# 動物實驗申請表

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申請單號: LAC-2017-0323　　日期: 2017-12-01



一、 計畫主持人：李爾博 Gilbert Aaron Lee　職稱：研究員　連絡電話：0958880131

共同主持人：黃綉文　職稱：研究員　連絡電話：

二、 單位：附醫研究部

實驗地點：北醫實驗動物中心　實驗內容：

三、 計畫/課程/試驗名稱：

(中文) 探討介白素15號系統於腦缺血後免疫反應的功能以及建立腦中風免疫療法

(英文) The role of IL-15 system in the immune response to cerebral ischemia-reperfusion and in the development of stroke immunotherapy

計劃類別：醫學研究類

四、 經費來源：科技部

五、 執行期限：2017-12-01 至 2019-12-01

延續型計劃：LAC-2017-0109

六、負責進行動物實驗之相關人員資料:

| 姓名 | 職稱 | 參與實驗期限 | 具有動物實驗相關技術與經驗年數 | 佐證資料上傳 | 附影像光碟 |
|---|---|---|---|---|---|
| 李爾博 | 研究員 | 2017/12/1-2019/12/01 | 安樂死 腦中風手術 10年動物實驗經驗 | P1132_M2299.pdf | |
| 毛馨儀 | 技佐 | 2017/12/1-2019/12/01 | 安樂死 腦部分析 | P1132_M2300.pdf | |
| 黃婉真 | 技佐 | 2017/12/1-2019/12/01 | 安樂死 腦部分析 | P1132_M2301.pdf | |

七、實驗所需之動物:

| 動物別/品系 | 使用量 | 動物來源 | 動物飼養場所 | 是否需要繁殖 |
|---|---|---|---|---|
| 小鼠:小鼠 / IL-15Flox | 100 | 其他: 北醫實驗動物中心 | 北醫實驗動物中心 | 是 |
| 小鼠:小鼠 / IL-15FLOX-GFAP-cre | 100 | 其他: 北醫實驗動物中心 | 北醫實驗動物中心 | 是 |
| 小鼠:小鼠 / IL-15KO | 50 | 其他: 北醫實驗動物中心 | 北醫實驗動物中心 | 是 |
| 小鼠:小鼠 / C57BL/6 | 90 | 國家實驗動物中心:<br>北醫實驗動物中心 | 北醫實驗動物中心 | 否 |
| 小鼠:小鼠 / Rag2KO | 50 | 樂斯科生物科技:<br>北醫實驗動物中心 | 北醫實驗動物中心 | 是 |

註a： 保育類野生動物請加註，並另依野生動物保育法相關規定辦理。

註b： 1.動物來源可能為國內外合法繁殖場(例如國家實驗動物中心，樂斯科生物科技有限公司，美國JAX實驗室・・・等)、其他國內外研究機構之轉讓予贈與(例如美國或歐洲的大學，EMMA・・・等)、小型私人繁殖場及野外捕捉等，請說明動物來源請說明動物來源，再由照護委員會(小組)評估適當性與合法性。

2.自野外捕捉隻動物請加註，並另加說明來源地區、隔離檢疫方式及隔離期間；取自民間市場者，必要時需比照辦理。

註c： 如動物飼養於非本機構之其他場所，需提供該場所所屬機構名稱、地址及該場所核准營運之證明文件(租借場地進行)或審核通過之動物實驗申請表(委託或合作)。

# 動物實驗申請表

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註d： 如需要繁殖「實驗動物(只供應做科學應用目的使用者)」，請填寫附錄一。

八、動物飼養場所類別:

本校動物中心

九、請簡述本研究之目的:

1. 我們會探討星狀膠細胞的IL-15如何影響腦中風小鼠的腦部的NK細胞和CD8 T細胞的活性與功能。
2. 我們將利用抗介白素 15 號抗體(neutralizing anti-IL-15 antibody)來發展腦中風免疫療法，並利用核磁共振影像系統來探討此抗體的療效與機制，以評估抗介白素 15 號抗體是否具備轉譯於腦中風臨床治療的可能性。

十、請以動物實驗應用3Rs之替代及減量原則,說明動物實驗試驗設計、實驗動物需求、動物種別及數量之必要性:

(一)動物科學應用替代方式說明:

1.研究內容:

藥劑、疫苗開發

2.是否進行實驗前替代方式評估?

是 , 此抗體的有效劑量有在細胞培養測試過，並且在腦中風小鼠有保護的效果。

3.使用活體動物之必要性?

已確實了解動物保護法第15條第1項及非活體動物替代方式之相關規範。

已確實遵守3R原則(替代、減量、精緻化)進行實驗設計及實驗前評估。

因此研究要探討抗體對免疫反應的影，因此需要使用活體小鼠來評估此反應。

(二)法源依據:

農委會-動物用藥品檢驗標準。

(三)參考文獻:1. Adipocyte IL-15 regulates local and systemic NK cell development J. Immuol. 193:1747-58. 此為IL-15Flox的文獻，目前沒有文獻報導IL-15Flox-GFAP-cre的小鼠。
2. Non-invasive tracking of CD4+ T cells with a paramagnetic and fluorescent nanoparticle in brain ischemia. Journal of Cerebral Blood Flow & Metabolism 2016, Vol. 36(8) 1464–1476. 此為文獻可以用MRI contrast agent標定免疫細胞，並追蹤免疫細胞在腦中風小鼠的位置。
3.A novel indication of platonin, a therapeutic immunomodulating medicine, on neuroprotection aganst ischemic stroke in mice.Scientific Reports 7:42277 此為文獻可以用rotar rod和openfield assay來測試motor function。
(四)詳細說明動物實驗試驗設計(動物分組方法、每組使用動物數量等):

1. 分析腦中風受損區域以及motor function
需要IL-15Flox和IL-15Flox-GFAP-CRE基因型老鼠各8隻，3個reperfusion時間點(3h, 48h, 7天)。共需male Flox=24隻，male IL-15Flox-GFAP-CRE 24隻
2. 分析腦部免疫細胞需要IL-15Flox和IL-15Flox-GFAP-CRE基因型老鼠各8隻，3個reperfusion時間點(3h, 48h, 7天)。共需male Flox=24隻，maleIL-15Flox-GFAP-CRE 24隻。
3. 分析星狀膠細胞IL-15在IL-15Flox和IL-15Flox-GFAP-CRE 的表現。從5個小鼠成腦約可純化出1百萬顆astrocyte，此細胞數目可以用ELISA檢測到IL-15約50pg/ml，預計做三次獨立實驗，所以共需female Flox=15隻, female IL-15Flox-GFAP-CRE=15隻。
4. 分析anti-IL-15 antibody 對NK細胞在腦中風IL-15KO小鼠的影響。控制組1組，實驗組兩組(有MRI contrast agent標定的NK細胞、沒標定的NK細胞)每組8隻，共需24隻IL-15KO male mice
6. 分析anti-IL-15 antibody 對CD8 T 細胞在腦中風Rag2KO小鼠的影響。控制組1組，實驗組兩組(有MRI contrast agent標定的CD8 T細胞、沒標定的CD8 T細胞)每組8隻，共需24隻Rag2KO male mice
7. 純化小鼠脾臟NK細胞和CD8T細胞來執行實驗3和4，其中還需要檢測細胞功能、細胞增生等實驗，因此需要WT小鼠各10隻，共20隻。

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8.分析 anti-IL-15 antibody 對WT小鼠在中風後，motor function recovery以及免疫反應的影響。控制組1組，實驗組1組，每組10隻，共需20隻WT 小鼠。
9. 分析anti-IL-15 antibody 對WT小鼠在中風後，其腦部影像T2、ADC、FA map的影響。控制組1組，實驗組1組，每組10隻，共需20隻WT小鼠。
因小鼠腦中風手術相當困難，成功率約80%，因此會於預計使用的實驗動物總量多預估一些數量。

*IL-15KO是小鼠全身的IL-15基因剔除，IL-15Flox-GFAP-cre是只有astrocyte的IL-15基因剔除。
*在之前的腦中風初步測試，小鼠執行完MRI scan後行為能力或活動力會更差(可能scan的流程或其他麻醉劑的效果)，無法準確的評估其motor function的功能，因此希望還是分兩組實驗來測試。
*腦中風手術地點為動物中心2樓或1樓

十一、請以動物實驗應用3Rs之精緻化原則,詳細說明實驗中所進行之動物實驗內容:

(一)實驗物質之投予、採樣方法及頻率:

Cerebral ischemia-reperfusion 手術
Briefly, we use 0.8% chloral hydrate to anesthetize the mice. The trunk of the right middle cerebral artery (MCA) was ligated with a 10-0 suture. Right common carotid arteries was then occluded using nontraumatic aneurysm clips. After 40 minutes of ischemia, the aneurysm clips and the suture were removed to restore the blood flow in two arteries.
接受腦中風手術之小鼠將於後續7天內被每天觀察是否產生嚴重不適如抽筋、癱瘓。也會觀察小鼠Analgesia quality, mucous membrane color、breathing patter等狀況，若有上述狀況就會立即安樂死，
若無異常現象，將以二氧化碳進行安樂死後，收集腦和脾臟進行分析。上述所有的實驗分組皆會接受腦中風手術。
需使用5% chloral hydrate 的原因是此麻醉效果已證實當大鼠接受腦中風的傷害後，其腦梗死區域會大於使用isofluorane當麻醉劑。另外chloral hydrate 的麻醉效果時間也比avertin長(avertin約25分鐘)，約可長達45分鐘。
因執行腦中風手術的時間較長(約45~1h)，因此需使用0.8%chloral hydrate為較理想的麻醉劑劑。未來若有經費購買氣麻機，會再換成isofluorane當麻醉劑。
實驗中動物之健康變化:

(二)動物之保定、禁食、禁水、限制行動(如代謝籠、跑步機、行為實驗)的方法及時間:

無

(三)麻醉(鎮靜)方法、劑量、投藥、手術方式與麻醉(手術)後的照護:

常用麻醉藥物-1：無 劑量：0.8% chloral hydrate 投藥方式：i.p.

其他麻醉藥物：0.8% chloral hydrate 附件 :

手術方式：小鼠腦中風手術

麻醉（手術）後的照護：

利用電毯維持體溫，直到甦醒

(四)如何使動物之緊迫或疼痛降至最低(例如使用鎮靜劑或止痛劑、添加環境豐富化物件等，並依疼痛標準級別與實驗目的，描述動物疼痛處理方式):
Carprofen (4mg/Kg)

| 疼痛分級： | 本實驗所涉及之操作項目 |
|---|---|
| Category C<br><br>1.動物進行不會造成痛苦或緊迫的操作。2.動物進行只造成短暫或輕微痛苦及緊迫的操作。<br>※這些操作不需使用到止痛藥。 | • 對動物進行抓取、秤重、短期保定或一般身體檢查<br>• 對21日齡以內的小鼠進行剪尾<br>• 對繁殖的動物以及繁殖後不使用的後代進行安樂死<br>• 麻醉下進行灌流<br>• 動物安樂死後採取組織 |

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| | |
|---|---|
| Category D<br><br>動物進行可能產生疼痛或壓力的操作，且<br>會給予適當之止痛、麻醉或鎮定藥。 | • 存活手術 |

(五)實驗預期結束之時機，以及動物出現何種異常與痛苦症狀時提前人道中止實驗:

實驗預期結束之時機（實驗終點）：

接受腦中風手術之小鼠將於後續7天內被每天觀察是否產生嚴重不適如抽筋、癱瘓。也會觀察小鼠Analgesia quality, mucous membrane color、breathing patter等狀況，若有上述狀況就會立即安樂死。
動物出現何種異常與痛苦症狀時提前人道中止實驗：

獸醫師意見(人道因素)，體重下降超過15%，無法正常走動，無法正常攝食／飲水，明顯對刺激之反應力下降

十二、請說明實驗結束後動物之處置方式(如復原處置、安樂死、屍體、處理方法、轉讓...等;若為轉讓請提供計劃實驗申請書):
* 安樂死: CO2
* 動物屍體依動物中心規定處理。

十三、有無進行危險性實驗，如生命危險(含感染性物質、致癌藥物)、放射線及化學危險(含有毒物)實驗:

操作場所：

否

(一)實驗之危險性屬於:

1、進行危險物品實驗施用之方法、途徑及場所：

2、針對實驗人員、實驗動物以及飼養環境所採行之保護措施：

3、實驗廢棄物與屍體之處理方式：

(二)如屬生物危險實驗，請陳述：

是否有生命安全委員會之核准資料：無, 理由：

(三)如屬放射線或毒性化學危險實驗，請說明本案向主管機關之申請狀況:

無(實驗內容不涉及放射線或毒性化學危險之操作)。

十四、動物實驗人道管理替代、減量及精緻化(3R)說明

(若有申請補助計畫須檢附3R說明時，請填寫說明。)

本研究計畫涉及動物實驗，已考量「替代(Replace)」、「減量(Reduce)」及「精緻化(Refine)」之3R精神，將實驗設計最佳化，並說明如下：

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（一）、3R原則

本實驗計畫已經本人及機構內「實驗動物照護及使用委員會(或小組)」詳實審查，無其他替代方案。

本實驗計畫已經本人及機構內「實驗動物照護及使用委員會(或小組)」詳實審查，已使用最少數量動物。

本實驗計畫已經本人及機構內「實驗動物照護及使用委員會(或小組)」詳實審查，已做到精緻化，或動物福利最佳化。包含:

已考慮並要求執行動物疼痛評估

已考慮並要求執行適當減輕動物痛苦的方式(如:麻醉劑,止痛劑,設定人道安樂死時機)

（二）、教育訓練

為促進3R精神之落實，本研究實際負責進行動物實驗之相關人員之教育與訓練經歷：

實驗動物人道管理(例如:動物福利、3R原則)。

實驗專業訓練

（三）、使用動物來源：

為確保本研究計劃實驗品質與效益，本實驗之動物來源為：

AAALAC認證繁殖機構

（四）、監督機制：

為確保實驗品質與效益，本研究激化相關實驗之監督機制為:

「實驗動物照護及使用委員會(或小組)」，隸屬機構層級為校級委員會

召集人職稱教授

已設置專責專職獸醫師，並參與計畫審查及動物照護與管理。

（五）、行政院農業委員會最近一次實地查核本機構「動物科學應用」之評比紀錄：

(請參考附件)

十五　申請人聲明

1.申請人保證以上所填資料完全屬實，因填報不實而生之後果，申請人願負完全之責任。

2.若本委員會或經委員會委託之獸醫師發現本案中實驗動物處於極度不適狀態，基於維護動物福祉之精神，申請人同意立即將實驗動物安樂死。

申請人，是否同意此項聲明? 是，申請人同意此項申明

申請人保證以上所填資料完全屬實，並確認此申請案之執行與運作符合「動物保護法」及相關法規之規定。

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## 附件一、

一、請說明本研究計劃須繁殖動物的理由：

1. 將IL-15FLOX和IL-15FLOX-GFAP-cre的交配才能產生出星狀膠細胞IL-15基因剔除小鼠。
2. 需使用IL-15KO 小鼠才能追蹤NK在腦中免疫反應的角色
3. 需使用RagKO 小鼠才能追蹤CD8 T細胞在腦中免疫反應的角色

| 品種 | 品系 | 繁殖動物總量 | 使用於實驗的子代數量 |
|---|---|---|---|
| 小鼠 | IL-15Flox | 100 | 63 |
| 小鼠 | IL-15Flox-GFAP-cre | 100 | 63 |
| 小鼠 | IL-15KO | 50 | 25 |
| 小鼠 | Rag2KO | 50 | 25 |

三、動物繁殖之負責單位：

由實驗室人員負責，請說明其對動物飼養之背景與訓練:

李爾博過去已有10年的動物實驗經驗，過去在北醫動物中心已成功繁殖出astrocyte IL-15 conditional knockout 小鼠。

四、請說明種原動物與子代的淘汰策略：

1. IL-15Flox與IL-15Flox-GFAP-cre小鼠互配，其公的子代(48隻)會留下做實驗，母的子代會留下(22隻)，作為配種與部分elisa的實驗。此兩品系的互配方式預計是第一年用5隻公鼠10隻母鼠做配種，第二年用3公鼠6母鼠配種。
2. IL-15KO

小鼠為公母互配，其公的子代(25隻)會留下做實驗，另外母的子代(留下10隻)作為配種。預計用3公6母做交配。

3.Rag2KO

小鼠為公母互配，其公的子代(25隻)會留下做實驗，另外母的子代(留下10隻)作為配種。預計用3公6母做交配。

因為腦中風實驗皆為公鼠，所以以上品系(IL-15Flox, IL-15Flox-GFAP-cre, IL-15KO, Rag2KO)生產的子代只有50%的機率會是實驗所用，所以會預先在3周離乳前淘汰多餘的母鼠子代。

五、未使用於實驗的動物之處置方法：

種原：

安樂死

# 動物實驗申請表

「本表請留存於貴機構實驗動物照護及使用委員會(或小組)備查，勿須報送本會;惟如使用猿猴、犬、貓進行科學應用時，應提供審核通過之申請表影本列為年度監督報告之附件。」

申請單號: LAC-2017-0323　　日期: 2017-12-01



子代：
安樂死

六、是否為基因改造動物?
是請填寫下列問題:

(一)請說明動物是否有任何特殊表現型或先天性異常?
否

(二)是否需特殊照養?
否

(三)請說明篩選基因採用方法與採樣時間:
利用PCR方式進行篩選，採樣時間為3-4周的小鼠

The Journal of Immunology

# Different NK Cell Developmental Events Require Different Levels of IL-15 *Trans* -Presentation



Gilbert Aaron Lee, Yae-Huei Liou, Szu-Wen Wang, Kai-Liang Ko, Si-Tse Jiang and Nan-Shih Liao

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# Different NK Cell Developmental Events Require Different Levels of IL-15 *Trans*-Presentation

**Gilbert Aaron Lee,*,† Yae-Huei Liou,† Szu-Wen Wang,† Kai-Liang Ko,† Si-Tse Jiang,† and Nan-Shih Liao†**

**NK cell development requires IL-15, which is "*trans*-presented" to IL-15Rβγ on NK cells by IL-15Rα on other cells. In this study, we report that different levels of IL-15 *trans*-presentation are required for different NK cell developmental events to reach full maturation status. Because the IL-15Rα intracellular domain has the capacity to recruit signaling molecules, we generated knockin and transgenic (Tg) mice that lack the intracellular domain to assess the role of the IL-15 *trans*-presentation level independent of the function of this domain. The level of IL-15Rα on various cells of these mice follows the order WT > Tg6 > knockin > Tg1 ≥ knockout. Bone marrow (BM)–derived dendritic cells prepared from these mice induced Stat5 phosphorylation in NK cells. The level of phospho-Stat5 correlated with the level of IL-15Rα on BMDCs, thus offering the opportunity to study quantitative effects of IL-15 *trans*-presentation on NK cell development in vivo. We found that NK cell homeostasis, mature NK cell differentiation, and acquisition of Ly49 receptor and effector functions require different levels of IL-15 *trans*-presentation input to achieve full status. All NK cell developmental events examined were quantitatively regulated by the IL-15Rα level of BM-derived and radiation-resistant accessory cells, but not by IL-15Rα of NK cells. We also found that IL-15Rα of radiation-resistant cells was more potent than IL-15Rα of BM-derived accessory cells in support of stage 2 to stage 3 splenic mNK differentiation. In summary, each examined developmental event required a particular level of IL-15 *trans*-presentation by accessory cells.** ***The Journal of Immunology*****, 2011, 187: 1212–1221.**

Natural killer cell development in mice involves multiple steps to reach full maturation (1). The earliest cells committed to NK lineage are NK precursors (NKp) that do not express known NK markers, such as NKR-P1C (NK1.1) or Ly49 receptors (Ly49Rs), but express IL-15Rβ (CD122) and the common γ-chain ($\gamma_c$) (2). Transition from NKp to immature NK cells (iNK) is characterized by the expression of NK1.1 and CD94/NKG2 and the subsequent acquisition of Ly49Rs. Expression of DX5, CD11b, and CD43 indicates differentiation into mature NK (mNK) cells. At this stage NK cells acquire the full capacity to launch effector functions, such as cytotoxicity and IFN-γ production, upon stimulation through NK receptors (3). These NK cell developmental events are regulated by cell-intrinsic mechanisms as well as extrinsic signals. One critical extrinsic signal is IL-15. The absence of IL-15 or its high-affinity receptor IL-15Rα results in dramatic defects in NK cell homeostasis, mNK differentiation, and the acquisition of Ly49Rs and effector functions (4, 5).

IL-15 delivers signals through a mode termed "*trans*-presentation" in which the IL-15/IL-15Rα complex expressed on one cell triggers signaling through IL-15Rβ and $\gamma_c$ on a neighboring cell (6). Previous studies indicate that NK cell homeostasis and mNK differentiation require IL-15Rα of bone marrow (BM)–derived as well as radiation-resistant accessory cells, whereas acquisition of Ly49Rs only requires IL-15Rα of BM-derived accessory cells (7–10). Overexpression of IL-15Rα by dendritic cells (DCs) in *Il15ra*$^{-/-}$ mice fully supports developing NK cells to acquire effector functions (11). These findings indicate that IL-15 *trans*-presentation provided by accessory cells is important for NK development.

In addition to *trans*-presentation of the ligand, IL-15Rα possesses other functions. Its intracellular (IC) domain has signaling potential (12) and is required for the recycling of IL-15Rα in the T cell line Kit-225 (13). This finding is consistent with the observation that the IL-15Rα level on the surface of CD8$^+$ T cells is reduced in mice whose IL-15Rα IC domain has been replaced with the IL-2Rα IC domain (14). These studies indicate that the IL-15Rα IC domain not only transmits signals, but also maintains an optimal level of IL-15Rα on the cell surface. However, the role of accessory cell IL-15Rα IC domain in NK cell development remains elusive.

The study of protein function by comparing wild-type (WT) and gene knockout (KO) cells or animals elucidates protein function in a none-or-all setting. Accumulative evidence indicates that protein quantity matters for decision making in biological processes (15–17). For NK cells, the functional responsiveness positively correlates with the quantity of signal input via inhibitory Ly49Rs during development (18, 19). NK cell homeostasis positively correlates with the level of $\gamma_c$ on NK cells (20, 21) and is upregulated by IL-15–expressing CD11c$^{high}$ DCs expanded with Flt3 ligands in vivo. However, the finding that an increase in IL-15–expressing DCs did not affect NK cell differentiation (22) suggests

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAC, bacterial artificial chromosome; BM, bone marrow; BMDC, BM-derived dendritic cell; BMDM, BM-derived macrophage; $\gamma_c$, common γ-chain; DC, dendritic cell; eGFP, enhanced GFP; ES, embryonic stem; IC, intracellular; iNK, immature NK; KI, knockin; KO, knockout; Ly49Rs, Ly49 receptors; MFI, mean fluorescence intensity; mNK, mature NK; NKp, NK precursor; P/I, PMA plus ionophore; Tg, transgenic; WT, wild type.



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a differential regulation of NK cell developmental events by different types of IL-15–expressing cells. Therefore, it remains elusive whether NK cell developmental events other than homeostasis are quantitatively controlled by IL-15 signals. In this study, we demonstrate that for each developmental event examined, that is, homeostasis, mature NK cell differentiation, acquisition of Ly49Rs, cytotoxicity, and IFN-γ productivity, NK cells need to receive a particular level of IL-15 *trans*-presentation by accessory cells to achieve the intended developmental fate.

## Materials and Methods

### *Mice*

C57BL/6J (B6/J), B6.SJL-*Ptprc*$^{a}$ *Pepc*$^{b}$/BoyJ (CD45.1$^{+}$ B6/J), and B6.Cg-Tg (ACTFLPe)9205Dym/J (FLPe) mice were purchased from The Jackson Laboratory and bred in our animal facility. *Il15ra*$^{-/-}$ mice (KO) were generated in this laboratory and backcrossed to B6/J for at least 27 generations (23). CD45.1$^{+}$ KO mice were generated by breeding. Ubiquitin promoter-driven enhanced GFP (eGFP) transgenic (Tg) mice on B6/J background (eGFP$^{+}$ B6 mice) were provided by Dr. C.-K.L. Wang (National Laboratory Animal Center, Taiwan). All mice used were 8–12 wk of age. All experiments involving mice were approved by the Institutional Animal Care and Use Committee at Academia Sinica and conformed to the relevant regulations.

### *Construction of Il15ra$^{\Delta IC}$/TCRa$^{IC}$ transgene and gene-targeting construct*

A modified *Il15ra* transgene was constructed by replacing the IL-15Rα IC domain with the TCRα IC domain in a mouse bacterial artificial chromosome (BAC) clone (RP24-148D16). A 43-bp DNA segment encoding the TCRα IC domain, FLAG, a stop codon, and carrying an additional A nucleotide (5′-AGGCTGTGGTCCAGTGATTACAAGGATGACGACGATAAGTAGA-3′, hatched box in Supplemental Fig. 1*A*) was inserted into exon 6 at a site immediately 3′ to the coding sequence of the transmembrane domain following the manual of the Counter Selection BAC Modification Kit (Gene Bridges, Dresden, Germany).

The *Il15ra* targeting construct was generated by insertion of a neomycin selection cassette flanked by FRT sites into intron 5 of *Il15ra* in the modified BAC clone described above using the Quick and Easy BAC Modification Kit (Gene Bridges).

### *Generation of chimeric Il15ra$^{\Delta IC}$/TCRa$^{IC}$ knockin and Tg mice*

For generation of *Il15ra*$^{\Delta IC}$/*TCRa*$^{IC}$ knockin (KI) mice, the construct described in the previous section was excised from the BAC backbone by NotI digestion, and transfected into mouse embryonic stem (ES) cells (B6/J strain) by electroporation. Genomic DNA of G418-selected ES clones was digested with StuI and analyzed by Southern blotting using a probe yielding a 5.3-kb band for the WT allele versus a 4.4-kb band for the targeted allele generated by homologous recombination (Supplemental Fig. 1*B*). ES cell clones with *Il15ra*$^{\Delta IC}$/*TCRa*$^{IC}$ were injected into B6/J-*Tyr*$^{c\text{-}2J}$ blastocysts. The resulting male KI chimeric mice were bred with B6/J-*Tyr*$^{c\text{-}2J}$ females, and the progeny were analyzed for germline transmission of the KI allele by PCR (data not shown). Mice carrying the KI allele were mated with FLPe Tg mice to remove the Frt-flanked neomycin cassette. KI heterozygous mice were then intercrossed to generate *Il15ra*$^{\Delta IC}$/*TCRa*$^{IC}$ KI mice. The KI allele contains a DNA sequence 43 bp longer than WT due to the insertion in exon 6. PCR of the KI allele confirmed its larger size as compared with the WT allele (Supplemental Fig. 1*C*).

To generate *Il15ra*$^{\Delta IC}$/*TCRa*$^{IC}$ Tg mice, the ∼54-kb BAC fragment containing the modified *Il15ra* was microinjected into the KO zygotes. As the KO mice retain WT *Il15ra* exon 6, the BF primer (5′-CCGTCCTGGGCAAGAATGGAAAT-3′) and BR primer (5′-GAGGGCCTCTTGGAATCGTTATG-3′) amplified both WT and Tg alleles from the genomic DNA of *Il15ra*$^{\Delta IC}$/*TCRa*$^{IC}$ Tg mice. The PCR product of Tg allele is larger than the WT product. Two founders with different levels of transgene were identified; the lower one was named Tg1, and the higher one Tg6 (Supplemental Fig. 1*D*).

### *Flow cytometry*

Cell surface molecules were stained with specific mAbs following standard protocols and analyzed on a LSRII flow cytometer (BD Biosciences, San Jose, CA). The following mAbs conjugated with fluorochrome or biotin were used: CD3 (2C11), CD8α (53-6.7), CD19 (6D5), NK1.1 (PK136), NKp46 (29A1.4), CD49b (DX5), Gr-1 (RB6-8C5), CD45.1 (A20), CD45.2 (104), F4/80 (BM8), CD11b (M1/70), Ly49A (YE1/48.10.6), Ly49G2 (4D11), Ly49D (4E5), Ly49I (YLI-90), Ly49C/I (5E6), Ly49H (3D10), IL-15Rα (BAF551), CD107a (1D4B), IFN-γ (XMG1.2), and phospho-Stat5 (C11C5) (obtained from eBioscience, San Diego, CA; BioLegend, San Diego, CA; R&D Systems, Minneapolis, MN; or Cell Signaling, Danvers, MA).

### *Preparation of BM-derived DCs, BM-derived macrophages, and in vitro IL-15* trans*-presentation assay*

BM-derived DCs (BMDCs) were generated, as described previously (24). Briefly, BM cells were cultured in culture medium containing 200 U/ml GM-CSF for 10 d and used as resting BMDCs. The proportion of CD11c$^{high}$ MHC-II$^{+}$ cells was on average 70–75%. Stimulated BMDCs were prepared by treatment with poly I:C (25 μg/ml) for 19 h. RPMI 1640 supplemented with 2 mM L-glutamine, 20 mM HEPES-NaOH, penicillin-streptomycin (2 U/ml), 50 mM 2-ME, and 10% FCS was used as culture medium.

BM-derived macrophages (BMDMs) were generated, as described previously (25). Briefly, BM cells were cultured in conditioned medium made of DMEM, 10% FBS, and 20% of L929 supernatant as a source of M-CSF for 7 d. BMDMs were then stimulated with LPS (1 μg/ml) in DMEM containing 10% FBS and M-CSF (1 ng/ml) for 24 h. The proportion of CD11b$^{+}$F4/80$^{+}$ cells was on average 95%.

NK cells (CD3$^{-}$CD19$^{-}$NK1.1$^{+}$) from the spleen of eGFP$^{+}$ B6 mice were sorted by FACS (Vantage SE/Diva; BD Biosciences). Poly I:C–stimulated BMDCs and eGFP$^{+}$ NK cells were mixed at 1:1 ratio, cultured for 30 min, and then stained intracellularly with anti–phospho-Stat5 Ab following standard protocols. mAb (5 μg/ml) to block IL-2Rβ (TM-β1; eBioscience) or to neutralize IL-2 (S4B6, homemade) was added to cultures, as indicated.

### *Detection of IL-15Rα*

For ELISA, BMDCs ($4 \times 10^{6}$ per condition) were lysed in lysis buffer (50 mM HEPES, 120 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, leupeptin (10 μg/ml), aprotinin [1 μg/ml]) and measured for IL-15Rα using the DuoSet kit (R&D Systems), according to the manufacturer's instructions. Cell surface IL-15Rα expression was analyzed by flow cytometry.

### *IFN-γ and CD107a mobilization assays*

Splenocytes of WT, KO, KI, and Tg mice were incubated in the absence or presence of plate-bound anti-NK1.1 Ab (20 μg/ml) or PMA (250 ng/ml) plus ionophore (A23187, 1 μg/ml) (P/I) for 5 h at 37°C. Culture medium contained 5 μg/ml anti-CD107a mAb, 6 μg/ml monensin (Sigma-Aldrich), and 50 U/ml murine rIL-2. Brefeldin A (10 μg/ml) was added for the last 4 h of culturing. Cells were stained intracellularly with anti–IFN-γ mAb or isotype control following the standard protocol, and analyzed via flow cytometry.

### *BM chimera*

BM cells from WT, KO, KI, and congenic CD45.1$^{+}$ KO mice were depleted of T and NK cells by complement-mediated lysis with anti-Thy1 (J1J) and anti-NK1.1 (PK136) mAbs. Indicated types of BM cells were mixed at 1:1 ratio and injected i.v. into lethally irradiated (1000 rad, $^{137}$Cs source) recipient mice. Chimeras were analyzed 9–12 wk later.

### *Statistical analysis*

Unpaired *t* test was applied to all data analyses using GraphPad Prism (GraphPad). The error bars represent the SEM.

## Results

### *The level of IL-15Rα on BMDCs determines the level of phospho-Stat5 induction in NK cells*

To separate the function of the IL-15Rα IC domain from the function of the level of IL-15 *trans*-presentation, we generated one KI and two Tg mouse lines carrying chimeric *Il15ra*, whose IC domain was replaced with the TCRα IC domain. We first examined the expression of IL-15Rα by *Il15ra*$^{\Delta IC}$/*TCRa*$^{IC}$ KI and Tg BMDCs, as IL-15Rα on BMDCs prime NK cells for IFN-γ production and cytotoxicity (26, 27). The level of IL-15Rα on KI BMDCs was 3–3.5 times lower compared with WT BMDCs under either resting or poly I:C–stimulated conditions (Fig. 1*A*), whereas the amounts of IL-15Rα in KI and WT BMDC lysates were comparable (Supplemental Fig. 1*E*, 1*F*). In the case of resting Tg1 BMDCs, the level of IL-15Rα on the cell surface (Fig. 1*A*) or in cell lysate was below

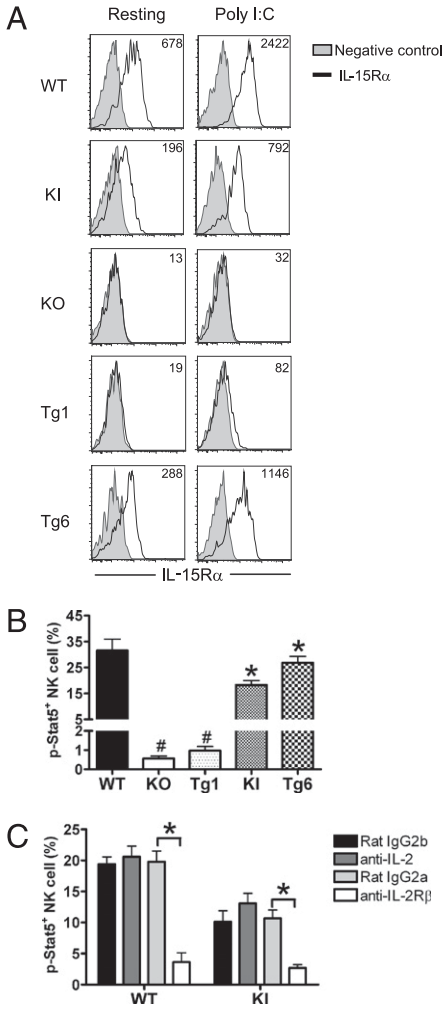


**FIGURE 1.** The level of IL-15Rα on BMDCs determines the level of Stat5 phosphorylation in NK cells. *A*, Expression of IL-15Rα on resting or poly I:C–stimulated CD11c$^+$MHC-II$^{high}$ BMDCs from *Il15ra* mutant mice. Cell surface IL-15Rα was detected by biotinylated anti-murine IL-15Rα mAb followed with streptavidin-allophycocyanin. Staining process without using the anti-murine IL-15Rα mAb is presented as negative control. The number in each panel is the Δ mean fluorescence intensity (MFI) of surface IL-15Rα. A representative of four experiments is shown. *B*, Induction of phospho-Stat5$^+$ NK cells by poly I:C–stimulated BMDCs. eGFP$^+$ NK cells (eGFP$^+$CD3$^-$CD19$^-$NK1.1$^+$) were analyzed for the percentage of phospho-Stat5$^+$ cells. Data points represent the average of four experiments. $^{\#}$Significant difference between indicated *Il15ra* genotypes and WT. $p < 0.05$. *Significant difference between indicated *Il15ra* genotypes and KO. $p < 0.05$. *C*, IL-2Rβ–blocking mAb, but not IL-2–neutralizing mAb, inhibited induction of Stat5 phosphorylation in NK cells by DCs. Data points represent the average of three experiments. $*p < 0.05$.

detection. Although poly I:C stimulation upregulated both surface and lysate IL-15Rα, the levels were the lowest among the KI and Tg cells (Supplemental Fig. 1*E*, 1*F*). For Tg6 BMDCs, surface IL-15Rα was 2-fold lower, whereas lysate IL-15Rα was 2-fold higher compared with those of WT cells with or without poly I:C stimulation (Fig. 1*A*, Supplemental Fig. 1*E*, 1*F*). Together, these results indicate that the IL-15Rα IC domain did not affect receptor expression, but was required for the optimal expression of IL-15Rα on the cell surface. The level of surface IL-15Rα follows the order of WT > Tg6 > KI > Tg1 ≥ KO. The same order was observed for IL-15Rα on splenic CD11b$^+$ DCs, F4/80$^+$CD11b$^+$ macrophages as well as on BMDMs and stromal cells. The level of surface IL-15Rα on splenic CD8αα$^+$ DCs follows the order of WT > Tg6 ≈ KI > Tg1 ≥ KO (Supplemental Fig. 2*A*).

We next examined whether the IL-15Rα level on BMDCs affects the input of IL-15 *trans*-presentation to NK cells. After coculturing of BMDCs and NK cells for 30 min, WT BMDCs induced Stat5 phosphorylation in 30% of NK cells, whereas KO BMDCs barely induced Stat5 phosphorylation in NK cells. Tg1 BMDCs poorly induced Stat5 phosphorylation, whereas KI and Tg6 BMDCs induced Stat5 phosphorylation in 18 and 26% of NK cells, respectively (Fig. 1*B*). The induction of phospho-Stat5 in NK cells by WT or KI BMDCs was inhibited by IL-2Rβ–blocking mAb, but not by IL-2–neutralizing mAb (Fig. 1*C*). These results indicate that the level of IL-15Rα on BMDCs determined the level of phospho-Stat5 in NK cells in an IL-2Rβ–dependent manner. Therefore, the *Il15ra* mutant mice display a wide range in the level of IL-15Rα, allowing us to study quantitative effects of IL-15 *trans*-presentation on NK cell development in vivo.

### *The level of IL-15* trans*-presentation by accessory cells positively regulates NK cell homeostasis in the absence of the IL-15Rα IC domain*

We first examined homeostasis of NK cells at precursor (NKp, Lin$^-$CD122$^+$NK1.1$^-$DX5$^-$), immature (iNK, CD3$^-$CD19$^-$NK1.1$^+$DX5$^-$), and mature (mNK, CD3$^-$CD19$^-$NK1.1$^+$DX5$^+$) stages in *Il15ra*$^{\Delta IC}$/*TCRa*$^{IC}$ KI and Tg mice. Because WT, KO, KI, and the two Tg mice lines harbor a similar total number of cells in the BM and spleen (Supplemental Fig. 2*B*), the percentage of NK cells in total cells reflects NK cellularity. Whereas the percentages of NKP were comparable among all mouse types examined, iNK cells and mNK cells in the BM and spleen of KO mice were significantly reduced in comparison with WT mice (Fig. 2*A*, WT versus KO). For BM iNK cells, there was no recovery in Tg1 mice, but intermediate recovery in KI mice and full recovery in Tg6 mice (Fig. 2*A*). By contrast, splenic iNK cells fully recovered in all KI and Tg mice (Fig. 2*A*). In contrast, both BM and splenic mNK showed progressive recovery in Tg1, KI, and Tg6 mice (Fig. 2*A*). These results indicate a positive correlation between the level of surface IL-15Rα and the restoration of iNK and mNK cells in the absence of the IC domain (Figs. 1*A*, 2*A*). The observation that splenic iNK cells, but not mNK cells, recovered to WT level in KI and Tg1 mice suggests that splenic iNK cells require less IL-15Rα than mNK cells to achieve normal homeostasis.

We next examined whether the IL-15Rα level on accessory cells affects homeostasis of iNK and mNK cells. To assess BM-derived accessory cells, we constructed mixed BM chimeras by transplanting CD45.1$^+$ KO BM plus CD45.2$^+$ WT, KI, or KO BM into KO recipients. This system allows evaluation of the effect of IL-15Rα provided only by CD45.2$^+$ BM-derived accessory cells. We found that the KI BM-derived accessory cells were as potent as their WT counterparts to support iNK cell homeostasis, but were less potent than the WT counterpart to support mNK cell homeostasis (Fig. 2*B*, KI + KO→KO versus WT + KO→KO). To assess radiation-resistant accessory cells, we used chimeric mice made of KO BM and WT, KI, or KO recipients. KI and WT radiation-resistant cells supported iNK homeostasis to a similar extent, whereas KI radiation-resistant cells were less potent than their WT counterparts in support of mNK cells (Fig. 2*C*, KO + KO→KI versus KO + KO→WT). Taken together, these results indicate that the IL-15Rα level on BM-derived and on radiation-resistant accessory cells positively regulates mNK homeostasis. The observation that both types of accessory cells from KI mice fully restored iNK, but not mNK cells suggests that iNK cells require a lower level of IL-15 *trans*-presentation than mNK cells to achieve normal homeostasis.

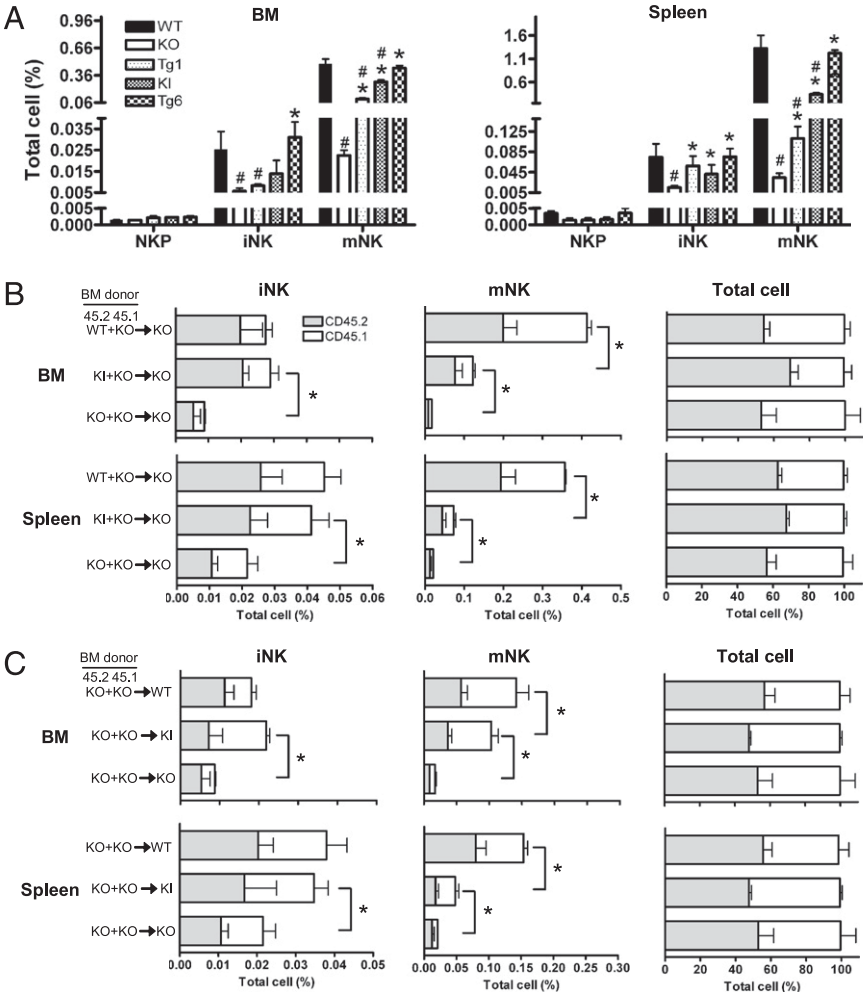


**FIGURE 2.** The level of IL-15Rα on accessory cell positively regulates NK cell homeostasis in the absence of the IL-15Rα IC domain. *A*, The composition of NKP, iNK, and mNK populations in the BM and spleen of *Il15ra* mutant mice. Lineage-negative ($Lin^-$) cells were negative for CD19, Gr-1, CD11b, CD11c, CD3, CD4, CD8, and Ter-119; $n$ = 5/group. #Significant difference between indicated *Il15ra* genotypes and WT. $p < 0.05$. *Significant difference between indicated *Il15ra* genotypes and KO. $p < 0.05$. *B* and *C*, Percentages of iNK, mNK, and $CD45^+$ cells derived from $CD45.2^+$ and $CD45.1^+$ donor BM were analyzed in the BM and spleen of the indicated BM chimera. $CD45.1^+$ and $CD45.2^+$ iNK cells were gated by $CD45.1^+CD3^-CD19^-NK1.1^+DX5^-$ and $CD45.1^-CD3^-CD19^-NK1.1^+DX5^-$, respectively. $CD45.1^+$ and $CD45.2^+$ mNK cells were gated by $CD45.1^+CD3^-CD19^-NK1.1^+DX5^+$ and $CD45.1^-CD3^-CD19^-NK1.1^+DX5^+$, respectively. $n$ = 5/group. *$p < 0.05$.

*The level of IL-15* trans*-presentation by accessory cells positively regulates mNK cell differentiation in the absence of the IL-15Rα IC domain*

The differentiation status of mNK cells can be further distinguished by the expression of CD11b and CD27. The earliest mNK cells are $CD27^+CD11b^{low}$ (stage 1), which give rise to the $CD27^+CD11b^+$ population (stage 2), and then to the most mature $CD27^{low}CD11b^+$ population (stage 3). Stage 1 and stage 2 mNK cells are present in the BM and spleen, whereas stage 3 mNK cells are present in the spleen (11, 28, 29). We found that the majority of mNK cells in KO mice are at stage 1 (Fig. 3*A*). The differentiation of mNK cells into stage 2 was fully restored in all KI and Tg mice, whereas the differentiation into stage 3 was progressively restored in Tg1, KI, and Tg6 mice (Fig. 3*A*). These results indicate a positive regulation of mNK differentiation by the level of IL-15Rα in the absence of the IC domain (Figs. 1*A*, 3*A*) and suggest that the threshold of the IL-15Rα level required for stage 1 to stage 2 mNK differentiation is lower than that for stage 2 to stage 3 mNK differentiation.

We next analyzed the role of NK cell IL-15Rα in mNK differentiation using WT + KO→WT chimera. Mature NK cells derived from either WT or KO BM showed a similar distribution of mNK subpopulations (Fig. 3*B*), indicating that IL-15Rα on NK cells is not essential for mNK differentiation.

We then examined the role of IL-15Rα level on accessory cells in mNK differentiation using mixed BM chimeras. As mNK cells derived from $CD45.1^+$ and from $CD45.2^+$ BM showed a similar distribution of CD11b and CD27 regardless of the *Il15ra* genotype (data not shown), we examined total mNK cells ($CD45.1^+$ and $CD45.2^+$) of the mixed BM chimeras to maximize the number of cells for analysis. For stage 1 to stage 2 mNK differentiation in the BM and spleen, KI accessory cells derived from either BM (Fig. 3*C*, KI + KO→KO versus WT + KO→KO) or recipient (Fig. 3*C*, KO + KO→KI versus KO + KO→WT) were less supportive than their WT counterparts. For stage 2 to stage 3 mNK differentiation in the spleen, IL-15Rα of BM-derived and radiation-resistant accessory cells are both required (Fig. 3*B*, WT + KO→WT versus Fig. 3*C*, KO + KO→WT and WT + KO→KO). The KI radiation-resistant cells were less potent than their WT counterparts (Fig. 3*C*, KO + KO→KI versus KO + KO→WT). We also found that WT radiation-resistant accessory cells were much more potent than WT BM-derived accessory cells (Fig. 3*C*, KO + KO→WT versus WT + KO→KO).

Taken together, these results indicate that IL-15Rα level on both BM-derived and radiation-resistant accessory cells positively regulates stage 1 to stage 2 mNK differentiation in the BM and spleen, and stage 2 to stage 3 mNK cell differentiation in the spleen. Moreover, IL-15Rα of radiation-resistant cells was more potent than IL-15Rα of BM-derived accessory cells in support of the latter event.

*The level of IL-15* trans*-presentation by accessory cells positively regulates acquisition of Ly49Rs in the absence of the IL-15Rα IC domain*

Acquisition of Ly49Rs occurs progressively during iNK to mNK differentiation, which equips NK cells with the capability to recognize self-MHC-I–expressing cells. Previous studies reported

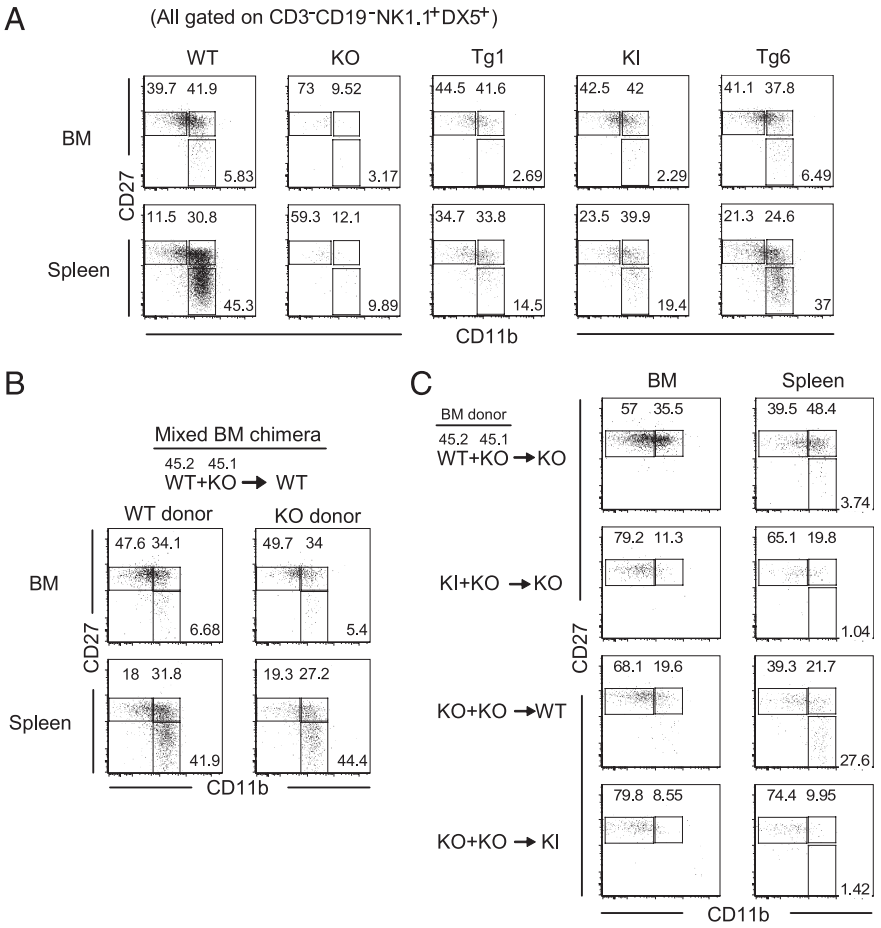


**FIGURE 3.** The level of IL-15Rα on accessory cells positively regulates mNK cell differentiation in the absence of the IL-15Rα IC domain. *A*, CD27 and CD11b expression on mNK cells ($CD3^-CD19^-NK1.1^+DX5^+$) of intact mice. *B* and *C*, CD27 and CD11b expression on mNK cells derived from donor BM cells of mixed BM chimera. mNK cells derived from WT and KO donor are gated by $CD45.1^-CD3^-CD19^-NK1.1^+DX5^+$ and $CD45.1^+CD3^-CD19^-NK1.1^+DX5^+$, respectively. $n = 5$/group; representative flow cytometric plots of five experiments are shown.

that the acquisition of Ly49Rs is defective in *Il15ra*$^{-/-}$ and *Il15*$^{-/-}$ mice, and only requires IL-15Rα of BM-derived cells (10, 30). We examined Ly49Rs acquisition by BM and splenic NK cells in *Il15ra*$^{\Delta IC}$/*TCRa*$^{IC}$ KI and Tg mice. NK cells of all mice types examined expressed a similar level of Ly49Rs (Supplemental Table I), whereas the percentage of $Ly49R^+$ cells was significantly reduced in the KO mice (Fig. 4*A*). The level of $Ly49A^+$ and $Ly49D^+$ cells recovered to WT level in all KI and Tg mice (Fig. 4*A*), whereas the level of other $Ly49R^+$ cells recovered progressively in Tg1, KI, and Tg6 mice, reaching WT level in Tg6 mice (Fig. 4*A*). These results indicate that the level of IL-15Rα positively regulates Ly49Rs acquisition by NK cells in the absence of the IL-15Rα IC domain, and suggest that the level of IL-15Rα required for acquisition of Ly49A and Ly49D is lower than that required for the acquisition of the examined Ly49Rs.

We next investigated whether the level of IL-15Rα on BM-derived accessory cells affects Ly49Rs acquisition. In order to avoid influence by IL-15Rα of NK cells, we examined Ly49Rs acquisition of $CD45.1^+$ KO NK cells in the mixed BM chimera groups. In mixed BM chimeras with WT recipient, IL-15Rα on BM-derived accessory cells was necessary for splenic NK cells to acquire each Ly49Rs examined, with the exception of Ly49A and Ly49D; however, IL-15Rα was dispensable for Ly49 acquisition by NK cells in the BM niche, except for Ly49H (Fig. 4*B*, *upper panels*). The latter result indicates that the level of IL-15Rα on radiation-resistant accessory cells was sufficient to support Ly49Rs acquisition by NK cells. In order to avoid any influence from recipient's IL-15Rα, we constructed mixed BM chimeras in KO recipients and analyzed Ly49 acquisition by $CD45.1^+$ KO NK cells (Fig. 4*B*, *lower panels*). The IL-15Rα level on BM-derived accessory cells (WT versus KI versus KO) clearly showed a positive correlation to NK Ly49Rs acquisition in the spleen as well as in the BM, except for Ly49A (Fig. 4*B*, *lower panels*).

We then examined the role of IL-15Rα on radiation-resistant accessory cells in Ly49Rs acquisition. In the presence of WT BM-derived cells, IL-15Rα on radiation-resistant accessory cells was only needed for the full acquisition of Ly49C/I and Ly49I in the spleen (Fig. 4*C*, *upper panels*). In the absence of IL-15Rα from BM-derived cells, IL-15Rα on radiation-resistant accessory cells was required for acquisition of all Ly49Rs examined (Fig. 4*C*, *lower panels*). Compared with WT radiation-resistant accessory cells, the KI counterparts exerted an intermediate level of support for all Ly49Rs, except Ly49A in both BM and spleen niches (Fig. 4*C*, *lower panels*).

Taken together, these results indicate that Ly49Rs acquisition by NK cells in the BM requires IL-15 *trans*-presentation by either BM-derived or radiation-resistant accessory cells. By contrast, Ly49Rs acquisition by NK cells in the spleen requires IL-15 *trans*-presentation by both types of accessory cells, especially the input from BM-derived accessory cells. The reduced supporting effect of KI accessory cells in comparison with that of WT accessory cells indicates that the level of IL-15 *trans*-presentation regulates Ly49Rs acquisition by NK cells. An exception is Ly49A, as KI accessory cells restored it to WT level. This observation suggests that the level of IL-15 *trans*-presentation required for Ly49A acquisition is the lowest among all Ly49Rs examined.

### *The level of IL-15* trans*-presentation by accessory cells positively regulates the acquisition of effector functions by NK cells in the absence of the IL-15Rα IC domain*

NK cells from *Rag*$^{-/-}$*Il15*$^{-/-}$ mice display reduced cytotoxicity toward YAC-1 targets and reduced IFN-γ production in response

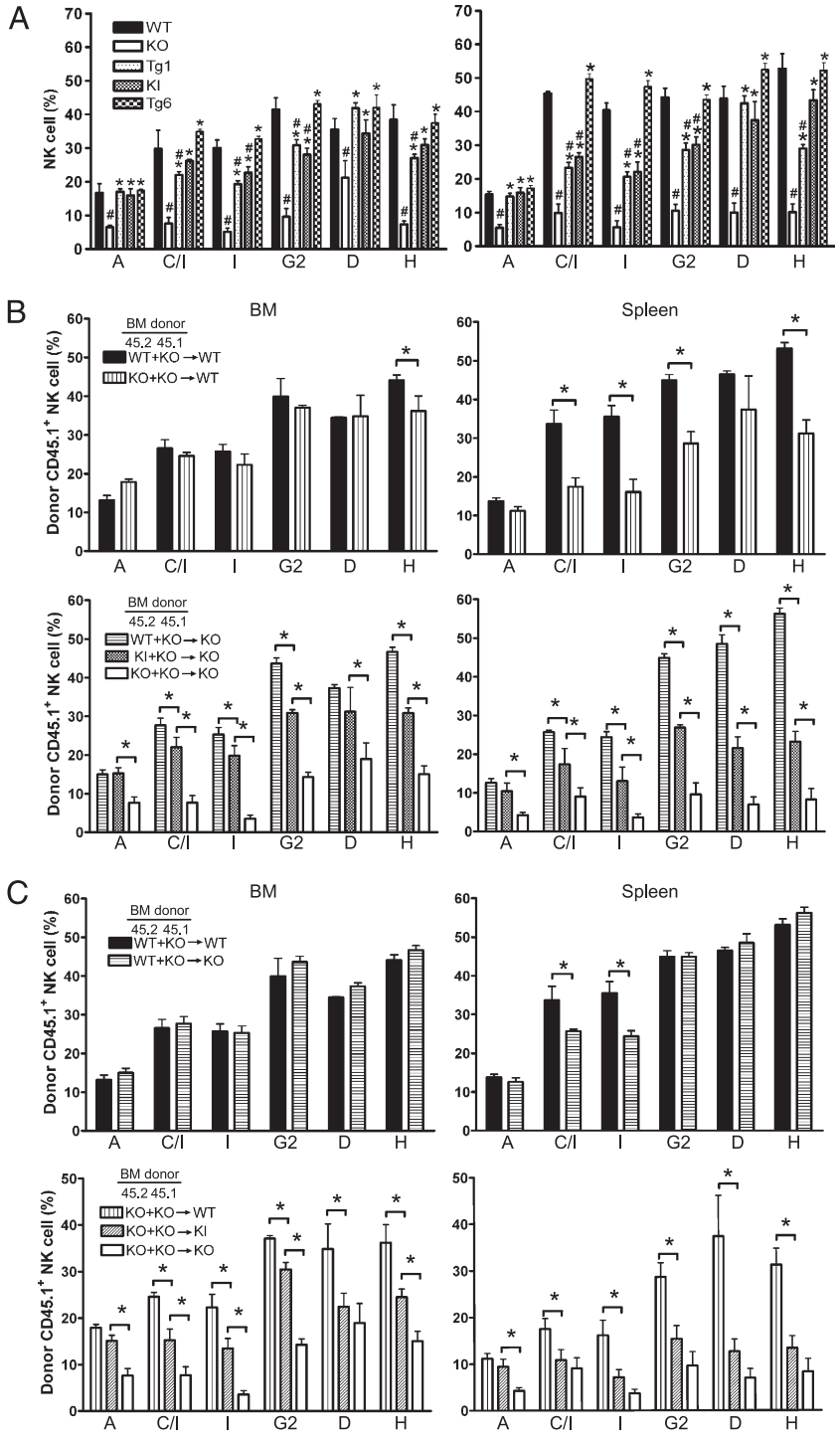


**FIGURE 4.** The level of IL-15Rα on accessory cells positively regulates Ly49Rs acquisition in the absence of the IL-15Rα IC domain. *A*, Ly49Rs expression on NK cells ($CD3^-CD19^-NK1.1^+$) of intact mice. $n = 5$/group. #Significant difference between indicated *Il15ra* genotypes and WT. $p < 0.05$. *Significant difference between indicated *Il15ra* genotypes and KO. $p < 0.05$. *B* and *C*, Ly49 receptors expression on $CD45.1^+$ NK cells ($CD45.1^+CD3^-CD19^-NK1.1^+$) of mixed BM chimera. $n = 4$/group. $*p < 0.05$.

to IL-2/IL-12 or PMA plus ionomycin stimulation (5), indicating a role of IL-15 in the acquisition of NK cell effector function. We examined the functional capacity of splenic NK cells in $Il15ra^{\Delta IC}$/$TCRa^{IC}$ KI and Tg mice ex vivo. Because isolated NK cells respond poorly to NK1.1 stimulation in vitro (Supplemental Fig. 3*A*), we examined the NK cell response in total splenocyte culture. As LPS- or CpG-activated murine DCs *trans*-present IL-15 to prime NK cells (26, 27, 31, 32), we first determined whether IL-15Rα on splenocytes affects the NK cell response in the absence of microbial stimulation. WT and KO NK cells expressed a similar level of surface NK1.1 before stimulation (Supplemental Table I). $CD45.2^+$ WT, $CD45.1^+$ KO, or WT + KO splenocytes were stimulated with immobilized anti-NK1.1 mAb or P/I. After stimulation, the percentages of IFN-$\gamma^+$ and $CD107a^+$ cells in KO NK cells in the mixed culture were similar to those in the KO culture (Supplemental Fig. 3*B*, *c* versus *d* and *g* versus *h*), whereas the percentages of IFN-$\gamma^+$ and $CD107a^+$ cells in WT NK cells in the mixed culture were similar to those in the WT culture (Supplemental Fig. 3*B*, *b* versus *a* and *f* versus *e*). These results indicate that the measured NK cell responses in total splenocytes were not affected by IL-15Rα on splenocytes. We thus used this assay to examine the functional status of NK cells developed in the KI and Tg mice. The level of surface NK1.1 on NK cells in WT, KI, and Tg mice was comparable (Supplemental Table I). The induction of IFN-$\gamma^+CD107a^+$ NK cells in response to NK1.1 triggering was reduced in the KO mice, whereas it was progres-

sively restored in Tg1, KI, and Tg6 mice (Fig. 5*A*). The level of IC IFN-γ was also restored (Fig. 5*B*). These results indicate that the level of IL-15Rα positively regulates IFN-γ production and degranulation in the absence of the IL-15Rα IC domain. The impaired response of KO NK cells to NK1.1 stimulation could be due to defects in effector function development or in NK1.1-triggered signaling. Therefore, we analyzed NK cell responses to P/I, which bypasses the NK1.1 receptor, yet activates protein

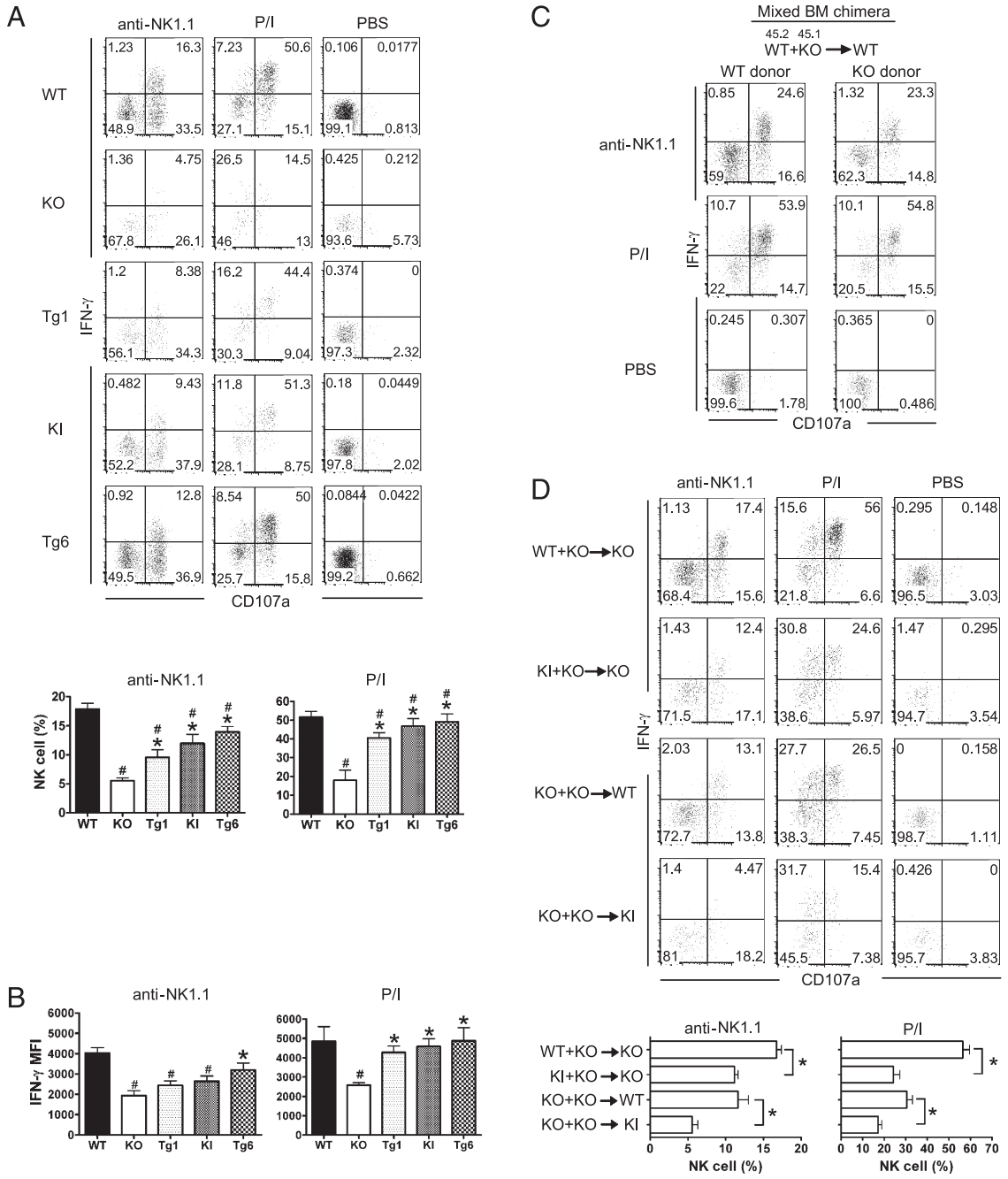


**FIGURE 5.** The level of IL-15Rα on accessory cells positively regulates the acquisition of effector function in the absence of the IL-15Rα IC domain. *A*, Degranulation and IFN-γ production in splenic NK cell from *Il15ra* mutant mice in response to anti-NK1.1 and P/I stimulation. NK cells were gated as $NK1.1^+CD3^-CD19^-$, except in anti-NK1.1 stimulation, in which they were gated as $NKP46^+CD3^-CD19^-$ cells. Numbers shown in each quadrant gate represent the frequency of $CD107a^+$ or $IFN\text{-}\gamma^+$ cells in NK cells. Representative flow cytometric plots of five experiments are shown. *Lower panels*, Bar graphs indicate percentage of $CD107a^+IFN\text{-}\gamma^+$ cell in total NK cells of indicated *Il15ra* genotype mice. #Significant difference between indicated *Il15ra* genotypes and WT. $p < 0.05$. *Significant difference between indicated *Il15ra* genotypes and KO. $p < 0.05$. *B*, MFI of IFN-γ in activated NK cells from *Il15ra* mutant mice after anti-NK1.1 and P/I stimulation; $n = 4$/group. #Significant difference between indicated *Il15ra* genotypes and WT. $p < 0.05$. *Significant difference between indicated *Il15ra* genotypes and KO. $p < 0.05$. *C*, Degranulation and IFN-γ production in splenic NK cells of indicated mixed BM chimera. In the anti-NK1.1 group, NK cells derived from WT and KO donors were gated by $CD45.2^+NKP46^+CD3^-CD19^-$ and $CD45.2^-NKP46^+CD3^-CD19^-$, respectively. In the P/I and PBS groups, NK cells derived from WT and KO donors were gated by $CD45.1^-NK1.1^+CD3^-CD19^-$ and $CD45.1^+NK1.1^+CD3^-CD19^-$, respectively. Representative dot plots of five experiments are shown. *D*, NK cells were gated as described in *A*. Representative dot plots of five experiments are shown. The bar graphs indicate percentage of $CD107a^+IFN\text{-}\gamma^+$ cells in total NK cells of indicated mixed BM chimera groups. *Significant difference between indicated BM chimera groups. $p < 0.05$.

kinase C and increases IC calcium directly. The percentage of CD107a$^+$IFN-γ$^+$ NK cells was reduced in KO NK cells, nearly restored to WT level in Tg1 mice, and fully restored in KI and Tg6 mice (Fig. 5*A*). Full restoration of IC IFN-γ level also occurred in Tg1, KI, and Tg6 mice (Fig. 5*B*). These results indicate that the level of IL-15Rα regulates the acquisition of NK cell effector function in the absence of the IC domain.

We next examined which type of cell's IL-15Rα affects the acquisition of effector function by NK cells. We analyzed WT + KO→WT chimera to assess the role of IL-15Rα on NK cells. The percentages of IFN-γ$^+$ and CD107a$^+$ cells induced by NK1.1 or P/I stimulation were similar in NK cells derived from WT and KO BM (Fig. 5*C*), indicating that IL-15Rα on NK cells is not required for the acquisition of NK cell effector function. We next analyzed the role of the IL-15Rα level on accessory cells. In the NK1.1-triggered response, the percentages of IFN-γ$^+$ and CD107a$^+$ NK cells were lower in chimeras made with KI accessory cells derived from either BM (Fig. 5*D*, KI + KO→KO versus WT + KO→KO) or recipient (KO + KO→KI versus KO + KO→WT) as compared with those in chimeras made with WT accessory cells. Similarly, reduced responses to P/I stimulation, especially with regard to induction of IFN-γ$^+$CD107a$^+$ NK cells, also occurred in chimeras made of KI accessory cells (Fig. 5*D*). Taken together, these results indicate that IL-15 *trans*-presentation level of BM-derived and radiation-resistant accessory cells positively regulates the acquisition of IFN-γ production and degranulation capacity during NK cell development.

## Discussion

IL-15 and IL-15Rα are critical for NK cell development. In this study, we generated mice expressing various levels of surface IL-15Rα in the absence of the IL-15Rα IC domain to investigate the effect of the IL-15 *trans*-presentation level on NK cell development. The level of IL-15Rα on resting and poly I:C–activated BMDCs followed the order WT > Tg6 > KI > Tg1 ≥ KO. The level of phospho-Stat5 in NK cells induced by the various types of BMDCs also followed the same order as the level of surface IL-15Rα. Although Tg1 cells expressed a very low level of surface IL-15Rα and induced Stat5 phosphorylation poorly in NK cells, they still supported certain NK cell developmental events in vivo, such as restoring Ly49A and D acquisition and P/I-stimulated IFN-γ production to WT level. In contrast, the level of IL-15Rα in Tg6 cells was sufficient to restore NK cell homeostasis, Ly49C/I, G2 and H acquisition, and mNK cell differentiation to WT level (Table I). These results indicate that different NK cell developmental events require different levels of IL-15 *trans*-presentation.

Splenic iNK cells defined as CD3$^-$CD19$^-$NK1.1$^+$DX5$^-$ most likely contain the NKR-expressing LTi cells (NKR-LTi) (33–37). Because there is no surface marker to identify splenic NKR-LTi cells, except using the RORγt fate map mice (36, 37), we could not readily distinguish them from iNK cells in the spleen. However, if the splenic NKR-LTi cells are IL-15 independent as the intestinal NKR-LTi cells (34, 35), we think that their presence would not mask the relationship between the IL-15 system and iNK cells, because their numbers in the spleen of various *Il15ra* mutant mice shall be the same.

Levels of two other molecules are known to quantitatively affect NK cell development. One is the input from Ly49 inhibitory receptors, which positively regulates NK cell functional responsiveness (18, 38). It has been proposed that the threshold of inhibitory input required for degranulation is lower than that for IFN-γ production (19). Another is the amount of $\gamma_c$ on NK cells, which correlates with NK cell homeostasis and acquisition of Ly49D (20, 21). In the current study, we demonstrate that the level of IL-15Rα on accessory cells positively regulates multiple NK cell developmental events. Together, these studies strengthen the view that not only the presence, but also the level of a relevant regulatory protein is critical for determining the outcome of a NK cell development process. This concept of protein threshold most likely applies to the regulation of other biological processes.

The requirement for functional maturation of NK cells is not fully understood. A recent study demonstrated that the abilities of NK cell IFN-γ production and degranulation are positively regulated by the strength of interaction between MHC class I molecules and the corresponding inhibitory Ly49Rs (18, 38). In this study, we found that the IL-15 system affects the Ly49R repertoire, but not the level of Ly49R (Supplemental Table 1) or MHC-I (data not shown). Moreover, the percentage of IFN-γ$^+$ NK cells was reduced in both of Ly49C/I$^+$ and Ly49C/I$^-$ KO NK subsets in response to NK1.1 or P/I stimulation (data not shown). These observations suggest that the regulation of the acquisition of NK cell effector function by IL-15Rα is independent of MHC-I/Ly49 interaction. Therefore, in addition to the input from inhibitory Ly49R, IL-15 signaling is also required for the developing NK cells to acquire effector function.

NK cell development is dependent on IL-15Rα of accessory cells, but not of NK cells. Previous studies using BM chimeras

Table I. Summary of NK cell developmental events in *Il15ra* mutant mice

| | Mice | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | KO | | Tg1 | | KI | | Tg6[a] |
| Surface IL-15Rα level | KO | ≤ | Tg1 | < | KI | < | Tg6 |
| Homeostasis[b] | − | | + | | ++ | | ++++ |
| Ly49 acquisition[c] | | | | | | | |
| Ly49A, D | − | | ++++ | | ++++ | | ++++ |
| Ly49C/I, G2, H | − | | ++ | | ++ | | ++++ |
| mNK differentiation[d] | + | | +++ | | +++ | | ++++ |
| IFN-γ production[e] | | | | | | | |
| Anti-NK1.1 | + | | ++ | | ++ | | +++ |
| P/I | ++ | | ++++ | | ++++ | | ++++ |

[a]The MFI of IL-15Rα on Tg6 BMDC is 50% of that on WT cells.
[b]The percentage of total splenic NK cells, as previously described.
[c]The percentage of indicated Ly49 expression in total splenic NK cells, as previously described.
[d]The percentage of mNK cells in total splenic NK cells was analyzed.
[e]The percentage of IFN-γ$^+$ NK cell in total splenic NK cells after anti-NK1.1 or P/I stimulation, as previously described.
−, Below 25% of WT level; +, 25–50% of WT level; ++, 50–70% of WT level; +++, 70–90% of WT level; ++++, 90–100% of WT level.

Table II. The role of accessory cell IL-15Rα in NK cell development

| | BM-Derived Cell | Radiation-Resistant Cell |
|---|---|---|
| Homeostasis[a] | + | + |
| Ly49 receptor acquisition[a] | + | + |
| mNK | | |
| Stage 1 | + | + |
| Stage 2 | + | + |
| Stage 3[b] | + | + |
| IFN-γ production | | |
| Anti-NK1.1 | + | + |
| P/I | + | + |

[a]The supportive effect provided by IL-15Rα of BM-derived accessory cells is more dominant than that provided by IL-15Rα of radiation-resistant accessory cells.
[b]The supportive effect provided by a single type of accessory cells is greater for radiation-resistant accessory cells than for BM-derived accessory cells.
+, IL-15Rα expressed by these accessory cells has supportive effects.

demonstrated that IL-15Rα of NK cell is dispensable for NK cell homeostasis and Ly49 acquisition (7, 11). In this study, we found that IL-15Rα of NK cells is dispensable for mNK cell differentiation and effector function acquisition. We also found that all NK cell developmental events examined were quantitatively regulated by the level of IL-15Rα on accessory cells. Previous conditional KO mice study indicates that IL-15Rα expressed by $CD11c^+$ cells or macrophages is required for the progression of mNK cells to stage 3 ($CD3^-NK1.1^+CD27^{low}CD11b^+$) (39). However, the role of IL-15Rα expression by radiation-resistant cells in mNK cell differentiation is not clear. In this study, we demonstrate that IL-15Rα of BM-derived and radiation-resistant accessory cells are both required to support stage 2 to stage 3 mNK cell differentiation in the spleen (Table II). We also found that the potency in supporting stage 2 to stage 3 mNK cell differentiation by a single type of accessory cells is greater for radiation-resistant than for BM-derived accessory cells. A recent study demonstrated that IL-15Rα expressed by radiation-resistant cells is dispensable with regard to Ly49Rs acquisition (10). However, we found that IL-15Rα of both BM-derived and radiation-resistant accessory cells is required (Table II). The reason for this difference is not clear.

In summary, this study provides new information pertaining to the function of IL-15 for NK cell development. First, NK cell homeostasis, mNK differentiation, and acquisition of Ly49Rs and effector functions during NK cell development are quantitatively regulated by the IL-15 *trans*-presentation level. Secondly, the IL-15Rα IC domain of accessory cells affects the level of surface IL-15Rα and subsequently the level of IL-15 *trans*-presentation received by NK cells. Lastly, IL-15Rα of radiation-resistant accessory cells is required for the acquisition of Ly49Rs and effector function, and is more potent than IL-15Rα of BM-derived accessory cells in support of stage 2 to stage 3 mNK cell differentiation.

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## Disclosures

The authors have no financial conflicts of interest.

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# 聲明書

本人李爾博請毛馨儀、黃婉真技佐，協助進行小鼠剪尾巴實驗、動物安樂死、取小鼠腦和脾臟。由本人負責指導實驗步驟，使毛馨儀、黃婉真具備執行操作該實驗能力，並本人為此人員之實驗操作技術背書。

李爾博 2017.12.22

# 聲明書

本人李爾博請毛馨儀、黃婉真技佐，協助進行小鼠剪尾巴實驗、動物安樂死、取小鼠腦和脾臟。由本人負責指導實驗步驟，使毛馨儀、黃婉真具備執行操作該實驗能力，並本人為此人員之實驗操作技術背書。

李爾博 2017.12.22

正 本

實驗動物中心 收發文號第 1051659Z 號 105年 11月 23日 18 時 單位自存，請妥善保管

檔　　號：
保存年限：

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受文者：臺北醫學大學

發文日期：中華民國105年11月22日
發文字號：動保管字第10532552000號
速別：普通件
密等及解密條件或保密期限：
附件：如主旨

裝

主旨：檢送105年度動物科學應用機構實地查核評比結果表、綜合評述意見及原函影本各1份，請查照惠辦。

說明：依據行政院農業委員會105年11月18日農牧字第1050043599號函辦理。

訂

正本：荷商台醫股份有限公司台灣分公司、衛生福利部國家中醫藥研究所、財團法人臺灣基督長老教會馬偕紀念社會事業基金會馬偕紀念醫院、國立臺灣師範大學、新光醫療財團法人新光吳火獅紀念醫院、臺北醫學大學、臺北市立大學、太景生物科技股份有限公司、中國文化大學、中央研究院、振興醫療財團法人振興醫院、衛生福利部食品藥物管理署、亞諾法生技股份有限公司、臺北市立萬芳醫院、實踐大學、台灣微脂體股份有限公司、瑞寶基因股份有限公司、瑞德生物科技有限公司、柏登生醫股份有限公司、祥圃實業股份有限公司
副本：行政院農業委員會

線

處長 嚴一峯

本案依分層負責規定授權業務主管決行

檔　　號：
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裝

主旨：檢送105年度動物科學應用機構實地查核評比結果表、貴轄機構查核結果及綜合評述意見各1份，請查照惠辦。

說明：

訂

一、有關105年度81家受查動物科學應用機構及查核評比結果表(如附件1)、查核結果及綜合評述意見(如附件2)，請貴單位於105年11月30日前轉知所轄受查機構請其依查核小組評述意見參考改進。另前項各機構評述意見為機構內部資料，請分案發送。

二、105年實地查核經評核「較差」之6家動物科學應用機構查核結果請貴單位確實督導3個月內提送書面改善資料，並進行複查後核轉本會。

線

三、另105年度查核評比結果，亦將於105年11月底前公告於動物保護資訊網(http://animal.coa.gov.tw/html/)實驗動物項下，請自行參閱。

正本：基隆市動物保護防疫所、臺北市動物保護處、新北市政府動物保護防疫處、桃園市政府動物保護防疫處、臺中市動物保護防疫處、臺南市動物防疫保護處、高雄市動物保護處、新竹縣家畜疾病防治所、新竹市動物保護及防疫所、苗栗縣動物保護防疫所、彰化縣動物防疫所、雲林縣動植物防疫所、嘉義縣家畜疾病防治所、嘉義市政府建設處、屏東縣家畜疾病防治所、宜蘭縣動植物防疫所、花蓮縣動植物防疫所、澎湖縣家畜疾病防治所

副本：科技部、衛生福利部、教育部、經濟部、行政院環境保護署、本會畜牧處、本會動植物防



2016/11/18
CYAA10532552000

# 105年度81家受查機構及查核結果一覽表-依受查機構所屬(縣)市排序

| 序號 | 動物保護主管機關 | 編號 | 機構名稱 | 優 | 良 | 尚可 | 較差 | 動物房舍所屬縣(市) |
|---|---|---|---|---|---|---|---|---|
| 1 | 基隆市動物保護防疫所 | 006 | 國立臺灣海洋大學 | | | ● | | 基隆市 |
| 2 | 新北市政府動物保護防疫處 | 071 | 財團法人亞東紀念醫院 | | ● | | | 新北市 |
| 3 | | 128 | 瑞立化學製藥股份有限公司 | | ● | | | 新北市 |
| 4 | | 152 | 輔仁大學學校財團法人輔仁大學 | | ● | | | 新北市 |
| 5 | | 232 | 馬偕學校財團法人馬偕醫學院 | ● | | | | 新北市 |
| 6 | | 234 | 濁水溪生物科技股份有限公司 | | | ● | | 桃園市 |
| 7 | 臺北市動物保護處 | 013 | 荷商台醫股份有限公司台灣分公司 | | | ● | | 臺北市 |
| 8 | | 020 | 衛生福利部國家中醫藥研究所 | | | | ● | 臺北市 |
| 9 | | 041 | 財團法人臺灣基督長老教會馬偕紀念社會事業基金會馬偕紀念醫院 | | ● | | | 新北市 |
| 10 | | 043 | 國立臺灣師範大學 | | | | ● | 臺北市 |
| 11 | | 046 | 新光醫療財團法人新光吳火獅紀念醫院 | | | ● | | 臺北市 |
| 12 | | 051 | 臺北醫學大學 | | | ● | | 臺北市 |
| 13 | | 052 | 臺北市立大學 | | | | ● | 臺北市 |
| 14 | | 075 | 太景生物科技股份有限公司 | | ● | | | 臺北市 |
| 15 | | 087 | 中國文化大學 | | | ● | | 臺北市 |
| 16 | | 101 | 中央研究院 | | | ● | | 臺北市 |
| 17 | | 111 | 振興醫療財團法人振興醫院 | ● | | | | 臺北市 |
| 18 | | 165 | 衛生福利部食品藥物管理署 | | ● | | | 臺北市 |
| 19 | | 166 | 亞諾法生技股份有限公司 | | | ● | | 桃園市 |
| 20 | | 169 | 臺北市立萬芳醫院-委託財團法人臺北醫學大學辦理 | | ● | | | 臺北市 |
| 21 | | 176 | 實踐大學 | | | ● | | 臺北市 |
| 22 | | 177 | 台灣微脂體股份有限公司 | ● | | | | 臺北市 |
| 23 | | 237 | 瑞寶基因股份有限公司 | | ● | | | 桃園市<br>屏東縣 |
| 24 | | 247 | 瑞德生物科技有限公司 | | | ● | | 新竹縣 |
| 25 | | 266 | 柏登生醫股份有限公司 | | | ● | | 苗栗縣 |
| 26 | | 270 | 祥圃實業股份有限公司 | | | ● | | 苗栗縣<br>雲林縣 |