Mcl1*. The insights into regulatory mechanism Notch signaling utilize to control macrophage effector functions and apoptosis is discussed.

Materials and Methods

Mice, BM-derived macrophages and RAW264.7 cell line

Female C57BL6 mice (aged between 10–12 weeks) were purchased from the National Laboratory Animal Centre (Mahidol University, Salaya, Thailand). All procedures involving laboratory animals were conducted according to guidelines issued by Chulalongkorn University. MyD88^{-/-} mice back-crossed onto the C57/BL6 background were kindly provided by Dr. Shizuo Akira and the experiments involving these mice were conducted at the Max Planck Institute for Infection Biology according to German protection law [7]. Mice were sacrificed, and femoral bone marrows were isolated. BM-derived macrophages were generated as described elsewhere [8]. In brief, cells were washed from BM cavity and incubated for 7 days in RPMI 1640 media supplemented with 10% fetal bovine serum purchased from HyClone Laboratory (Logan, UT), 100 μ M gentamycin and 20% L929-conditioned media. Media were changed every 2 days and cells were harvested at the end of culture using 5 mM EDTA in phosphate buffer saline (PBS). Cells were confirmed to be of macrophage/monocyte lineage by cell surface staining with anti-CD11b and anti-Mac1 mAbs and by analysis using flow cytometry. RAW264.7 cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 μ M gentamycin and L-glutamine.

For overexpression of Nic in RAW264.7, pcDNA3 containing DNA corresponding to intracellular Notch (amino acid 1754-2556) or pcDNA3 empty vector control were used to transfect using FuGene6 (Roche) and selected in G418 to obtain stably transfected cell line.

Reagents and antibodies

LPS from *Escherichia coli* O26:B6, polyinosinic:polycytidylic acid were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic TLR2 agonist Pam₃Cys lipopeptide was purchased from EMC microcollections GmbH (Germany). TLR agonist CpG DNA has been described elsewhere [9]. Mouse r-IFN γ was purchased from R&D Systems (Minneapolis, MN) and kept at -80°C . The γ -secretase inhibitor, IL-CHO, has been described elsewhere [10]. IL-CHO was dissolved in DMSO at a concentration of 5 mM and aliquots were kept at -80°C . TriZol reagent for total RNA isolation was purchased from Life Technologies (Grand Island, NY). The mAb for cell surface staining, i.e. FITC-conjugated anti-Mac1 mAb, PE-conjugated anti-CD11b, mAb and biotinylated anti-mouse MHC class II (IA^b) mAb were purchased from Caltag (Carlsbad, CA). Rabbit polyclonal Ab against Notch1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal Ab against cleaved Notch1 (Val1744) was purchased from Cell Signaling Technology (Boston, MA). The mAb against β actin was purchased from Chemicon International (Temecula, CA). Secondary Ab conjugated with HRP was purchased from Amersham Biosciences (Piscataway, NJ).

RT-PCR

Total RNA was isolated from cells treated as indicated using TriZol reagent according to the manufacturer's instruction. RNA (500 ng) was used to generate cDNA using random hexamer primers

and RevertAid M-MuLV reverse transcriptase (Fermentas; ON, Canada). Obtained cDNAs were used as templates to amplify *Notch1-4*, *Hes1*, *IL-6*, *IL-10* and *-actin*. The primer pairs used in this study are as follows.

Primer	Sequence (5' to 3')
Murine Notch1 forward	GTGAGGGTGATGTCAATG
Murine Notch1 reverse	TGAAGTTGAGGGAGCAGT
Murine Notch2 forward	TGGAGGTAAATGAATGCCAGAGC
Murine Notch2 reverse	TGTAGCGATTGATGCCGTCC
Murine Notch3 forward	ACACTGGGAGTTCTCTGT
Murine Notch3 reverse	GTCTGCTGGCATGGGATA
Murine Notch4 forward	CACCTCCTGCCATAACACCTTG
Murine Notch4 reverse	ACACAGTCATCTGGGTTTCATCTCAC
Murine Hes1 forward	CCGGTCTACACCAGCAACAGT
Murine Hes1 reverse	CACATGGAGTCCGAAGTGAGC
Murine IL-6 forward	CATGTTCTCTGGGAAATCGTGG
Murine IL-6 reverse	AACGCACTAGGTTTGCCGAGTA
Murine IL-10 forward	TCAAACAAAGGACCAGCTGGACAACATACTGC
Murine IL-10 reverse	CTGTCTAGGTCCTGGAGTCCAGCAGACTCAA
Murine Mcl1 forward	GACCGGCTCCAAGGACTC
Murine Mcl1 reverse	TGTCCAGTTTCCGGAGCAT
β actin forward	ACCAACTGGGACGACATGGAGAA
β actin reverse	GTGGTGGTGAAGCTGTAGCC

The PCR reactions were carried out using Taq DNA polymerase (Fermentas). The PCR products were analyzed on 1.5% agarose gel by electrophoresis, except those for *Hes1* which were analyzed on 5% polyacrylamide gel, and visualized after staining with ethidium bromide using gel documentation system (BioRad; Hercules, CA).

Real Time RT-PCR

A quantitative real time RT-PCR assay was developed for detection and quantification of *Deltex* transcripts using β -actin as endogenous control using total RNA isolated from cells treated as indicated using Trizol reagent (Invitrogen Life Technologies). Primers used in this study are as followed: *Deltex* forward 5' GTAAGGCTTCAAGGGG TCGCT 3', *Deltex* reverse 5' CTCAGCTTGATGCGTGTATAGCC3', β actin forward 5' ACCAACTGGGACGACATGGAGAA 3', β actin reverse 5' GTGGTGGTGAAGCTGTAGCC 3'. PCR amplification was performed with 2x QuantiTect SYBR Green PCR Master Mix with 0.5 μ M primers, 2 μ l cDNA and nuclease-free water according to the manufacturer's protocol (Qiagen). The PCR conditions are as follows: 95°C for 15 min, 95°C for 15 sec, 55°C for 30 sec and 60°C for 30 sec, followed by repeating of 40 cycles. Levels of mRNA were measured by a Light Cycler (Roche

Molecular Biochemicals, Indianapolis, IN). Levels of mRNA were expressed as threshold cycle (C_T) and used comparative C_T method for analysis. For relative quantification, expression of *Deltex* as target gene was normalized by expression of β -actin relative to a calibrator. The amount of target was given by $2^{-\Delta\Delta C_T}$.

Western blot

Cells treated as indicated were harvested and cell lysates were prepared as described previously [11]. Amounts of proteins were measured using BCA protein assay kit (Pierce; Rockford, IL). Cell lysates (30 g) were separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis using Protein III system (Bio-Rad). After gel separation, proteins were transferred onto PVDF membrane (Amersham Biosciences) and blocked in PBS containing 3% nonfat dry milk and 0.05% Tween 20. Blots were probed with rabbit anti-Notch1 Ab at 1:1000 dilution, rabbit anti-cleaved Notch1 Ab at 1:1000 dilution or anti- β actin mAb at 1:5000 dilution, followed by washing and probing with HRP-conjugated donkey anti-rabbit IgG Ab or sheep anti-mouse IgG Ab at 1:4000 dilution. After washing, signals were detected using ECL Western blotting analysis system (GE Healthcare).

Measurement of nitrites

To measure the amount of NO produced by macrophages, BM-derived macrophages were treated as indicated. At the end of culture, culture supernatants were harvested by centrifugation and kept at -80°C . Culture supernatants were subjected to assay for NO production using Griess reagents as described previously [12].

Measurement of TNF by ELISA

Quantitative ELISA was carried out using mouse TNF α ELISA test kit purchased from eBioscience (San Diego, CA). Culture supernatants were collected after treating cells as indicated and kept at -80°C until subjected to ELISA. ELISA was carried out according to manufacturer's instructions.

Bacterial Infections

BCG (Copenhagen) or MTB were cultured in liquid media M7H9 supplemented with 0.05% Tween20 and 10% OADC until OD reached 0.6-0.8. Bacterial clumped were disrupted by passing through 26G needle 10 times before using for BMDM infection. After 4 hrs of infection, extracellular bacteria were washed off by rinsing with cold PBS twice and complete media were added. Infected BMDM were subjected for total RNA isolation and crystal violet assay. For CFU assay, BMDM were lysed in PBS containing 0.1% TritonX100. Supernatant were plated onto M7H11 agar supplemented with 10% OADC [13]. Colonies were counted at day 21 to determine CFU. For Annexin V binding assay, RAW264.7 were infected as described and cells were harvested and stained according to the manufacturer's instruction. Cells were analyzed by FACS (BD Biosciences).

Statistical analysis

Student's paired t-test was used to determine the statistical significant of the data obtained and $p < 0.05$ represented a statistical significant difference.

Results

Generating Bone Marrow Derived Macrophages (BMDM)

Female mice strain C57BL/6 aged between 8-10 weeks old were sacrificed upon arrival and cells in femoral bone marrows were flushed out by RPMI1640 media. Cells were incubated with RPMI1640 media conditioned with 20% L929-filter sterile culture supernatant and 5% horse serum. Media were replaced at day 4 and incubated for 7 days. Adherent cells were harvested at the end of one week culture using 5 mM EDTA in phosphate buffered saline and analyzed for cell surface expression of macrophage-lineage specific markers, F4/80 and CD11b, and MHC class II by flow cytometry (Figure 1). More than 95% were found to be CD11b⁺ and Mac1⁺ and upregulation of MHC class II was observed in the majority of cell population.

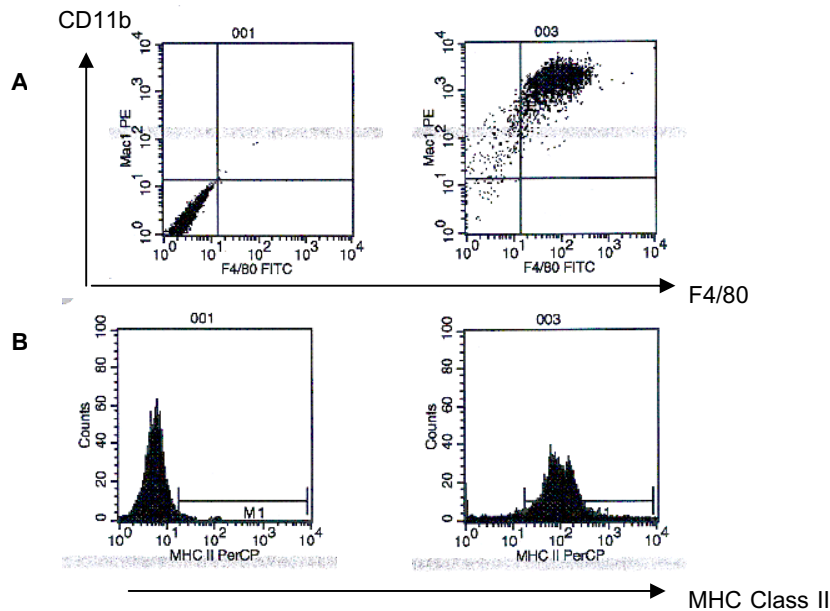


Figure1. Expression of macrophage-specific cell surface markers in bone marrow-derived stem cells cultured in the presence of L929 conditioned media. Cells stained with A. isotype control antibody, B. antibodies for F4/80, CD11b (Mac1) and MHC class II.

Examining expression profile of Notch in primary macrophages and RAW264.7 macrophage cell line activated by TLR agonists

To address whether Notch receptors are expressed in macrophages during LPS/IFN γ stimulation, we examined expression of Notch1-4 in RAW264.7 and BMDM by semi-quantitative RT-PCR. As shown in Figure 2, Notch1, 2 and 4 were readily detected in resting RAW264.7 and BMDM. Upon activation by LPS with or without IFN γ , Notch1 was upregulated in both RAW264.7 and BMDM, while Notch2 was seen upregulated only in BMDM. In contrast, Notch4 was downregulated in time-dependent manner. Expression of Notch3 was not detected in our study.

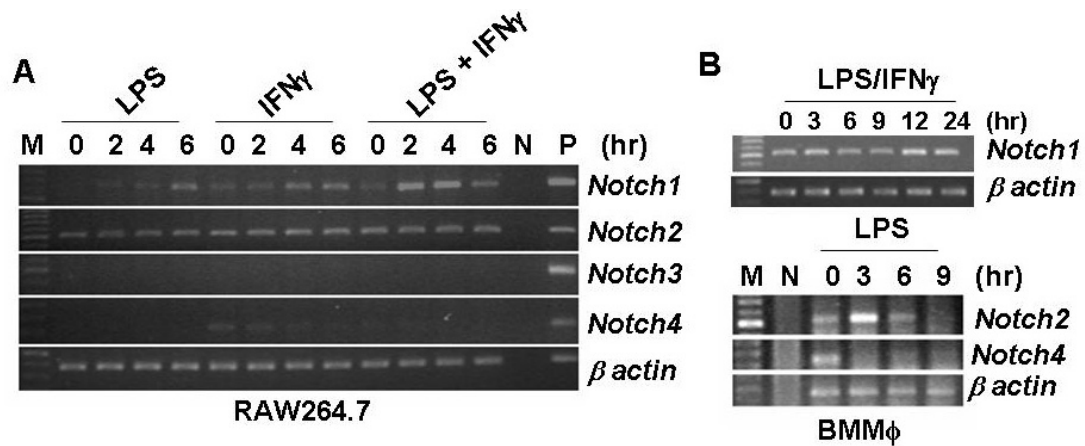


Figure 2. Expression profiles of Notch receptors in RAW264.7 and BMDM upon stimulation with LPS/IFN γ as examined by semi-quantitative RT-PCR. Negative control (N) was performed by using total RNA without reverse transcriptase.

In order to examine an effect of stimulation of macrophages on expression of Notch1 upon stimulation through TLRs, we used 4 different agonists, i.e. bacterial peptidoglycan (TLR2), poly I:C (TLR3), bacterial lipopolysaccharide (TLR4) and unmethylated CpG DNA (TLR9). All agonists used in this study were found to upregulate expression of Notch1 starting at 6 hr after treatment and the expression persisted up until 12 hr (Figure 3). This effect was similarly observed in RAW264.7 cell line and peritoneal macrophages (data not shown). Since expression of Notch receptors does not guarantee that Notch signaling is activated, we performed two additional assays, i.e. detecting cleaved Notch1 and measuring expression of Notch target genes, *Hes1* and *Deltex1*, to determine whether Notch signaling was activated upon LPS/IFN γ stimulation. As shown in Figure 4, cleaved Notch at Val1744 was detected upon stimulation of BMDM. Increased expression of *Hes1* was detected only in stimulated RAW264.7 but not in BMDM. On the other hand, increased *Deltex* expression as measured was detected only in BMDM but not in RAW264.7.

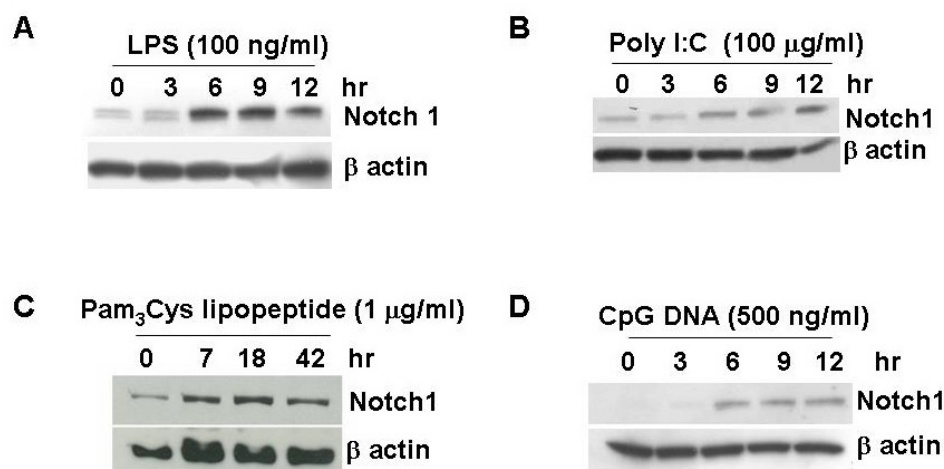


Figure 3. Notch1 is upregulated upon stimulation through TLRs. BMDM were stimulated by LPS (A), PolyI:C (B), Pam3Cys lipopeptide (C) and CpG DNA (D) for the indicated durations and expression of Notch1 was detected by Western blot.

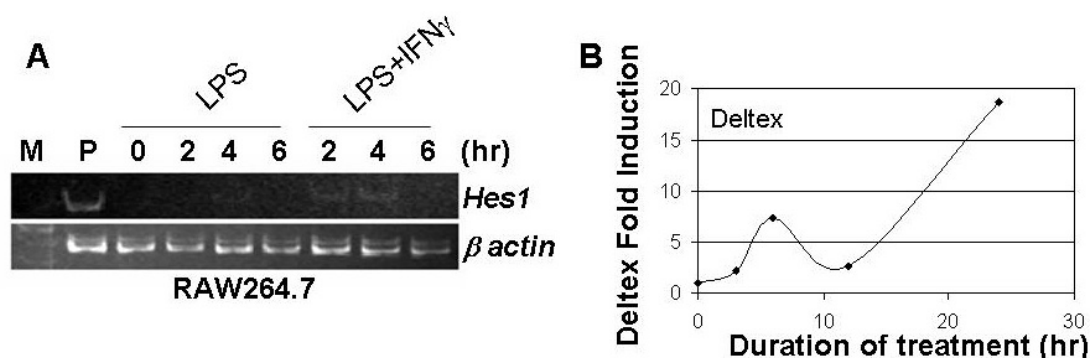


Figure 4. Notch Signaling is activated upon LPS/IFN γ stimulation. RAW264.7 (A) or BMDM (B) were stimulated by LPS/IFN γ as indicated. Expressions of Hes1 and Deltex were detected by semiquantitative RT-PCR (A) and real time RT-PCR (B), respectively.

Signaling molecules involved in TLR signaling has been extensively studied. MyD88 is one of adaptor molecules utilized by this signal pathway in signal transduction leading to effective innate immune responses such as NF κ B activation, expression of pro-inflammatory molecules. To elucidate the signal from TLR engagement to Notch1 upregulation, BMDM from MyD88 KO mice were stimulated with TLR2 or TLR4 agonists and Notch1 expression was examined. As shown in Figure 5, TLR4-mediated Notch1 upregulation was MyD88-independent in an early phase (up to 6 hr), but became MyD88 dependent at later time point. On the other hand, TLR2-mediated Notch1 upregulation was completely abrogated in Myd88 deficient BMDM, suggesting that TLR2 is dependent on MyD88 to upregulate Notch1.

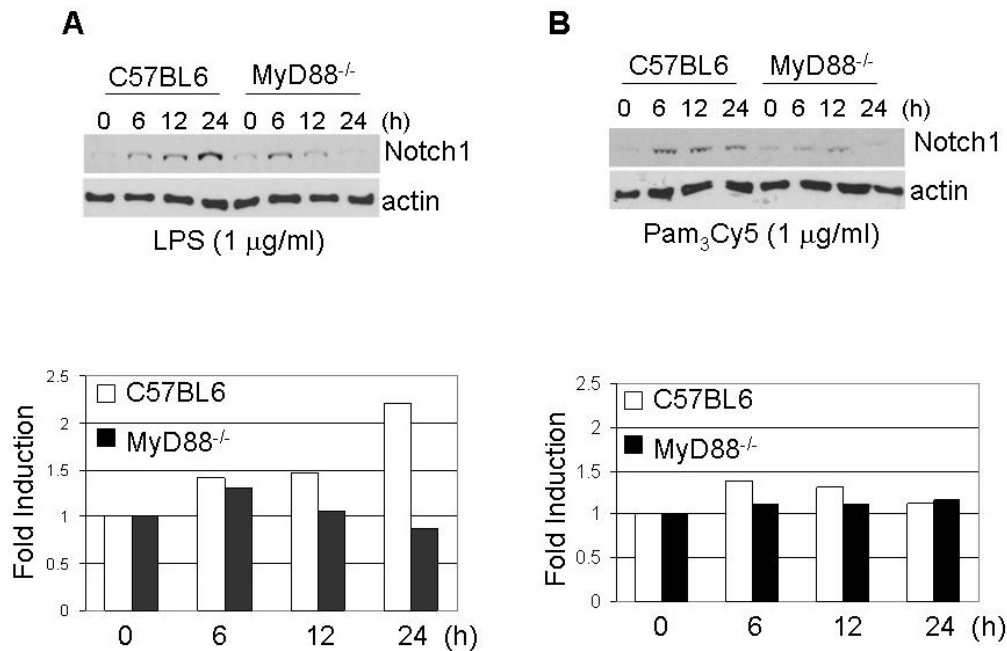


Figure 5. Notch1 upregulation in MyD88 deficient BMDM upon TLR stimulation. BMDM derived from C57BL/6 or MyD88 KO mice were treated as indicated using LPS (A) or Pam3Cys5 (B). Expression of Notch1 was detected by Western blot and the band intensities of two independent experiments were quantitated using QuantiOne software. The expression level of Notch1 was normalized to β actin.

GSI treatment inhibits Notch signaling and affects effector functions of macrophages

In order to elucidate the role Notch signaling play in stimulated macrophages, pharmacological approach was employed. GSI, IL-CHO, was designed to inhibit enzymatic activity of γ -secretase and the processing of its substrates. It has been used in pervious studies to suppress Notch signaling both in vitro and in vivo. When BMDM were treated with IL-CHO, cleaved Notch1 was no longer detected, implying that Notch1 processing by γ -secretase was suppressed (Figure 6). In addition, Notch1 upregulation was completely abrogated, confirming previous report on self regulatory nature of Notch signaling (Figure 6). In addition, prolong treatment of BMDM with IL-CHO did not decrease cell viability as judged by morphological observation. Therefore, IL-CHO was used to characterize the role Notch signaling plays in activated macrophages.

Upon activation by LPS/IFN γ , macrophages generally upregulate proinflammatory cytokines such as TNF α and IL-6, and produce antimicrobial chemical intermediates such as nitric oxide and reactive oxygen species. We examined the effect of IL-CHO treatment on expressions of TNF α , iNos, IL-6 and IL-10 by RT-PCR. As shown in Figure 7, IL-CHO treatment affected mRNA expression of these effector molecules in macrophages in a complicated manner. While iNos and IL-10 were upregulated, IL-10 was upregulated and delayed in TNF α downmodulation was observed. To confirm these results, we

measured amount of nitric oxide and $\text{TNF}\alpha$ produced during IL-CHO treatment, and found that both were lower in IL-CHO treated cells, compared with vehicle treated control.

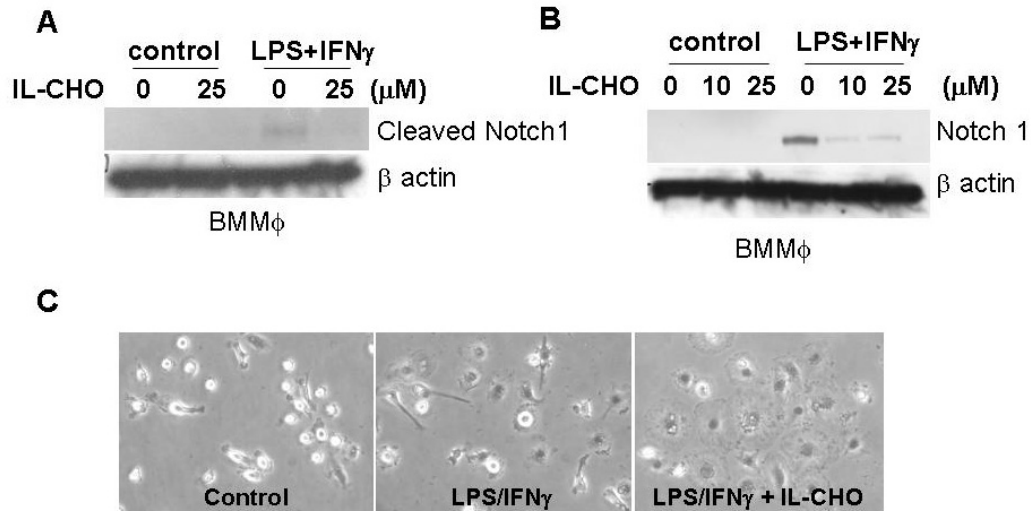


Figure 6. Treatment of BMDM with IL-CHO abrogated Notch1 upregulation. BMDM were pretreated with IL-CHO or vehicle control DMSO for 1 hr before being stimulated with LPS/ $\text{IFN}\gamma$. Cleaved Notch1 (A) and Notch1 (B) were detected by Western blot. Morphological changes of BMDM in prolonged culture (72 hr) were shown in C as observed under inverted light microscope.

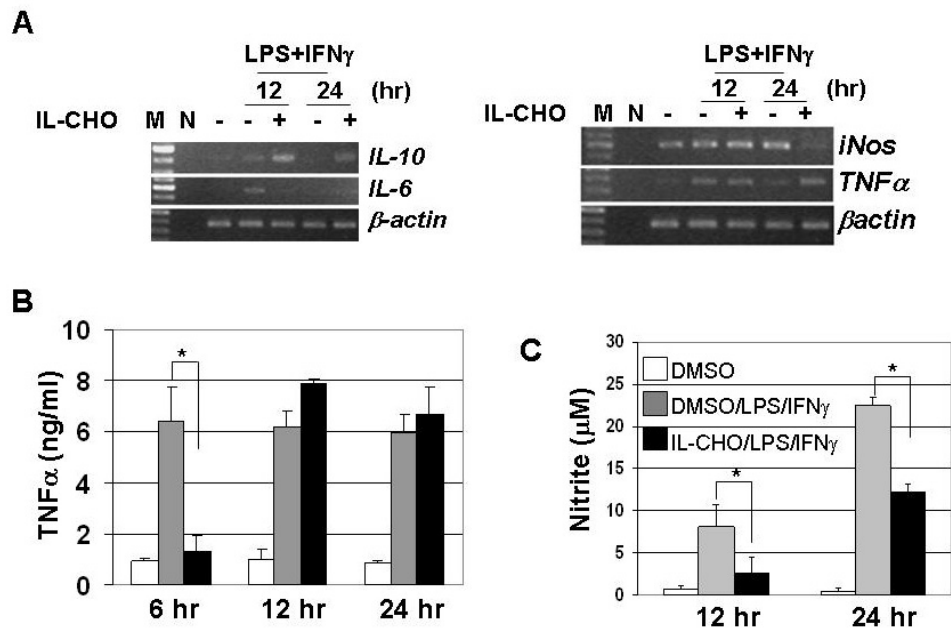


Figure 7. Effects of IL-CHO treatment on cytokines and nitric oxide production in BMDM. BMDM were treated as indicated and mRNA (A) or culture supernatants (B) were collected. RT-PCR was performed to examine expression of IL-10, IL-10, iNos, $\text{TNF}\alpha$. Amounts of $\text{TNF}\alpha$ and nitric oxide production were measured by ELISA and Griess reaction, respectively. Statistically significance (* $p < 0.05$) was indicated.

In addition, when cell surface expression of MHC class II was examined, IL-CHO treatment enhanced MHC class II expression (Figure 8). Taken together, GSI treatment profoundly affects effector functions of macrophages.

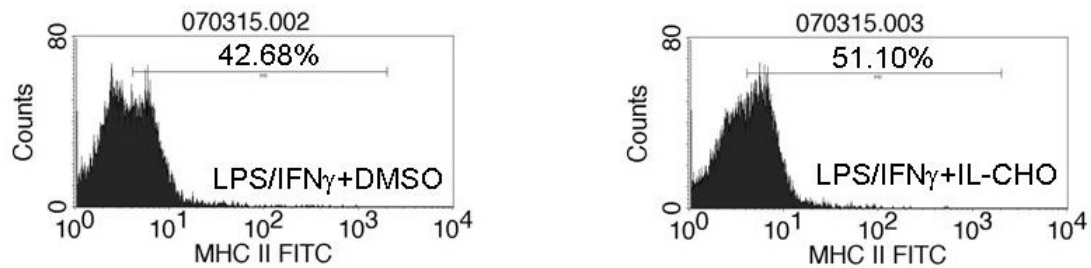


Figure 8. IL-CHO treatment enhanced surface expression of MHC Class II. BMDM were treated as indicated and cell surface expression of MHC Class II was analyzed by FACS.

Overexpression of Truncated Notch Intracellular Domain in RAW264.7

Since γ -secretase inhibitor may inhibit processing of other substrates besides Notch receptors, the effects observed in the previous experiment may not be entirely dependent on Notch receptors. To address whether the effect of GSI treatment is dependent on Notch signaling, genetic approach to overexpress truncated intracellular Notch1 (Nic) was employed. The truncated Notch1 mimics Notch receptor which is processed by γ -secretase upon ligand engagement and exhibits constitutively active phenotypes. Stably transfected RAW264.7 cell lines were generated and the expression of Nic was confirmed. As shown in Figure 9, RAW264.7 pcDNA-Nic expressed significantly higher Nic even under non-stimulated condition. Upon LPS stimulation, more Notch1 was observed in transfected cell line, but not control or empty vector transfected cells.

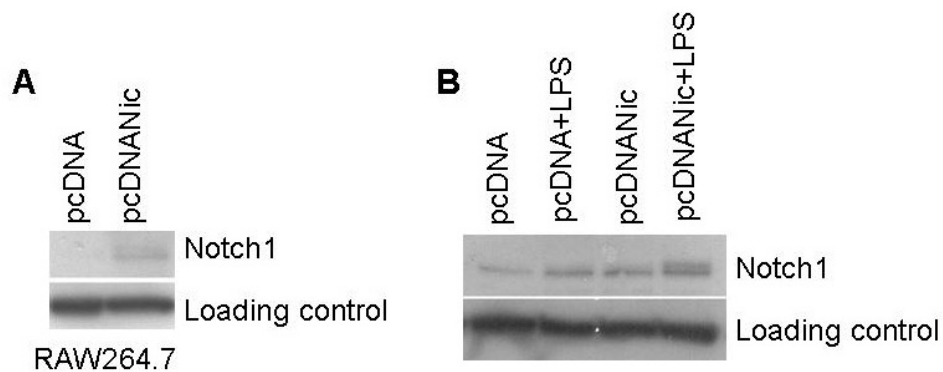


Figure 9. Overexpression of truncated Notch1 in RAW264.7. RAW264.7 was stably transfected with pcDNA3 empty vector or pcDNA3-Nic containing Nic and expression of Notch1 was detected by Western blot.

Stably transfected RAW264.7 cells were stimulated and examined nitric oxide and TNF α production in stimulated cells. Both nitric oxide and TNF α production dramatically increased in Nic overexpressing cells, compared with control or cells transfected with empty vector. This result is in contrast with those seen in GSI treated macrophages. Therefore, manipulating Notch level during activation of macrophages profoundly affects their effector function.

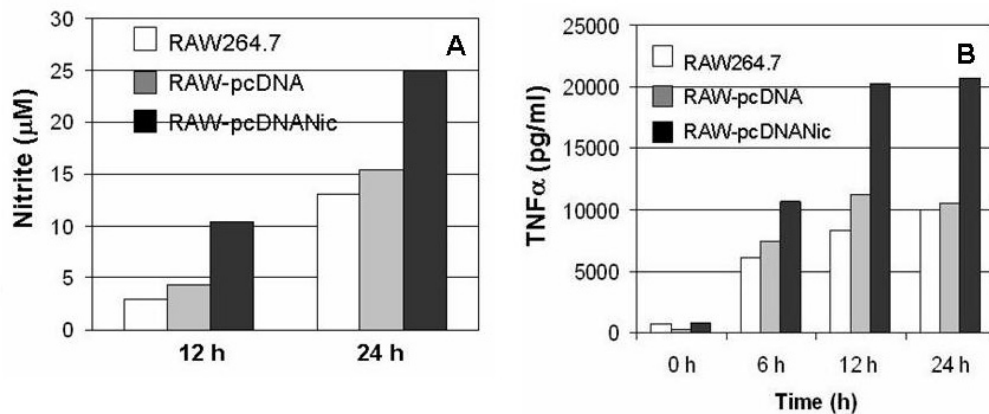


Figure 10. Overexpression of Nic increases nitric oxide and TNF α production in RAW264.7 cells. RAW264.7 cells stably transfected with pcDNA3 empty vector, pcDNA3Nic were activated as indicated. Nitric oxide and TNF α produced were assayed by Griess reaction and ELISA, respectively.

Notch1 is upregulated in macrophages during BCG Infection

To establish the involvement of Notch signaling during mycobacterial infection, we examined the expression pattern of Notch1 in mycobacteria infected macrophages. BMDM or RAW264.7 cell line was infected with attenuated BCG (Copenhagen) at multiplicity of infection (MOI) of 10:1 and expression of Notch1 was analyzed by Western blot. As shown in Figure 11, BCG infection led to upregulation of Notch1 and the level of expression was maintained until 72 hr post infection in BMDM. Infected RAW264.7 cell line also upregulated Notch1 but with slower kinetics, compared to that seen with BMDM. Infection with different MOI ranging from 5:1 to 40:1 did not result in different Notch1 upregulation pattern. In addition to live BCG, mycobacteria derived 19KDa lipopeptide also induced Notch1 upregulation in dose-dependent manner (Figure 3B). To address the requirement of TLR signaling adaptor molecule, MyD88, in this phenomenon, wild type or MyD88 deficient BMDM were infected with BCG and expression of Notch1 was examined. Compared to wild type BMDM, MyD88 deficient BMDM failed to upregulate Notch1 early on until 48 hr after infection. At 48 hr, Notch1 expression in both cells are at comparable level, suggesting that other signaling cascade is responsible for Notch1 expression during this stage. These results suggest that Notch signaling may be involved in responses against BCG infection mounted by macrophage and Notch upregulation in this situation is partially mediated by MyD88.

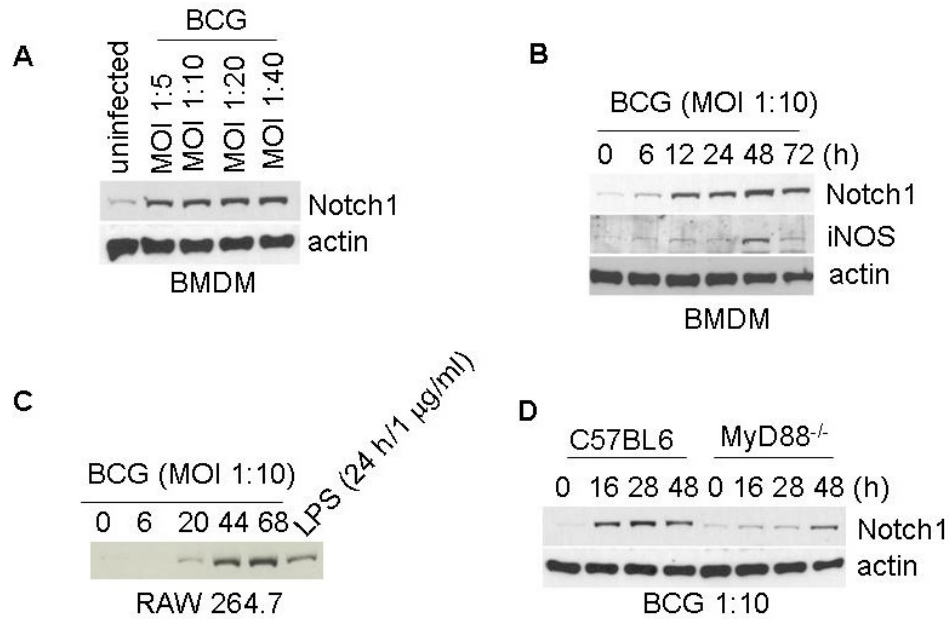


Figure 11. Notch1 expression in BMDM and RAW264.7 upon infection with BCG. (A) BMDM were infected with different MOI of BCG for 24 hr and expression of Notch1 was detected with Western blot. (B-C) BMDM or RAW264.7 cell line were infected with BCG at MOI of 1:10 for indicated times and Notch1 expression was detected by Western blot. (D) BMDM from C57BL/6 or MyD88KO mice were infected with BCG at MOI of 1:10 for indicated periods and expression of Notch1 was detected by Western blot.

To test whether infection with *Mycobacterium tuberculosis* (MTB), a causing agent of pulmonary tuberculosis, also induces Notch1 upregulation, laboratory strains of MTB, *i.e.* avirulent strain H37Ra and virulent strain H37Rv, were used to infect BMDM at MOI of 1:5 and expression of Notch1 was examined. As shown in Figure 12, both strains of MTB induced Notch1 upregulation with similar kinetics. Surprisingly, virulent strain of MTB seemed to induce less Notch1 upregulation than avirulent strain, implying that Notch1 may be one of the host target genes modified by MTB during infection.

Effects of IL-CHO treatment on BCG-infected BMDM

Since Notch1 is upregulated upon BCG and MTB infection as described above, we sought to address the role its play during infection. Treatment of BCG-infected BMDM completely abrogated Notch1 expression, similar to what have been observed previously when BMDM were co-treated with IL-CHO and LPS/IFN γ (Figure 12). Since we have observed decrease in nitric oxide production in stimulated BMDM pretreated with IL-CHO, we assayed for nitric oxide production in BCG-infected BMDM pretreated with IL-CHO. As expected, IL-CHO treatment decreased nitric oxide production. Decreasing one of potent anti-microbial defense molecule nitric oxide production often leads to defect in microbial

eradication. Therefore, we assayed for CFU in BCG-infected BMDM pretreated with IL-CHO. We unexpectedly found that IL-CHO treatment led to a rapid clearance of BCG at day 3 after infection (Figure 13). By day 7 post infection, the levels of CFU in mock treatment and IL-CHO treatment were comparable, consistent with avirulent attenuated nature of BCG.

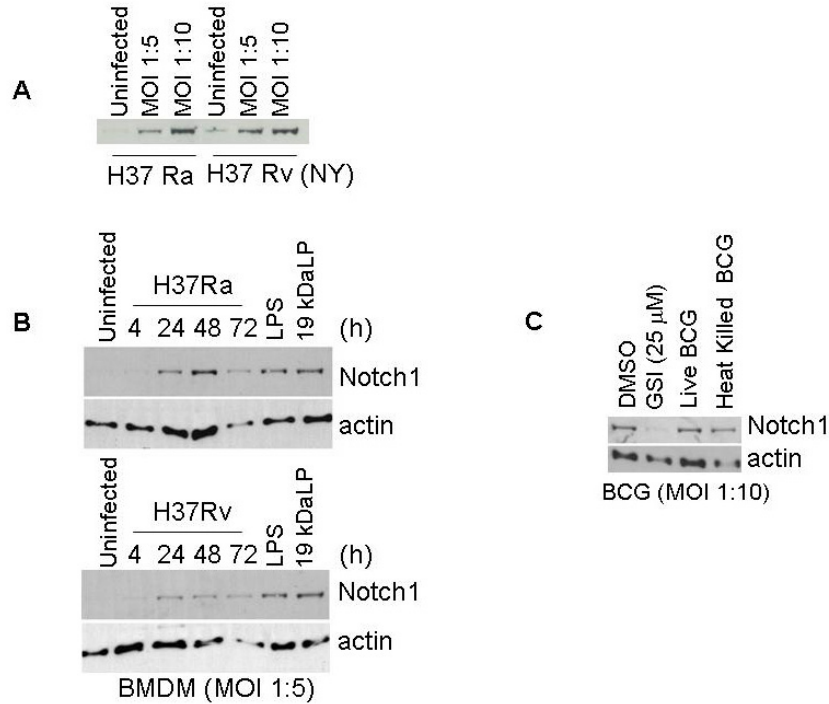


Figure 12. Infection with MTB induced Notch1 upregulation in BMDM and GSI treatment affects Notch1 upregulation by BCG infection. (A-B) BMDM were infected with different MOI of MTB for 24 hr (A) or at MOI of 1:5 for indicated times and expression of Notch1 was detected with Western blot. (C) BMDM were pretreated with DMSO or IL-CHO for 1 hr and infected with live BCG for 24 hr. Expression of Notch1 was detected by Western blot.

Apoptosis of infected macrophages is considered one of effective mechanisms of self defense. Upon apoptosis, phagocytosed bacteria are destroyed and apoptotic bodies containing bacterial antigens are engulfed by nearby dendritic cells. Engulfed apoptotic bodies are processed within DC and bacterial antigens are loaded onto MHC class I molecule and presented to CD8⁺ cytotoxic T cells, a process known as “cross presentation”. Antigen-stimulated CD8⁺ T cells are major players in mounting effective specific cell mediated immune response against infections with intracellular bacteria [14, 15].

Since we observed decrease CFU upon GSI treatment, we wonder whether macrophages died more when Notch signaling is suppressed. Crystal violet assay revealed that GSI treatment resulted in significant cell death at day 7 post infection (Figure 14). During apoptosis of MTB infected macrophages, anti-apoptotic gene *Mcl1* plays an important role in regulating this process. In an attempt to uncover genes regulated by Notch signaling, ChIP-on-chip assay was carried out using anti-Notch1 antibodies to

precipitate chromatin-protein complex (J. Schreiber and S. H. E. Kaufmann, personal communication). In this study, *Mcl1* was found to be one of the target genes of Notch signaling pathway. When expression of *Mcl1* was examined by RT-PCR, *Mcl1* was detected at 24 hr when cells were infected with BCG. In contrast, expression of *Mcl1* was completely abrogated when cells were pre-treated with GSI. In addition, Nic overexpressing RAW264.7 underwent less apoptosis upon infection with BCG, as assayed by AnnexinV binding. Taken together, these results strongly suggest that Notch signaling is involved in apoptosis of BCG (and MTB)-infected macrophages, partially through regulating anti-apoptotic gene *Mcl1* expression.

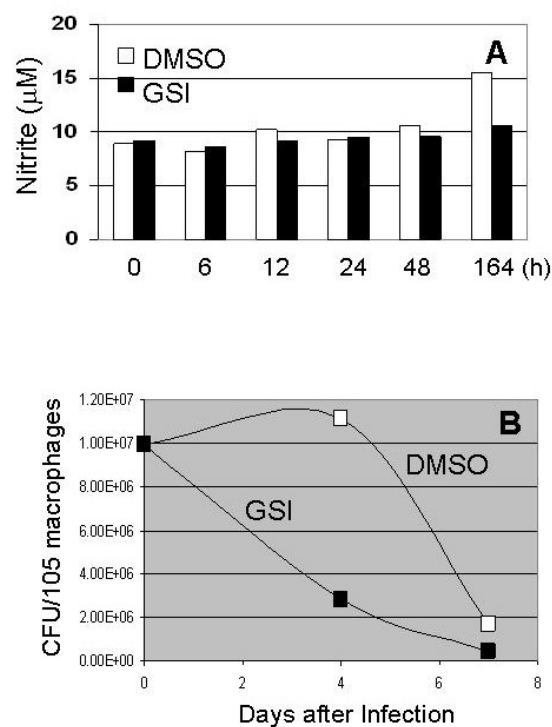


Figure 13. Nitric oxide production and CFU of BCG in infected macrophages. BMDM were infected with BCG (Copenhagen) at MOI of 1:10 for indicated times. Production of nitric oxide (A) and CFU (B) were determined as described in materials and methods.

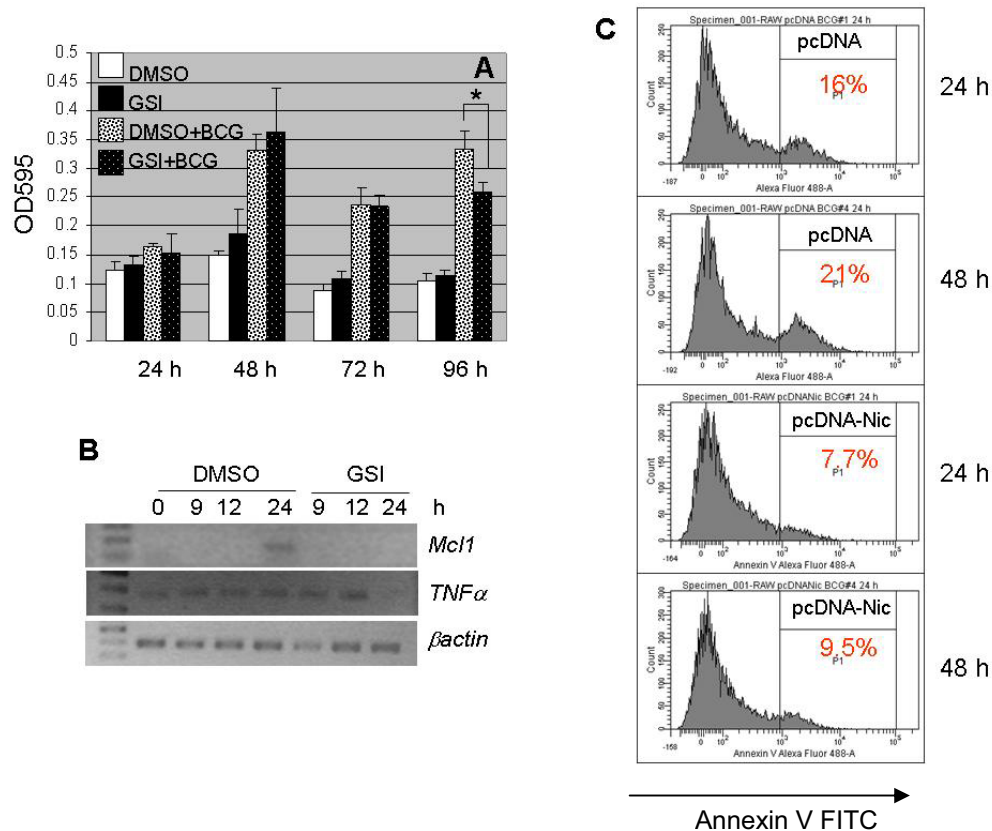


Figure 14. Apoptosis of BCG-infected macrophages. (A) BMDM pretreated with DMSO or IL-CHO were infected with BCG at MOI of 1:10 and cell viability was assayed using crystal violet assay. (B) BMDM pretreated with DMSO or IL-CHO were infected with BCG at MOI of 1:10 for indicated times. Expression of *McI1* and *TNFα* was determined by RT-PCR. (C) RAW264.7 stably transfected with control empty vector or pcDNA-Nic were infected with BCG at MOI of 1:10. Apoptotic marker phosphatidylserine was detected by Annexin V FITC binding assay and cells were analyzed by FACS.

Discussion

Notch receptors and ligands are expressed during development of myeloid lineage cells and their expression persists after maturation to monocytes or tissue macrophages[16]. A role for Notch signaling during cell fate decision of dendritic cells, monocytes/macrophages, and granulocytes has been reported [17, 18]. In addition to a crucial role during development, it has become apparent that Notch signaling also regulates the function of peripheral immune cells, in particular T lymphocytes [19]. It has been suggested that different TLR agonists can modulate the expression of Notch receptors and Notch ligands on antigen-presenting cells and, as a result, influence the outcomes of a given immune response. In this study, we examined effects of stimulating macrophages through TLRs on the expressions of Notch receptors. We found that all tested TLR agonists (TLR2, TLR3, TLR4 and TLR9) upregulate

Notch1 protein expression in both the macrophage-like cell line RAW264.7- and BM-derived primary macrophages. TLR2-mediated Notch1 upregulation was completely abrogated in MyD88^{-/-} macrophages, whilst TLR4 signaling did not rely on MyD88 for early Notch1 upregulation. Notch2 mRNA was also upregulated by the TLR4 agonist in primary macrophage cultures, but not in RAW264.7 cells. In addition, downregulation of Notch4 mRNA was seen in both cell types. Furthermore, both cleaved Notch1 protein and *Hes1* mRNA were readily detected upon macrophage stimulation, providing strong evidence that Notch signaling was activated. This result suggests that Notch signaling regulated biological activities of macrophages in response to activation by microbes through TLRs.

To verify the role of Notch signaling in regulating inflammatory responses triggered by macrophages, we tested effects of GSI on cytokine production, NO production and MHC class II expression. GSI treatment did not alter mRNA expression of *TNF α* but the amount of secreted protein was markedly decreased at the early stage of stimulation, suggesting that Notch signaling regulates *TNF α* production at the post-translational level. Expression of *IL-10* mRNA was increased in GSI-treated cells, suggesting that Notch signaling can inhibit production of IL-10 in macrophages. Interestingly, Notch signaling has been shown to be required for IL-10 production by T cells where the Notch receptor colocalizes with CD4 [20]. In contrast to IL-10, expression of *IL-6* mRNA was suppressed in GSI-treated cells, suggesting that Notch signaling is required for IL-6 expression. In fact, the binding partner of Notch, CBF-1, binds to and regulates the *IL-6* gene [21]. In addition to cytokines, decreased production of NO and increased expression of MHC class II were detected in GSI-treated macrophages. It appears that Notch plays a critical role in steering an immune response toward inflammation by regulating expression of various cytokines and effector molecules generated by macrophages.

Recently, Monsalve *et al.* [5] showed that increasing Notch1 expression upon stimulation with LPS plus rIFN γ in RAW264.7 cells modulates STAT1 transactivation capacity. Using an overexpression strategy, they also found decreased NO production, but increased ICAM1 and MHC class II expression by stable transfectants with a truncated active form of Notch [5]. The discrepancy between the results obtained in this study and those from Monsalve *et al.* [5] may lie in experimental conditions and the different approaches used. The major differences are that we used primary BMDM, not a cell line, for studying effects of GSI treatment. As shown here, expression of some Notch receptors was affected differentially in RAW264.7 cells and primary macrophages. In addition, we cannot rule out the possibility that GSI treatment affected other signaling pathways resulting in the phenotypes observed in our study.

Signaling through TLRs often causes activation of the NF- κ B pathway. Modulating Notch signaling also has a profound effect on the NF- κ B signaling cascade in T lymphocytes and hematopoietic precursor cells [11, 22, 23]. Therefore, it is tempting to speculate that Notch regulates activities of macrophages by interfering with the NF- κ B pathway. However, Monsalve *et al.* [5] observed unaltered NF- κ B reporter activity after treatment with LPS plus rIFN γ , when Notch is constitutively activated. Instead, they found that constitutively activated Notch1 decreases AP-1 and increases STAT1/3 reporter activity.

We further examined the role Notch signaling plays in regulating macrophage effector functions in bacterial infection settings. Intracellular bacteria, MTB, infect and survive within alveolar macrophages by modulating macrophages to their own advantage [24]. We found that both attenuated BCG and MTB upregulate Notch1 and this upregulation is dependent on MyD88. Suppressing Notch signaling by GSI, BCG-infected macrophages produced less nitric oxide. Surprisingly, inhibition of Notch signaling resulted in decreased CFU and viability of macrophages. Together with preliminary data obtained from chromatin immunoprecipitation microarray analysis, we uncovered a link between Notch and anti-apoptotic gene *Mcl1*. *Mcl1* is modulated in macrophages upon MTB infection. Suppression of Notch signaling decreased *Mcl1* expression. In contrast, overexpression of activated form of Notch protected macrophages from BCG infection. These results strongly suggest that Notch signaling regulates apoptosis upon BCG and probably MTB infection via direct regulation of *Mcl1*.

Our study reveals that Notch signaling is activated in LPS plus IFN γ -stimulated macrophages and Notch signaling regulates critical biological functions of activated macrophages. Notch signaling is a crucial regulator of biological functions in macrophages during the effector phase by promoting inflammatory responses and regulating apoptosis of macrophages. Consistent with this notion, components of Notch signaling are upregulated in lesions of chronic inflammation [25, 26]. It will be of interest to investigate whether Notch is involved in initiating inflammation by interacting with the TLR signaling cascade or with other signaling pathways involving TLRs. In addition, Notch signaling may provide a novel target for drug intervention during MTB infection.

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กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และสำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) ที่ให้การสนับสนุนโครงการวิจัยนี้จนเสร็จสมบูรณ์ ผู้วิจัยขอขอบคุณ Professor Barbara A. Osborne (University of Massachusetts at Amherst, USA) และ Professor Stefan H. E. Kaufmann (Max Planck Institute of Infection Biology, Germany) ที่ให้ความร่วมมือในงานวิจัยนี้และเอื้อเฟื้อพลาสมิด เชลล์คัลเจอร์ และ สัตว์ทดลอง ผู้วิจัยขอขอบคุณ ดร. สาธิต พิษณุางกูร (AFRIMS) ที่กรุณาเอื้อเฟื้อ CpG DNA และ รองศาสตราจารย์ ดร. อังคณา ฉายประเสริฐ (ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ ศิริราชพยาบาล มหาวิทยาลัยมหิดล) ที่กรุณาให้คำแนะนำเรื่องการเลี้ยงเชื้อ BCG สุดท้ายนี้ผู้วิจัยขอขอบคุณนักวิจัยที่ปรึกษา รองศาสตราจารย์ ดร. ศิริรัตน์ เร่งพิพัฒน์ และภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

Output ที่ได้จากโครงการ

1. เข้าร่วมเสนอผลงานใน Annual Meeting of American Association of Immunologist 2006 ณ เมืองบอสตัน สหรัฐอเมริกา ระหว่างวันที่ 12-16 พฤษภาคม 2549 โดยนำเสนอในรูปแบบโปสเตอร์ หัวข้อ Toll-like receptor (TLR) agonists upregulate Notch receptors in murine macrophages
2. เข้าร่วมเสนอผลงานใน Keystone Symposia Tuberculosis: From Lab Research to Field Trials 2007 ณ เมืองแวนคูเวอร์ แคนาดา ระหว่างวันที่ 20-25 มีนาคม 2550 โดยนำเสนอในรูปแบบโปสเตอร์ หัวข้อ Notch1 is upregulated during BCG infection in macrophages
3. Manuscript ที่ส่งไปพิจารณาเพื่อตีพิมพ์ใน European Journal of Immunology ซึ่งกำลังอยู่ระหว่างการ revision

ภาคผนวก

1. บทคัดย่อการไปเสนอผลงานใน Annual Meeting of American Association of Immunologist 2006
2. บทคัดย่อการไปเสนอผลงานใน Keystone Symposia Tuberculosis: From Lab Research to Field Trials 2007
3. Manuscript ที่กำลังส่งไปเพื่อพิจารณาตีพิมพ์และอยู่ระหว่างการ revision จาก European Journal of Immunology

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Notch Signaling is Activated by TLR Stimulation and Regulates Macrophage Functions

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Key words: macrophages, Notch signaling, γ -secretase inhibitor, Toll-like receptors

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Abbreviations: GSI, Gamma secretase inhibitor

Summary

Notch signaling is a well-conserved pathway involved in cell fate decisions, proliferation and apoptosis. We report on the involvement of Notch signaling in regulating gene expression in activated macrophages. TLR agonists such as bacterial lipopeptide, polyI:C, LPS and unmethylated CpG DNA all induced upregulation of Notch1 in primary and macrophage-like cell lines. Notch1 upregulation was dependent on the MyD88 pathway when stimulated through TLR2, but not TLR4. Activated Notch1 and expression of the Notch target gene, *Hes1*, were detected in activated macrophages, suggesting that Notch signaling was activated upon stimulation. Inhibiting processing of Notch receptor by γ -secretase using a γ -secretase inhibitor (GSI), the expression of Notch1 was downregulated to basal levels. This treatment significantly modulated expression of *TNF α* , *IL-6*, and *IL-10*. While expression of *IL-6* was suppressed, *IL-10* was induced upon GSI treatment. Production of *TNF α* decreased in GSI-treated cells at 6 h but reached levels comparable to control at 24 h. In addition, the amount of NO produced was significantly lower and the expression of MHC class II was upregulated in GSI-treated cells. Taken together, stimulation of macrophages through the TLR signaling cascade triggered activation of Notch signaling, which in turn regulated gene expression patterns involved in proinflammatory responses.

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Introduction

The innate immune response recognizes invasion of microorganisms using pattern recognition receptors (PRRs) through recognition of pathogen-associated molecular patterns (PAMPs) [1]. Toll-like receptors (TLRs), the mammalian orthologs of *Drosophila* Toll receptor, play a crucial role in recognizing PAMPs. There are more than 10 TLRs identified in mammals, each showing differential specificity for its ligands [2]. The signaling generated by engagement of TLRs with specific ligands involves the recruitment of TIR domain-containing adaptor proteins such as MyD88 and TIRAP/MAL, phosphorylation of secondary messengers which culminates in nuclear translocation of transcription factors such as NF- κ B [3]. This event leads to initiation of innate immune responses including inflammation through cytokine production and secretion of reactive mediators. The outcome of stimulation through the TLR signaling pathway can be varied depending on combination of receptor-ligand interactions. It is still largely unknown how individual TLR signals are eventually shaped for ligand-specific outcomes [2, 3].

Notch signaling is a well-conserved signaling pathway involved in cell fate decisions during development of various systems, including the hematopoietic and neuronal systems [4]. The Notch receptor is a heterodimeric transmembrane protein expressed on the cell surface of signal receiving cells. The extracellular domain is comprised of EGF-like repeats and is responsible for ligand binding. Receptor-ligand interactions lead to the cleavage of the transmembrane peptide by two proteolytic

enzymes, *i.e.* tumor necrosis factor α (TNF α)-converting enzyme (TACE) and γ -secretase. These events release the intracellular domain of Notch (NIC) and promote migration into the nucleus, where it forms a transactivation complex with the DNA-binding protein, RBP-J/CBF-1. The transactivation complex further recruits coactivators such as p300 via a scaffold protein MAML and drives the transcription of target genes, such as *Hes-1*, *CD25* as well as *Notch* itself [5]. Mammalian Notch receptors are encoded by four different genes, *i.e.* *Notch1-4*, and genes encoding five mammalian Notch ligands have been identified, *i.e.* *Jagged1*, *2*, *Delta-like 1*, *3* and *4*.

Notch receptors and ligands are expressed at various stages during hematopoiesis and the signal initiated by receptor–ligand interactions plays a crucial role in lineage commitment of certain cell lineages [4, 5]. Disruption of Notch signaling using either genetic or pharmacological approaches in T-lineage cells results in arrest of thymic development and functional aberrations in peripheral T lymphocytes [6–8]. Similarly, Notch signaling has been shown to influence various myeloid lineage choices [9, 10]. Analyses of expression patterns of Notch receptors/ligands in monocyte/macrophage lineage cells reveal that both Notch receptors and ligands are temporally expressed during development, and this expression persists even after completion of development [11–13]. Differential effects of TLR agonists in regulating the expression of Notch ligands have been described, and the importance of Notch ligand expression by antigen-presenting cells in regulating the outcomes of immune responses is highlighted by its influence on

Th1/Th2 immunity [7]. Furthermore, Notch signaling has been shown to directly regulate immune responses in the periphery such as production of several cytokines in T lymphocytes, including IFN γ , IL-4 and IL-10 [7, 14-16].

In addition to Notch ligands, Notch receptors are also expressed in mature macrophages and, recently, Notch signaling has been reported to be involved in regulating expression of genes involved in antigen presenting capacity and cytotoxicity [12, 17]. However, it is unclear whether activation through TLR regulates Notch expression and the role of Notch signaling in regulating cytokine production in this cell type has not been addressed. In this study, we investigate the effects of different TLR agonists on the expression of Notch receptors and the role Notch signaling plays in macrophages using pharmacological approaches to inhibit Notch signaling. We report that various TLR ligands similarly trigger upregulation of Notch1 in murine bone marrow (BM)-derived macrophages and the macrophage-like cell line RAW264.7, both in an MyD88-dependent and MyD88-independent manner. Furthermore, Notch signaling is activated by stimulation through TLR signaling. Using a γ -secretase inhibitor (GSI) to block Notch signaling we demonstrate that GSI blocks the upregulation of Notch induced by LPS/IFN γ stimulation. Expression of several cytokine genes and NO production at late stages of activation (12–24 h), were significantly altered by GSI treatment. These results suggest that Notch signaling triggered by TLR stimulation regulates gene expression in activated macrophages.

Results

Notch receptors are upregulated by activation of macrophages with LPS/IFN γ

It has been shown previously that mature macrophages express Notch1 and Notch 4 [12]. In order to investigate the effects of stimulation through TLR on the expression of Notch receptors, we treated the macrophage-like cell line RAW264.7 with LPS (TLR4 ligand), rIFN γ or LPS plus rIFN γ for 0 h, 2 h, 4 h, and 6 h and analyzed the expression of Notch1 by RT-PCR. As shown in Fig. 1A, stimulation with LPS, rIFN γ and LPS plus rIFN γ significantly triggered upregulation of Notch1 transcription, with stimulation by LPS plus rIFN γ being the strongest inducer. Four different Notch receptors are differentially expressed during hematopoiesis. Functional diversity and functional redundancy among these four receptors have been reported [18, 19]. Since Notch1 is upregulated by LPS treatment, we investigated effects of LPS on the expression of other Notch receptors in RAW264.7 cell line by RT-PCR. The expression of Notch2 was not affected by stimulation, while expression of Notch4 was downregulated by treatment with IFN γ . In BM-derived macrophages, similar upregulation of Notch1 was confirmed, while Notch2 was upregulated as early as 3 h after LPS treatment, in contrast to that seen in RAW264.7 cell line (Fig. 1B, Fig. 3A). The expression of Notch3 could not be detected at any time point during this experiment (data not shown). The expression of Notch4, on the other hand, was detected at 0 h, but readily downregulated at 3 h after treatment.

In addition, we examined the expression of the Notch target gene, *Hes1* as marker for activation of Notch signaling. As shown in Fig. 1C, treatment of RAW264.7 cells by LPS or LPS plus IFN γ both upregulated *Hes1* expression. Stimulation with LPS plus IFN γ induced more rapid upregulation of *Hes1* at 2 h post treatment, while LPS alone induced *Hes1* upregulation at 4 h post treatment. Taken together, treatment with LPS plus IFN γ affects Notch receptor expression in primary BM-derived macrophages and a macrophage-like cell line, and this stimulation also triggers activation of Notch signaling.

Macrophages change their functional phenotypes based on the local cytokine microenvironment [20]. They can be activated by treatment with LPS alone, IFN γ alone or a combination of LPS plus IFN γ . Each stimulus induces a different biological outcome, and the combination of both signals often shows synergistic effects in macrophages. Therefore, we determined effects of IFN γ on LPS-induced Notch1 upregulation in macrophages. Treatment by IFN γ alone for 18 h induced slight upregulation of Notch1 protein in BM-derived macrophages (Fig. 2A). Treatment with both IFN γ plus LPS simultaneously yielded an upregulation pattern of Notch1 similar to that in cells treated with LPS alone (Fig. 2A). In addition, the order of IFN γ or LPS addition did not influence the expression of Notch1, as compared to LPS treatment alone (Fig. 2B). NO was produced by activated macrophages (data not shown) verifying the biological synergy of LPS and IFN γ treatment. Taken together, LPS alone or in combination with IFN γ induced

upregulation of Notch1 in macrophages. These data also confirm and extend the PCR analyses shown in Fig. 1 by demonstrating the increase in Notch protein levels following treatment with LPS plus IFN γ .

Different TLR agonists upregulate Notch1 expression in macrophages in an MyD88-dependent and MyD88-independent fashion

Macrophages express abundant TLRs which enable them to sense the presence of pathogens via recognition of PAMPs and alert other immune cells to the insult. Because treatment with LPS (a TLR4 agonist as shown in Fig. 3A and above), influences the expression pattern of Notch1 in macrophages, we next examined the effects of treatment of BM-derived macrophages with other TLR agonists, e.g. synthetic lipopeptide Pam₃Cys lipopeptide (TLR2), polyI:C (TLR3) and CpG DNA (TLR9), on Notch1 expression. All TLR agonists tested here induced Notch1 upregulation at 6 h post treatment, similar to that seen when stimulated with LPS (Fig. 3). In addition, treatments of BM-derived macrophages and RAW264.7 cells with TLR agonists all induced Notch1 upregulation in a dose-dependent manner (data not shown). Therefore, stimulation through TLRs similarly upregulated Notch1 expression at the protein level.

Upon recognition of microbial products, TLRs transduce signals through multiple pathways by recruiting different adaptor molecules. MyD88 is one of the adaptor molecules utilized by multiple TLRs. While TLR2 agonists signal solely

through MyD88, TLR4 agonists signal via MyD88-dependent and MyD88-independent pathways [21]. We asked whether Notch1 upregulation mediated by stimulation through TLR2 and TLR4 in macrophages depends on MyD88. BM-derived macrophages from MyD88^{-/-} mice upregulated Notch1 at 6 h after stimulation with LPS, similar to WT controls. However, defects in sustaining Notch1 expression for longer periods were detected in MyD88^{-/-} BM-derived macrophages (Fig. 4A). In contrast, when BM-derived macrophages from MyD88^{-/-} mice were stimulated by TLR2-specific Pam₃Cys lipopeptide, upregulation of Notch1 was completely absent both at early and late time points (Fig. 4B). Therefore, Notch1 upregulation induced by signaling through TLR4 at an early stage was MyD88 independent whereas sustaining Notch1 expression over longer time periods was MyD88 dependent. On the other hand, TLR2-mediated Notch1 upregulation relied completely on the MyD88 adaptor molecule.

Effect of γ -secretase inhibitor on Notch1 expression

To investigate the role of Notch signaling in regulating biological function(s) of macrophages, we employed a pharmacological approach by GSI, IL-CHO [22]. Since all Notch receptors are proteolytically cleaved and activated by the multi-subunit enzyme, γ -secretase, the problem of functional redundancy among receptors can be overcome by using GSI [23]. Using GSI as a tool, several studies have reported phenotypes similar to Notch-loss-of-function mutations [8, 14, 24, 25].

Stimulation of macrophages with LPS plus rIFN γ generated cleaved Notch1 and upregulation of Notch1, confirming that Notch signaling is activated by this treatment (Fig. 5 A–B). Pretreatment with IL-CHO prior to LPS and IFN γ stimulation resulted in elimination of cleaved Notch1 and a marked decrease in Notch1 upregulation at concentrations between 10 and 25 μ M. This result is consistent with previous findings suggesting that transcription of Notch receptors is under the control of a feed-forward loop of Notch signaling itself [14]. To exclude cytotoxicity of IL-CHO treatment, we verified morphological changes of cells stimulated with LPS plus rIFN γ in the presence or absence of IL-CHO up to 36 h (Fig. 5C). We did not detect any obvious changes of cell death and analysis of cytotoxicity did not reveal any significant differences in viability (data not shown). However, cells cultured in the presence of IL-CHO appeared more flat and attached to the surface than cells cultured with vehicle control. Taken together, IL-CHO treatment completely abrogated Notch1 expression in activated macrophages at doses that were not toxic to cells.

Effects of IL-CHO on biological macrophage functions

To investigate the effects of interfering with Notch signaling using IL-CHO on biological macrophage functions, we examined the expression of several cytokines and effector molecules typically produced by activated macrophages upon stimulation with LPS plus rIFN γ . Treatment with IL-CHO of stimulated

macrophages did not affect mRNA expression of *TNF α* , *IL-10* and *iNos* at 6 h after stimulation (data not shown). However, effects of IL-CHO were seen at 12 h and 24 h after treatment. While the expression level of *IL-10* mRNA increased, *IL-6* mRNA significantly decreased in the presence of IL-CHO (Fig. 6A). When the abundance of secreted *TNF α* was measured at 6 h, 12 h and 24 h after stimulation, IL-CHO treatment modified *TNF α* production at 6 h but this effect was not seen at later time points (Fig. 6B). In addition, NO production was significantly suppressed in the presence of IL-CHO. Treatment with IL-CHO also enhanced cell surface expression of MHC class II (Fig. 6C–D). Hence, inhibition of Notch signaling using GSI altered biological functions of macrophages, suggesting a pivotal role for Notch signaling in innate immune responses by activated macrophages.

Discussion

Notch receptors and ligands are expressed during development of myeloid lineage cells and their expression persists after maturation to monocytes or tissue macrophages[26]. A role for Notch signaling during cell fate decision of dendritic cells, monocytes/macrophages, and granulocytes has been reported [27-29]. In addition to a crucial role during development, it has become apparent that Notch signaling also regulates the function of peripheral immune cells, in particular T lymphocytes [5]. It has been suggested that different TLR agonists can modulate the expression of Notch receptors and Notch ligands on antigen-presenting cells and, as

a result, influence the outcomes of a given immune response. In this study, we examined effects of stimulating macrophages through TLRs on the expressions of Notch receptors. We found that all TLR agonists (TLR2, TLR3, TLR4 and TLR9) tested upregulate Notch1 protein expression in both the macrophage-like cell line RAW264.7- and BM-derived primary macrophages. TLR2-mediated Notch1 upregulation was completely abrogated in MyD88^{-/-} macrophages, whilst TLR4 signaling did not rely on MyD88 for early Notch1 upregulation. Notch2 mRNA was also upregulated by the TLR4 agonist in primary macrophage cultures, but not in RAW264.7 cells. In addition, downregulation of Notch4 mRNA was seen in both cell types. Furthermore, both cleaved Notch1 protein and *Hes1* mRNA were readily detected upon macrophage stimulation, providing strong evidence that Notch signaling was activated. This result suggests that Notch signaling regulated biological activities of macrophages in response to activation by microbes through TLRs.

To verify the role of Notch signaling in regulating inflammatory responses triggered by macrophages, we tested effects of GSI on cytokine production, NO production and MHC class II expression. GSI treatment did not alter mRNA expression of *TNFα* but the amount of secreted protein was markedly decreased at the early stage of stimulation, suggesting that Notch signaling regulates *TNFα* production at the post-translational level. Expression of *IL-10* mRNA was increased in GSI-treated cells, suggesting that Notch signaling can inhibit production of IL-10

in macrophages. Interestingly, Notch signaling has been shown to be required for IL-10 production by T cells where the Notch receptor colocalizes with CD4 [16]. In contrast to IL-10, expression of *IL-6* mRNA was suppressed in GSI-treated cells, suggesting that Notch signaling is required for IL-6 expression. In fact, the binding partner of Notch, CBF-1, binds to and regulates the *IL-6* gene [30]. In addition to cytokines, decreased production of NO and increased expression of MHC class II were detected in GSI-treated macrophages. It appears that Notch plays a critical role in steering an immune response toward inflammation by regulating expression of various cytokines and effector molecules generated by macrophages.

Recently, Monsalve *et al.* [17] showed that increasing Notch1 expression upon stimulation with LPS plus rIFN γ in RAW264.7 cells modulates STAT1 transactivation capacity. Using an overexpression strategy, they also found decreased NO production, but increased ICAM1 and MHC class II expression by stable transfectants with a truncated active form of Notch [17]. The discrepancy between the results obtained in this study and those from Monsalve *et al.* [17] may lie in experimental conditions and the different approaches used. The major differences are that we used primary BM-derived macrophages, not a cell line, for studying effects of GSI treatment. As shown here, expression of some Notch receptors was affected differentially in RAW264.7 cells and primary macrophages. In addition, we cannot rule out the possibility that GSI treatment affected other signaling pathways resulting in the phenotypes observed in our study.

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6 Signaling through TLRs often causes activation of the NF- κ B pathway.
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9 Modulating Notch signaling also has a profound effect on the NF- κ B signaling
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11 cascade in T lymphocytes and hematopoietic precursor cells [14, 25, 31]. Therefore,
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13 it is tempting to speculate that Notch regulates activities of macrophages by
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15 interfering with the NF- κ B pathway. However, Monsalve *et al.* [17] observed
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17 unaltered NF- κ B reporter activity after treatment with LPS plus rIFN γ , when Notch
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19 is constitutively activated. Instead, they found that constitutively activated Notch1
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21 decreases AP-1 and increases STAT1/3 reporter activity.
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25 Notch signaling has been reported to directly regulate cytokine production in
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27 T lymphocytes, such as IFN γ , IL-4, IL-6 and IL-10 [7, 14, 16, 32]. Since
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29 macrophages also produce IL-6, IL-10 and IFN γ , it will be interesting to explore the
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31 role Notch signaling plays in regulating cytokine production in macrophages. The
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33 effects of GSI on cytokine production are reminiscent of those seen with
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35 overexpression of SOCS3 [33]. SOCS3 plays an important role in regulating
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37 expression of *TNF α* , *iNOS* and *IL-6* in macrophages, and its expression is induced
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39 by IL-10 [33, 34]. Expression of IL-6 and iNOS are negatively regulated by SOCS3
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41 at mRNA and protein levels, while TNF α expression is negatively regulated at the
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43 protein level [33]. Since mRNA of IL-10 increased in the presence of GSI, it will be
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45 of interest to explore the relationships between Notch signaling, IL-10 and SOCS3 in
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47 macrophages. In Th cells, Notch has been shown to colocalize with CD4 and to be
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49 required for IL-10 production [16]. However, GSI enhanced mRNA expression of
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IL-10 in stimulated macrophages, suggesting that Notch signaling inhibits IL-10 production in this cell type. Interestingly, chromatin remodeling studies revealed differences in DNaseI hypersensitivity sites in the promoter of IL-10 in T lymphocytes and macrophages, suggesting distinct regulatory mechanisms in these two cell types [35].

Our study reveals that Notch signaling is activated in LPS plus IFN γ -stimulated macrophages and Notch signaling regulates critical biological functions of activated macrophages. Notch signaling is a crucial regulator of biological functions in macrophages during the effector phase by promoting inflammatory responses. Consistent with this notion, components of Notch signaling are upregulated in lesions of chronic inflammation [36, 37]. It will be of interest to investigate whether Notch is involved in initiating inflammation by interacting with the TLR signaling cascade or with other signaling pathways involving TLRs.

Materials and methods

Mice, BM-derived macrophages and RAW264.7 cell line

Female C57BL6 mice (aged between 10–12 weeks) were purchased from the National Laboratory Animal Centre (Mahidol University, Salaya, Thailand). All procedures involving laboratory animals were conducted according to guidelines issued by Chulalongkorn University. MyD88^{-/-} mice back-crossed onto the C57/BL6 background were kindly provided by Dr. Shizuo Akira and the experiments involving these mice were conducted at the Max Planck Institute for Infection Biology according to German protection law [38]. Mice were sacrificed, and femoral bone marrows were isolated. BM-derived macrophages were generated as described elsewhere [39]. In brief, cells were washed from BM cavity and incubated for 7 days in RPMI 1640 media supplemented with 10% fetal bovine serum purchased from HyClone Laboratory (Logan, UT), 100 μ M gentamycin and 20% L929-conditioned media. Media were changed every 2 days and cells were harvested at the end of culture using 5 mM EDTA in phosphate buffer saline (PBS). Cells were confirmed to be of macrophage/monocyte lineage by cell surface staining with anti-CD11b and anti-Mac1 mAbs and by analysis using flow cytometry. More than 95% were found to be CD11b⁺ and Mac1⁺. RAW264.7 cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 μ M gentamycin and L-glutamine.

Reagents and antibodies

LPS from *Escherichia coli* O26:B6, polyinosinic:polycytidylic acid were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic TLR2 agonist Pam₃Cys lipopeptide was purchased from EMC microcollections GmbH (Germany). TLR agonist CpG DNA has been described elsewhere [40]. Mouse r-IFN γ was purchased from R&D Systems (Minneapolis, MN) and kept at -80°C . The γ -secretase inhibitor, IL-CHO, has been described elsewhere [22]. IL-CHO was dissolved in DMSO at a concentration of 5 mM and aliquots were kept at -80°C . TriZol reagent for total RNA isolation was purchased from Life Technologies (Grand Island, NY). The mAb for cell surface staining, i.e. FITC-conjugated anti-Mac1 mAb, PE-conjugated anti-CD11b, mAb and biotinylated anti-mouse MHC class II (IA^b) mAb were purchased from Caltag (Carlsbad, CA). Rabbit polyclonal Ab against Notch1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal Ab against cleaved Notch1 (Val1744) was purchased from Cell Signaling Technology (Boston, MA). The mAb against β actin was purchased from Chemicon International (Temecula, CA). Secondary Ab conjugated with HRP was purchased from Amersham Biosciences (Piscataway, NJ).

RT-PCR

Total RNA was isolated from cells treated as indicated using TriZol reagent according to the manufacturer's instruction. RNA (500 ng) was used to generate

cDNA using random hexamer primers and RevertAid M-MuLV reverse transcriptase (Fermentas; ON, Canada). Obtained cDNAs were used as templates to amplify *Notch1-4*, *Hes1*, *IL-6*, *IL-10* and β -actin. The primer pairs used in this study are as follows.

Primer	Sequence (5' to 3')
Murine Notch1 forward	GTGAGGGTGATGTCAATG
Murine Notch1 reverse	TGAAGTTGAGGGAGCAGT
Murine Notch2 forward	TGGAGGTAAATGAATGCCAGAGC
Murine Notch2 reverse	TGTAGCGATTGATGCCGTCC
Murine Notch3 forward	ACACTGGGAGTTCTCTGT
Murine Notch3 reverse	GTCTGCTGGCATGGGATA
Murine Notch4 forward	CACCTCCTGCCATAACACCTTG
Murine Notch4 reverse	ACACAGTCATCTGGGTTTCATCTCAC
Murine Hes1 forward	CCGGTCTACACCAGCAACAGT
Murine Hes1 reverse	CACATGGAGTCCGAAGTGAGC
Murine IL-6 forward	CATGTTCTCTGGGAAATCGTGG
Murine IL-6 reverse	AACGCACTAGGTTTGCCGAGTA
Murine IL-10 forward	TCAAACAAAGGACCAGCTGGACAACATACTGC
Murine IL-10 reverse	CTGTCTAGGTCCTGGAGTCCAGCAGACTCAA
β actin forward	ACCAACTGGGACGACATGGAGAA

β actin reverse	GTGGTGGTGAAGCTGTAGCC
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The PCR reactions were carried out using Taq DNA polymerase (Fermentas). The PCR products were analyzed on 1.5% agarose gel by electrophoresis, except those for *Hes1* which were analyzed on 5% polyacrylamide gel, and visualized after staining with ethidium bromide using gel documentation system (BioRad; Hercules, CA).

Western blot

Cells treated as indicated were harvested and cell lysates were prepared as described previously [14]. Amounts of proteins were measured using BCA protein assay kit (Pierce; Rockford, IL). Cell lysates (30 μg) were separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis using Protein III system (BioRad). After gel separation, proteins were transferred onto PVDF membrane (Amersham Biosciences) and blocked in PBS containing 3% nonfat dry milk and 0.05% Tween 20. Blots were probed with rabbit anti-Notch1 Ab at 1:1000 dilution, rabbit anti-cleaved Notch1 Ab at 1:1000 dilution or anti-βactin mAb at 1:5000 dilution, followed by washing and probing with HRP-conjugated donkey anti-rabbit IgG Ab or sheep anti-mouse IgG Ab at 1:4000 dilution. After washing, signals were detected using ECL Western blotting analysis system (Amersham Biosciences).

Measurement of nitrites

To measure the amount of NO produced by macrophages, BM-derived macrophages were treated as indicated. At the end of culture, culture supernatants were harvested by centrifugation and kept at -80°C . Culture supernatants were subjected to assay for NO production using Griess reagents as described previously [41].

Measurement of $\text{TNF}\alpha$ by ELISA

Quantitative ELISA was carried out using mouse $\text{TNF}\alpha$ ELISA test kit purchased from eBioscience (San Diego, CA). Culture supernatants were collected after treating cells as indicated and kept at -80°C until subjecting to ELISA. ELISA was carried out according to manufacturer's instructions.

Statistical analysis

To calculate the statistical differences between control and samples, Student's paired t-test was used. Values of $p < 0.05$ were considered significant.

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For Peer Review

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Figure Legends

Figure 1. Expression profiles of *Notch1-4* and *Hes1* in activated macrophages.

- A–B. RAW264.7 or BM macrophages were treated with LPS (100 ng/ml), rIFN γ (10 ng/ml) or LPS plus rIFN γ and total RNA was isolated at indicated times. Expressions of *Notch 1-4* were analyzed by RT-PCR. The β *actin* was used as loading control.
- C. RAW264.7 cells were treated with LPS or LPS plus rIFN γ for indicated times and total RNA was isolated. *Hes1* expression was analyzed by RT-PCR.

Figure 2. Notch1 is upregulated in macrophages stimulated with IFN γ and LPS.

- A. BM macrophages were left untreated or treated with LPS (100 ng/ml), rIFN γ (10 ng/ml) or LPS plus rIFN γ for 18 h. Cell lysates were analyzed for Notch1 expression by Western blot. The β *actin* was used as loading control.
- B. BM macrophages were left untreated or treated with rIFN γ (10 ng/ml) alone, LPS (100 ng/ml) alone for 12 h. For co-treatment with LPS plus rIFN γ , macrophages were pretreated with LPS (100 ng/ml) for 4 h and rIFN γ was added and incubated for a further 8 h, or pretreated with rIFN γ (10 ng/ml) for 4 h and LPS was added and further incubated for 8 h. Cell lysates were analyzed for Notch1 expression by Western blot. The β *actin* was used as loading control.

Figure 3. TLR agonists upregulate Notch1 expression in activated macrophages.

BM macrophages were treated with LPS (100 ng/ml) (A), poly I:C (100 µg/ml) (B), Pam3Cys lipopeptide (1 µg/ml) (C) or CpG DNA (500 ng/ml) (D) for indicated times and cell lysates were analyzed for Notch1 expression by Western blot. The β actin was used as loading control.

Figure 4. Upregulation of Notch1 in MyD88^{-/-} BM.

BM macrophages from C57BL6 WT mice or MyD88^{-/-} mice were stimulated with LPS (A) or Pam3Cys lipopeptide (B) for indicated times. Cell lysates were analyzed for Notch1 expression by Western blot.

Figure 5. Effects of GSI treatment on Notch1 expression and morphology of macrophages.

A–B. BM macrophages were pretreated with IL-CHO or DMSO mock control for 1 h, and treated with LPS (100 ng/ml) plus rIFN γ (10 ng/ml) for 18 h. Cell lysates were analyzed expression of cleaved Notch 1 (A) or Notch1 (B) by Western blot. The β actin was used as loading control

C. Morphology of BM macrophages treated as described above for 36 h.

Figure 6. Effects of GSI treatment on production of cytokines and NO and expression of MHC class II in macrophages.

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6 A. BM macrophages were pretreated with mock control DMSO or IL-CHO (25
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8 μM) for 1 h before stimulation with LPS and rIFN γ for indicated periods.
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10 Total RNA was isolated and expressions of *IL6*, *IL-10* were analyzed using
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12 RT-PCR. Expression of *β -actin* was used as loading control.
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16 B. BM macrophages were treated as described in A and culture supernatants
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18 were harvested at 6 h, 12 h and 24 h. The amount of TNF α in culture
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20 supernatant was measured by ELISA. The results shown are representative of
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22 two independent experiments carried out in duplicate. (* $p < 0.05$ between
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24 DMSO-treated and IL-CHO-treated samples).
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28 C. BM macrophages were pretreated as described in B. Culture supernatants
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30 were harvested and subjected to the assays for nitrite production using Griess
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32 reaction. The results correspond to representatives of the means $\pm$ SD of
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34 triplicate experiments. (* $p < 0.05$ between DMSO-treated and IL-CHO-treated
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36 samples).
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40 D. BM macrophages were treated as described in B for 24 h and cells were
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42 harvested and stained using biotinylated anti-MHC class II (I-A^b) and avidin-
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44 FITC. Expression of MHC class II was analyzed by flow cytometry.
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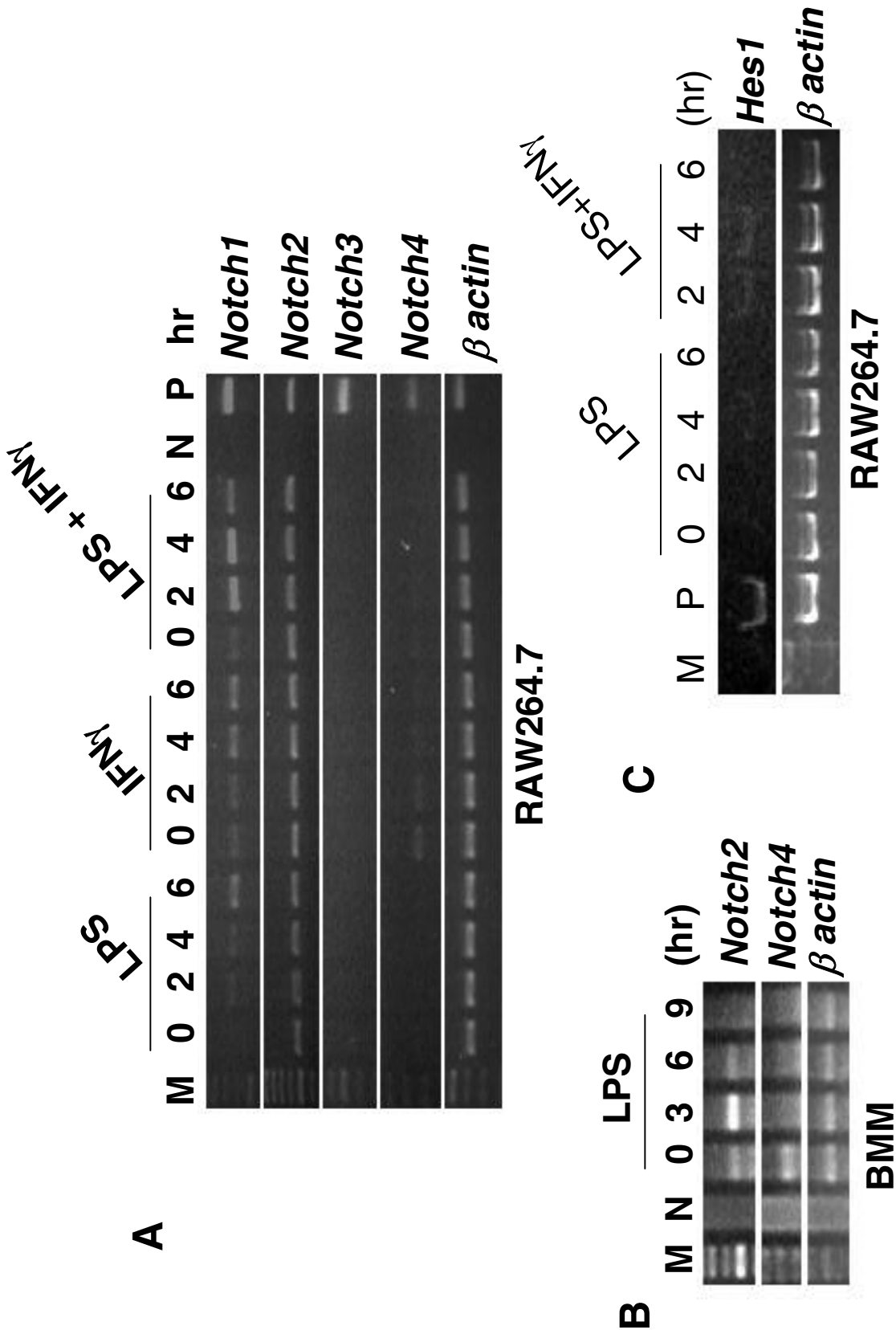


Figure 1
Palaga et al., 2006

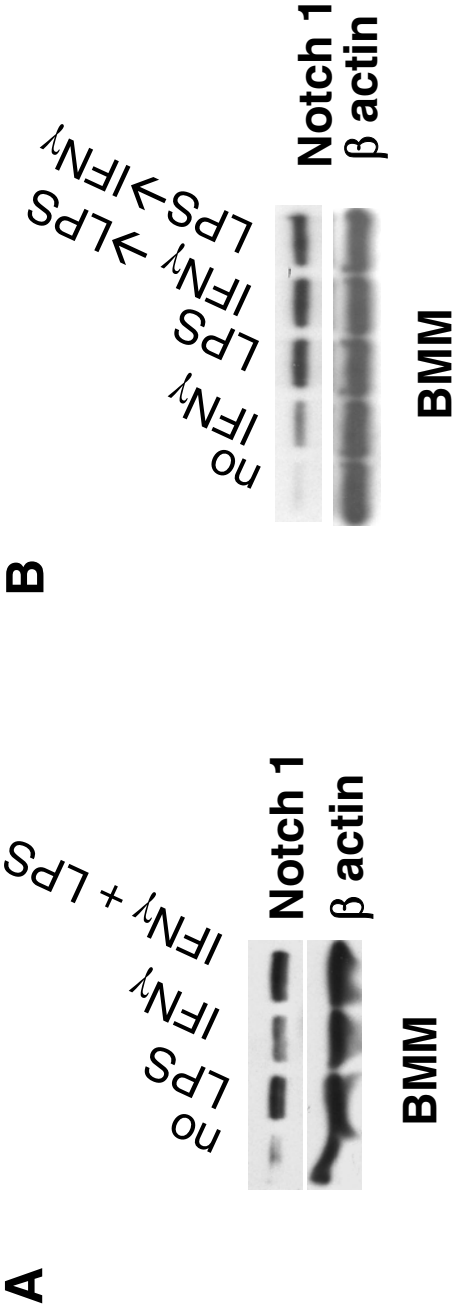


Figure 2
Palaga et al., 2006

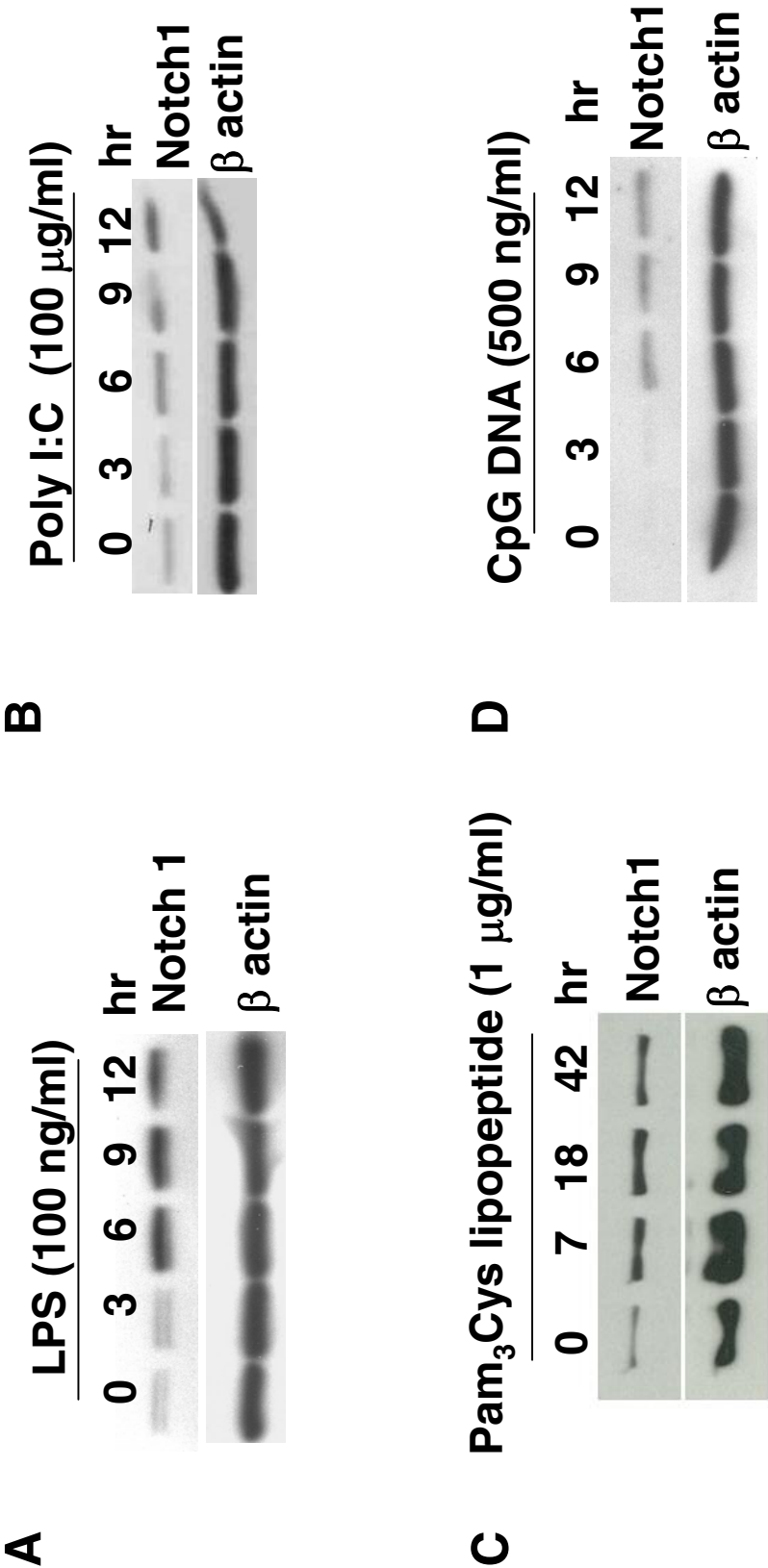


Figure 3
Palaga, et al., 2006

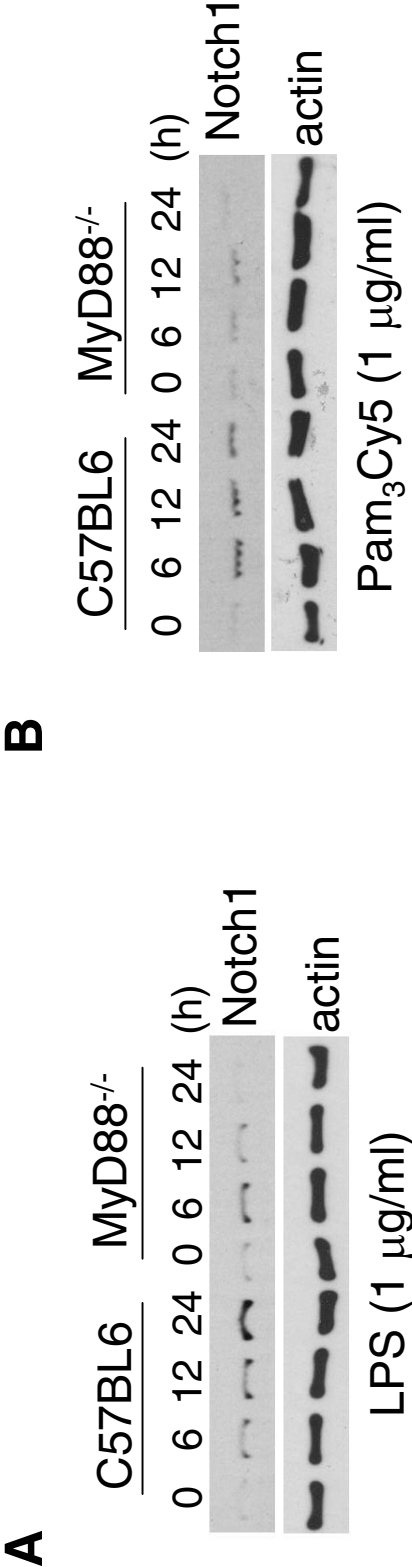
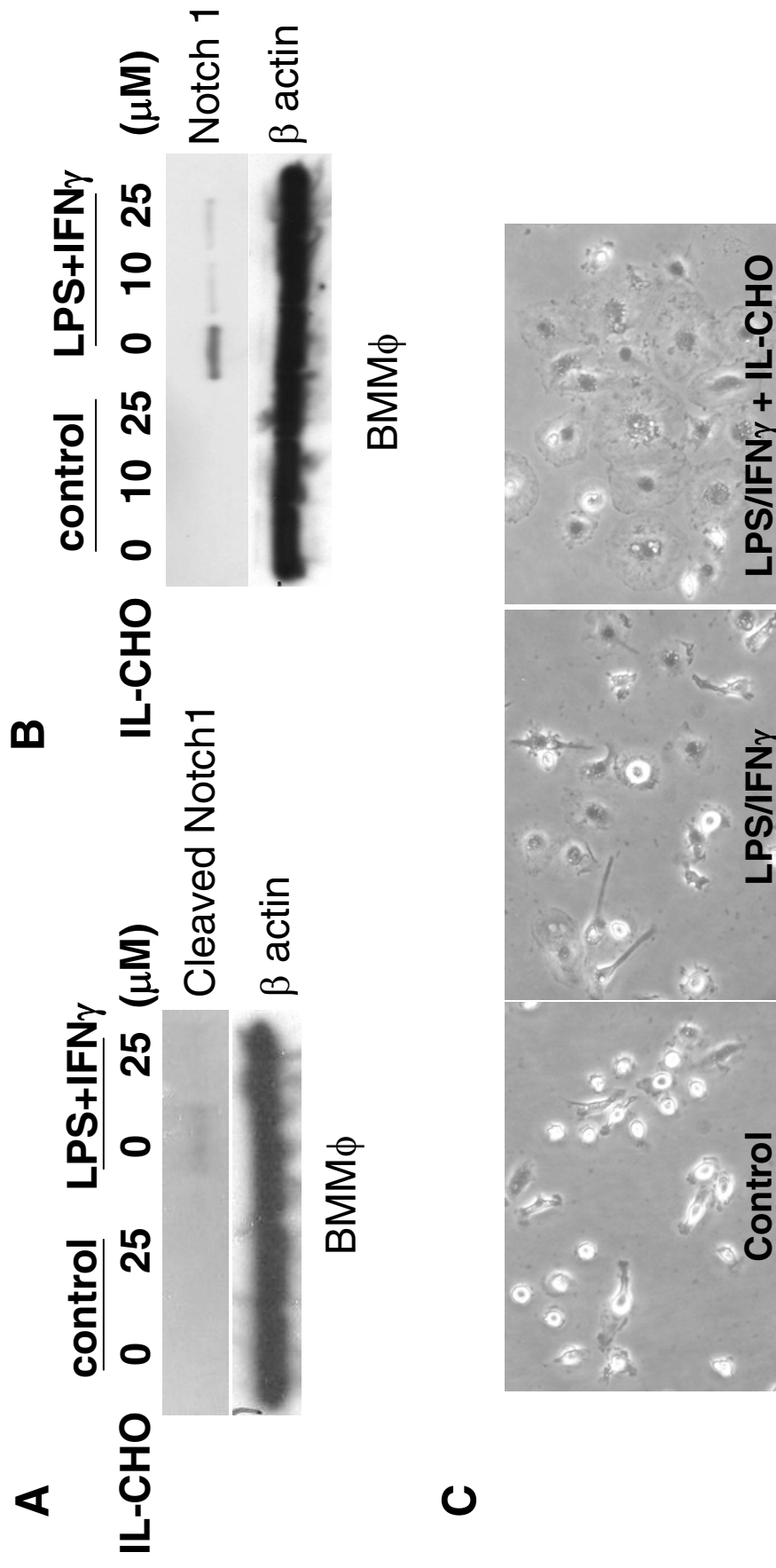


Figure 4
Palaga et al., 2006



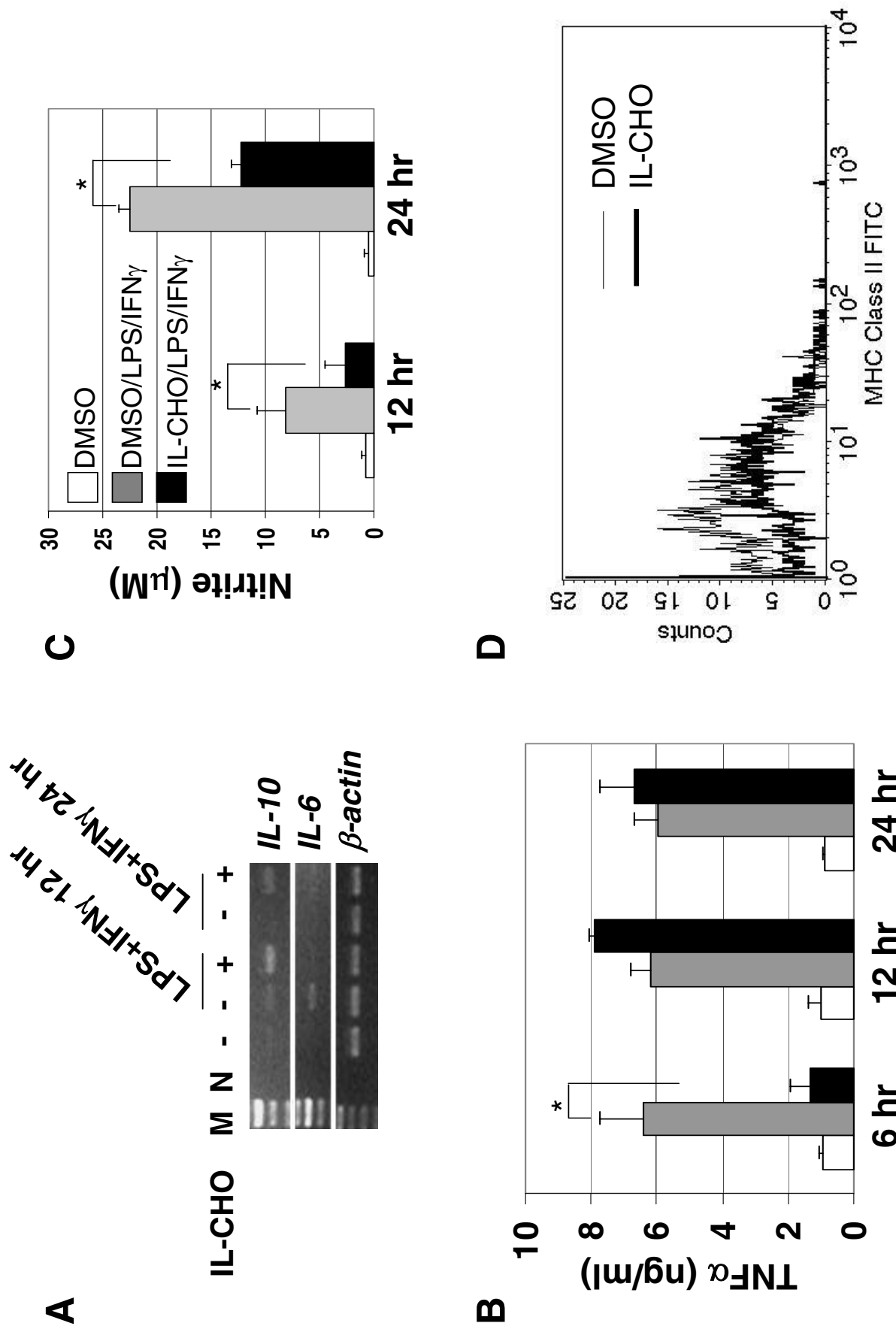


Figure 6
Palaga et al., 2006

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