IL-2 産生を促進し、Treg 細胞の増殖を誘導し、その Treg 細胞を介して気道炎症の抑制に関わることが明らかになった。

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Ｇ．知的財産権の出願・登録状況

１．特許取得

なし

２．実用新案登録

なし

３．その他

なし

厚生労働科学研究費補助金（免疫アレルギー疾患予防・治療研究事業）
分担研究報告書
新しい制御性T細胞体外増幅法による喘息治療法の開発
分担研究項目：Treg細胞の増幅誘導抗体の探索

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研究要旨：マスト細胞と$CD4^+$T細胞をIL-33の存在下で共培養を行うことにより、$CD4^+CD25^+Foxp3^+$ Treg細胞を選択的に増幅させることができる。そこで、マスト細胞と$CD4^+$T細胞の共培養により、選択的かつ効率的にTreg細胞を誘導するようなIL-33以外の因子の探索や培養条件の検討を行うことを目的とし、大腸菌由来のリコンビナントIL-33の代替として、IL-33受容体に対する刺激抗体の探索を試みた。その結果、入手しえた抗IL-33受容体抗体には、ヒトおよびマウスのマスト細胞を刺激しうるものはなく、リコンビナントIL-33の代替として使用できないことが明らかになった。

A．研究目的
当研究班では、これまでにin vitro培養系で制御性T細胞を選択的に効率良く増幅する方法として、IL-33の存在下で、T細胞とマスト細胞を共培養する系を確立した。培養に使用するIL-33は、ヒトIL-33遺伝子を導入した組換え体大腸菌から精製する。その過程で組換え体IL-33に混入する大腸菌由来の異物は、本培養系で増幅した制御性T細胞を生体に戻す際の弊害となりうる可能性が懸念される。そのため、大腸菌由来ではないIL-33に代替しうるものが必要になる。そこで、代替候補として、IL-33受容体に対する刺激抗体に注目し、そのような抗体をスクリーニングすることを目的とする。

B．方法
IL-33受容体に対する入手可能な抗体をマウスおよびヒトのマスト細胞に作用させ、マスト細胞からのサイトカイン産生を促進できるような刺激抗体を探す。マウスのIL-33受容体に対する抗体は、従来評価した市販のモノクローナル抗体３種（clone 245707, 245714及びDJ8）に加え、ドイツの研究者が作成したモノクローナル抗体１種（3E10）および市販のポリクローナル抗体１種を使用した。ヒトIL-33受容体に対する抗体は、市販のモノクローナル抗体３種（clone 97203, D14Lおよび14J07）を使用した。

C．結果
ドイツの研究者が作成した抗IL-33受容体抗体3E10は、IL-33受容体を発現するTh2細胞を活性化し、サイトカイン産生を増強できる刺激抗体と報告されていたものであったが、マスト細胞に対しては、マウスマスト細胞からIL-6及びIL-13産生を誘導しなかった。同様に、他のマウスIL-33受容体に対するモノクローナル抗体３種およびポリクローナル抗体１種、ヒトIL-33受容体に対するモノクローナル抗体３種についても、マウスおよびヒトマスト細胞を活性化するには至らなかった。ラット由来の受容体抗体3E10によるTh2細胞の活性化には、3E10を細胞に添加後、抗ラットIgG抗体をさらに加えて、3E10を架橋することで効率が上がることが報告されている。同様に、マウスおよびヒトマスト細胞においても、各種抗IL-33受容体抗体に対する二次抗体を添加し、架橋による影響の評価をおこなったが、マスト細胞の活性化を誘導する条件は見いだせなかった。

D．考察
入手しえた抗IL-33受容体抗体には、ヒトおよびマウスのマスト細胞を刺激しうるものはなく、この目的のための刺激抗体を新たに作製する、もしくは、組換え体IL-33を大腸菌で作製するのではなく、哺乳動物細胞株を使用して調製することにより、本課題の問題点である大腸菌由来の異物の混入を防ぐ方法といえる。

E．結論
使用した抗IL-33受容体抗体には、組換え体IL-33に代替できるようなマスト細胞を刺激できる至適な活性をもつものは現時点では見つからなかった。

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Ｇ．知的財産権の出願・登録状況
１．特許取得
なし
２．実用新案登録
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厚生労働科学研究費補助金（免疫アレルギー疾患予防・治療研究事業）
分担研究報告書
新しい制御性T細胞体外増幅法による喘息治療法の開発
分担研究項目：マスト細胞によるTreg細胞増幅誘導機構の解明

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研究要旨：マウスにIL-33を点鼻により吸入させると、好酸球の強い浸潤を伴う気道炎症が誘導される。その際、肺の所属リンパ節では、Treg細胞が顕著に増加し、また、このTreg細胞がIL-33依存的な気道炎症の抑制に重要であることも明らかになっている。そこで本研究では、IL-33を吸入させたマウスにおける所属リンパ節のTreg細胞数の変動と肺胞洗浄液中のTreg細胞数および炎症の程度との間に相関性が見られるかどうかを明らかにすることを目的とした。その結果、PBSを吸入させたマウスの所属リンパ節および肺胞洗浄液中においてもFoxP3陽性のTreg細胞が検出されたが、IL-33の吸入後には、肺内での好酸球の浸潤とともに、所属リンパ節と肺胞洗浄液内のTreg細胞数は増加することが明らかになった。

A．研究目的

マウスにIL-33を点鼻により吸入させると、好酸球の強い浸潤を伴う気道炎症が誘導される。その際、肺の所属リンパ節では、Treg細胞が顕著に増えることをこれまでに示してきた。一方、マスト細胞欠損マウスでは、IL-33吸入後、野生型マウスよりも、肺所属リンパ節でのTreg細胞が著名に少ないため、気道炎症が重症化することが示唆されていた。実際に、IL-33の存在下でT細胞とマスト細胞を共培養することで誘導したTreg細胞をマスト細胞欠損マウスにあらかじめ移入しておくことで、マスト細胞欠損マウスで観察されるIL-33による気道炎症の重症化を防ぐことができる。しかしながら、マスト細胞は通常、リンパ節では稀少であり、リンパ節ではなく、肺の炎症局所でTreg誘導に関わっている可能性が高い。しかしながら、IL-33吸入後の肺胞洗浄液中のリンパ球数は好酸球数に比べて遥かに少なく、従来の細胞内分子染色方法によるFoxp3陽性Treg細胞の検出は困難であった。そこで、Foxp3発現とともにGFPを発現するレポーターマウス（Foxp3/eGFPマウス）にIL-33を吸入させ、肺胞洗浄液中のFoxp3陽性Treg細胞数の変化と、肺の所属リンパ節でのTreg細胞数との相関を評価をする。

B．方法

Foxp3/eGFPマウスにPBSおよびIL-33を一日一回、三日間連続吸入し、四日目に肺胞洗浄液と肺の所属リンパ節細胞を回収した。肺胞洗浄液内、および、肺の所属リンパ節細胞中の$CD4^+CD25^+Foxp3^+$ Treg細胞の割合をEGFPの発現量を指標としてフローサイトメトリーによって解析した。

C．結果

PBSを吸入したFoxp3/eGFPマウスの肺胞洗浄液中においてもEGFP陽性Treg細胞が検出されたが、IL-33を吸入させると、PBS吸入群に比べて、肺胞洗浄液中と肺の所属リンパ節でEGFP陽性Treg細胞数が増えていることがわかった。

D．考察

IL-33吸入後、肺胞洗浄液中でのTreg細胞数の増加がみられ、マスト細胞は肺の炎症局所でのTreg細胞の増殖誘導に関わっている可能性が示唆された。その直接的な証明は、マスト細胞欠損マウスにFoxp3/eGFPマウスを交配して得られるマスト細胞欠損 Foxp3/eGFP レポーターマウスにIL-33を吸入させ、野生型Foxp3/eGFPマウスと比較検討する必要がある。

E．結論

IL-33吸入後、肺所属リンパ節細胞内のTreg細胞の変動は、肺所属リンパ節細胞内のTreg細胞の変動と相関することが明らかになった。

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G．知的財産権の出願・登録状況

１．特許取得

なし

２．実用新案登録

なし

３．その他

なし

Ⅳ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表　平成 23 年度(2011)

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Ⅴ. 研究成果の刊行物・別刷

（主なもの）

Paracrine IL-33 Stimulation Enhances Lipopolysaccharide-Mediated Macrophage Activation

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Abstract

Background: IL-33, a member of the IL-1 family of cytokines, provokes Th2-type inflammation accompanied by accumulation of eosinophils through IL-33R, which consists of ST2 and IL-1RAcP. We previously demonstrated that macrophages produce IL-33 in response to LPS. Some immune responses were shown to differ between ST2-deficient mice and soluble ST2-Fc fusion protein-treated mice. Even in anti-ST2 antibody (Ab)-treated mice, the phenotypes differed between distinct Ab clones, because the characterization of such Abs (i.e., depletion, agonistic or blocking Abs) was unclear in some cases.

Methodology/Principal Findings: To elucidate the precise role of IL-33, we newly generated neutralizing monoclonal Abs for IL-33. Exogenous IL-33 potentiated LPS-mediated cytokine production by macrophages. That LPS-mediated cytokine production by macrophages was suppressed by inhibition of endogenous IL-33 by the anti-IL-33 neutralizing mAbs.

Conclusions/Significance: Our findings suggest that LPS-mediated macrophage activation is accelerated by macrophage-derived paracrine IL-33 stimulation.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

IL-33 (also called IL-1F11, DVS27 and NF-HEV), which is a member of the IL-1 family of cytokines that includes IL-1 and IL-18, was identified as a ligand for ST2 (also called T1, DER-4, Fit-1 and IL-1R4) [1,2,3,4]. IL-33 is considered to be a cytokine that potently induces production of such Th2-cytokines as IL-5 and IL-13 by ST2-expressing immune cells such as Th2 cells [1,5,6], mast cells [7,8,9,10,11], eosinophils [6,12,13], basophils [12,13,14] and macrophages [15,16], and by stem-cell-like cells such as $CD34^+$ hematopoietic stem cells [17], natural helper cells [18] and nuocytes [19]. IL-33 is thereby thought to contribute to the development of Th2-cytokine-associated immune responses, including host defense against nematode infection and allergic diseases [2,3,4].

Indeed, administration of IL-33 to mice resulted in increased serum levels of Th2-cytokines such as IL-4, IL-5 and IL-13, as well as IgG1 and IgE, and development of inflammation accompanied by accumulation of eosinophils in the lung and gut [1]. Moreover, polymorphism of the ST2 and/or IL-33 genes was found in patients with asthma [20,21,22], atopic dermatitis [23], rhinitis [24] and rhinosinusitis [25]. The mRNA and/or protein levels of ST2, soluble ST2, which acts as a decoy receptor for IL-33, and IL-33 are increased in specimens from patients with allergic diseases such as asthma [26,27,28,29,30,31], conjunctivitis [31], rhinitis [24] and atopic dermatitis [32]. Therefore, these observations strongly suggest the importance of IL-33 and ST2 for the development of Th2-cytokine-associated allergic disorders.

However, based on the results of a study using mice treated with anti-ST2 Ab or soluble ST2-Fc fusion proteins and/or deficient in ST2, the roles of IL-33 and ST2 in the pathogenesis of certain immune diseases, including allergic airway inflammation, remain controversial [4]. Studies using ST2-deficient mice found that ovalbumin (OVA)-induced airway inflammation developed nor-

mally in ST2-deficient mice sensitized twice with OVA emulsified with alum [33,34,35], whereas it was attenuated in the case of a single sensitization [35]. On the other hand, mice treated with anti-ST2 mAb clone "3E10," which induced Th2 cell activation as an agonistic Ab, at least *in vitro* [36], without depleting ST2-expressing cells *in vivo* [37], and mice treated with soluble ST2 showed reduced development of OVA-induced airway inflammation, even though they were sensitized twice with OVA with alum [38,39]. Unlike in ST2-deficient mice [33,34,35], the development of OVA-induced airway inflammation was aggravated in mice injected with ST2-deficient OVA-specific TCR (DO11.10)-expressing Th2 cells in comparison with those injected with wild-type DO11.10 Th2 cells after OVA challenge [34]. That finding suggests that ST2 plays a negative role in Th2 cells, at least in that setting. On the other hand, it was shown that administration of anti-ST2 mAb "3E10" and soluble ST2-Fc fusion proteins to mice injected with DO11.10 Th2 cells resulted in attenuation of OVA-induced airway inflammation [38,40]. These seemingly contradictory observations could be explained on the basis of different roles for IL-33 and ST2 in distinct ST2-expressing cells. In support of that concept, IL-33 is able to enhance IFN-γ production by NK cells and iNKT cells [26], which are also involved in the pathogenesis of allergic airway inflammation [41,42]. Therefore, the precise roles of IL-33 and ST2 in different types of cells need to be elucidated.

We and others have demonstrated that IL-33 is able to enhance cytokine secretion by mast cells [7,9] and macrophages [43]. We also reported that both mast cells and macrophages can produce IL-33 after stimulation with IgE and LPS, respectively [44]. These observations suggest that IL-33 may be involved in the activation of these cells by autocrine/paracrine IL-33 release after such stimulation. In the present study, we used newly generated anti-IL-33 mAbs and demonstrated that activation of macrophages, but not mast cells, was modulated by paracrine IL-33 stimulation.

Materials and Methods

Mice

BALB/cA (BALB) mice, C57BL/6J (B6J) mice and C57BL/6N (B6N) mice were purchased from CLEA Japan and Sankyo Lab, respectively. B6J-TLR4$^{-/-}$ mice [45] and BALB-ST2$^{-/-}$ mice [46] were kindly provided by Drs. Tsuneyasu Kaisho (RIKEN, Japan) and Andrew N.J. McKenzie (MRC, Cambridge, UK), respectively. B6J-TRAF6$^{-/-}$ mice [47] and B6N-IL-33$^{-/-}$ mice [48] were generated as described elsewhere. All mice were housed under specific-pathogen-free conditions in our institutes (National Research Institute for Child Health and Development or The Institute of Medical Science, The University of Tokyo), and the animal protocols were approved by the Institutional Review Board of the National Research Institute for Child Health and Development (#06-10) and The Institute of Medical Science, The University of Tokyo (#A09-10).

Anti-mouse ST2 Abs

Anti-mouse ST2 mAb (clone 3E10) had been generated as described elsewhere [40]. FITC-conjugated and non-conjugated anti-mouse ST2 mAbs (clones DJ8 [49,50], 245707 and 245714) were obtained from MD Bioscience and R&D Systems, respectively.

Anti-IL-33 Abs

Anti-human/mouse IL-33 mAb (Nessy-1, Alexis), anti-mouse IL-33 mAb (518017, R&D Systems) and anti-mouse IL-33 polyAb (AF3626, R&D Systems) were used.

Generation of anti-mouse IL-33 mAbs

Anti-mouse IL-33 mAbs were generated and provided by Medical & Biological Laboratories Co., Ltd. (Nagano, Japan). cDNA encoding the mouse IL-33 corresponding to amino acids 109–266 was expressed in *E. coli* as an N-terminal tagged fusion protein. After purification of the fusion protein, the tagged sequence was cleaved enzymatically and removed by affinity purification. Five-week-old female C3H mice (Japan SLC, Hamamatsu) were immunized with the purified protein emulsified with Freund's complete adjuvant (Sigma-Aldrich) by injection into the footpads 5 times at 1-week intervals]. Three days after the final immunization, cells from the lymph nodes of the immunized mice were fused with P3-U1 mouse myeloma cells in the presence of 50% (w/v) polyethylene glycol (PEG4000) (Wako). Hybridomas were screened by ELISA and immunoblotting to identify those generating mAbs. Positive clones were subcloned two times by limiting dilution and rescreened by ELISA and immunoblotting. The mAbs were purified from the culture supernatant using Protein A-Sepharose (GE Healthcare). The eluted antibodies were analyzed by SDS-PAGE.

Bone marrow cell-derived and fetal liver cell-derived cultured mast cells

Mouse femoral bone marrow cell-derived cultured mast cells (BMCMCs) were generated as described elsewhere [7]. For generation of fetal liver cell-derived cultured mast cells (FLCMCs), livers were harvested from newborn TRAF6$^{+/+}$ and TRAF6$^{-/-}$ mice, and liver single-cell suspensions were prepared by grinding the tissues through a 70-μm nylon cell strainer (BD Falcon) with the plunger of a 5-ml disposable syringe. Bone marrow cells and fetal liver cells were cultured in the presence of 10 ng/ml rmIL-3 (PeproTech) for 6–8 weeks, at which time flow cytometry showed the cells to be a >98% c-kit$^+$ FcεRIα$^+$ population. Before using the cells, rmIL-3 was removed by washing. MCs (2×10^5 cells/well in 96-well flat-bottom plates) were cultured with 1 μg/ml IgE (SPE-7, Sigma), 30 or 100 ng/ml rmIL-33 (R&D Systems) and a combination of 1 μg/ml SPE-7 plus 100 ng/ml rmIL-33 in the presence and absence of 40 or 80 μg/ml anti-mouse ST2 mAb, anti-IL-33 Ab or isotype-matched control IgG for 24 h.

Thioglycolate (TGC)-induced macrophages

For collection of thioglycolate (TGC)-induced mouse peritoneal macrophages (TGC-macrophages), mice were injected intraperitoneally with 5 ml of 2% TGC (Nissui). Three days later, peritoneal exudate cells (PECs) were collected. TGC-macrophages (2×10^5 cells/well in 96-well flat-bottom plates) were incubated with 0–100 ng/ml LPS (*Salmonella enterica* serotype typhimurium; SIGMA) in the presence and absence of 40 μg/ml anti-ST2 mAb, anti-IL-33 mAb or isotype-matched control IgG for 24 or 48 h.

Flow cytometry

BMCMCs were incubated with anti-CD16/CD32 mAb (93, eBioscience; or 2.4G2, BD Biosciences) for 15 min on ice. The cells were then incubated with PE-conjugated anti-mouse FcεRIα (MAR-1, eBioscience), APC-conjugated anti-mouse c-Kit (2B8, eBioscience) and FITC-conjugated or non-conjugated anti-mouse ST2 mAb (DJ8, 3E10, 245707 or 245714) for 45 min on ice. After washing, the cells were incubated with mFITC-conjugated anti-rat IgG2b (RG7/11.1, BD Biosciences) or anti-rat IgG2a (RG7/1.30, BD Biosciences) as the second antibody for non-conjugated anti-mouse ST2 mAbs for 45 min on ice. The expression of ST2 on 7-amino actinomycin D-negative FcεRIα$^+$ c-Kit$^+$ BMCMCs was

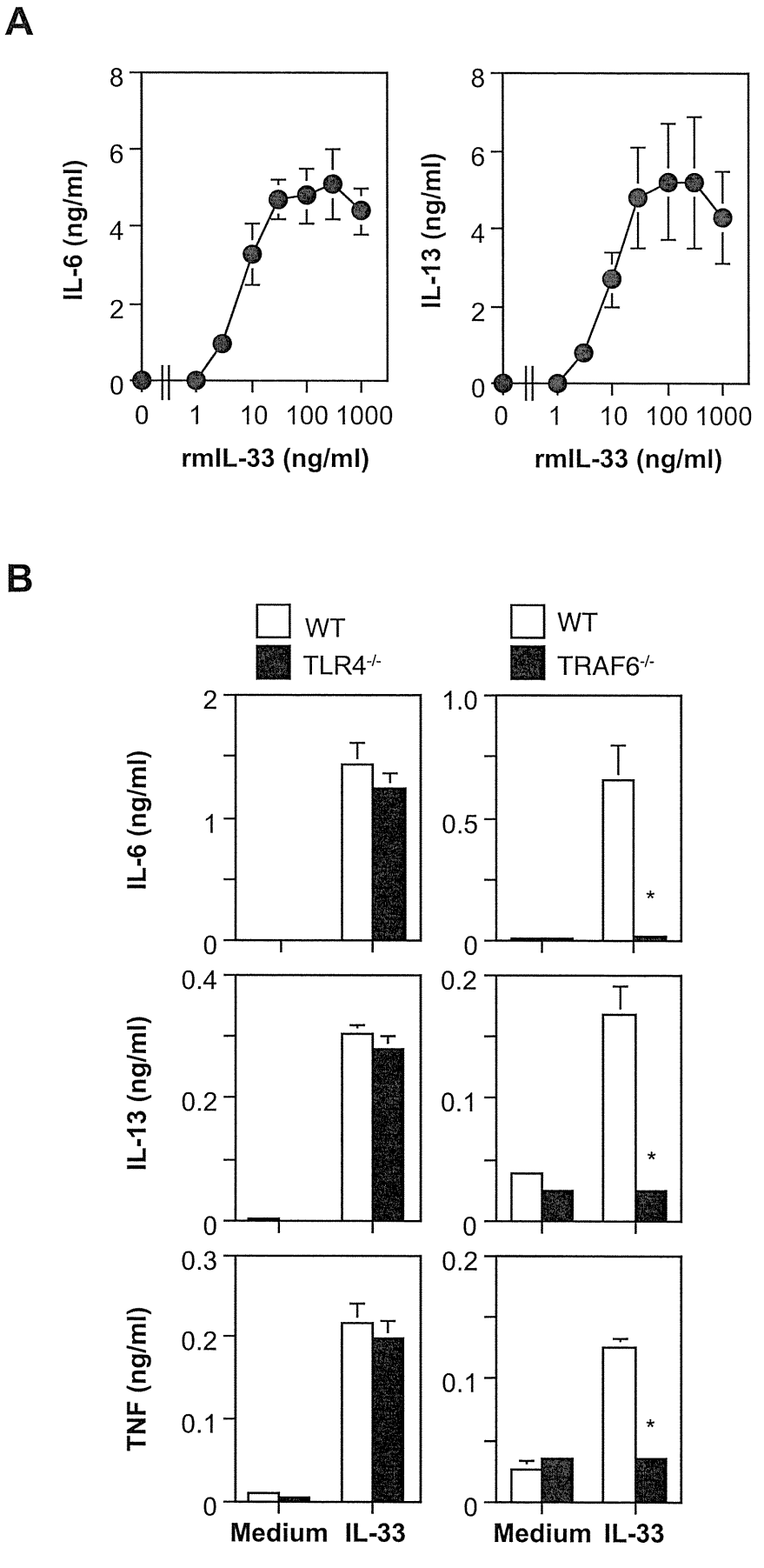
A
IL-6 (ng/ml)
rmIL-33 (ng/ml)
IL-13 (ng/ml)
rmIL-33 (ng/ml)
B
WT
TLR4-/-
WT
TRAF6-/-
IL-6 (ng/ml)
IL-13 (ng/ml)
TNF (ng/ml)
Medium
IL-33
Medium
IL-33

Figure 1. IL-33 induces TRAF6-dependent cytokine production by mast cells. BMCMCs obtained from B6J-WT mice (A) and B6J-WT and -TLR4$^{-/-}$ mice (B; left panels) and FLCMCs obtained from B6J-WT and -TRAF6$^{-/-}$ mice (B; right panels) were cultured in the presence of various concentration of rmIL-33 (A) or in the presence and absence of 100 ng/ml rmIL-33 for 6 h (for TNF measurement) and 24 h (for IL-6 and IL-13 measurement). The levels of IL-6, IL-13 and/or TNF in the culture supernatants were determined by ELISA. Data show the mean + SD (n = 3). *$p<0.05$ vs. WT.
doi:10.1371/journal.pone.0018404.g001

analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson).

Cell survival

TGC-induced peritoneal macrophages (1×10^6 cells/ml for FACS analysis and 2.5×10^5 cells/ml for lactate dehydrogenase [LDH] release assay, respectively) were cultured in the presence and absence of 100 ng/ml LPS for 0–48 h. Cell viability was assessed using a MEBCYTO-Apoptosis kit (MBL) or LDH assay kit (CytoTox 96; Promega) as described previously [44].

Cytokine ELISA

The levels of IL-6, IL-13 and TNF in culture supernatants were measured with mouse IL-6, IL-13 and TNF ELISA sets (eBioscience).

ELISPOT

The number of IL-33-secreting cells by ELISPOT assay was performed as described elsewhere [44]. Breifly, MultiScreen-IP plates (MAIPS4510; Millipore) were coated with anti-mouse IL-33 polyclonal Ab (R&D Systems; 2 μg/ml in PBS) as a capture Ab at 4°C overnight. After blocking with PBS containing 10% FCS, TGC-induced peritoneal macrophages (2×10^4/200 μl) were cultured in the presence or absence of 100 mg/ml LPS or 0.1 μg/ml PMA plus 1 μg/ml ionomycin at 37°C for 24 h or 48 h. After washing the wells, biotinylated anti-mouse/human IL-33 mAb (Nessy-1; Alexis Biochemicals, 400 ng/ml in PBS containing 10% FCS) as a detection Ab was applied and incubated at r.t. for 1 h. Then, after washing the wells, HRP-conjugated streptavidin (BD Biosciences) was added to the wells at r.t. for 1 h. AEC (Sigma) were used as substrates. Positive spots on Ab-coated plates were analyzed with NIH Image software.

Statistics

An unpaired Student's *t*-test, 2-tailed, was used for statistical evaluation of the results.

Results

Effects of anti-ST2 mAbs on cytokine production by BMCMCs

Several mAbs against mouse ST2, i.e., clones DJ8 [49,50], 3E10 [40], 245707 and 245714, have been generated to study the role(s) of ST2 in immune responses. It was recently demonstrated *in vitro* that IL-33-mediated cytokine production by macrophages was inhibited by addition of DJ8 [43], suggesting that DJ8 acts as a neutralizing Ab for IL-33 bioactivity. The crosslinking of ST2 by 3E10 enhanced Th2 cytokine production by Th2 cells *in vitro* [36], while the administration of 3E10 in mice resulted in the suppression of Th2 cell/cytokine-mediated allergic or viral airway inflammation [38,40,51] without depletion of ST2-expressing cells [37]. However, the effects of the other mAbs on IL-33-mediated immune cell activation remain unknown.

Recombinant mouse IL-33 (rmIL-33) can induce cytokine secretion by mouse bone marrow cell-derived cultured mast cells (BMCMCs) (Fig. 1A) dependent on MyD88, which is an essential adapter molecule for signal transduction of the TLR/IL-1R (TIR) superfamily [7]. As in the case of MyD88$^{-/-}$ BMCMCs [7] and ST2$^{-/-}$ BMCMCs (data not shown), IL-6, IL-13 and TNF production by FLCMCs deficient in TRAF6, which is a downstream molecule of MyD88, was impaired by rmIL-33 (derived from *E. coli*) (Fig. 1B). On the other hand, rmIL-33-mediated secretion of these cytokines was observed to be comparable in wild-type (WT) and TLR4$^{-/-}$ BMCMCs (Fig. 1), indicating that the biological activity of rmIL-33 was not influenced by contamination with endotoxin.

We next examined the effects of the anti-mouse ST2 mAbs on cytokine production by BMCMCs after IL-33 stimulation. Cytokine secretion by BMCMCs in response to 3–30 or 100 ng/mL rmIL-33 was profoundly or partially (nearly half maximum) inhibited in the presence of 40 μg/mL anti-ST2 mAb (DJ8), respectively (Fig. 2A). Therefore, we used 30 or 100 ng/mL rmIL-33 in the other neutralization studies. IL-33-mediated IL-6 and IL-13 production by WT BMCMCs was inhibited by addition of 245707 as well as DJ8, but not 3E10 or 245714 (Fig. 2B). Like rIL-33, it has been reported that crosslinking of ST2 by 3E10 promoted cytokine secretion by Th2 cells *in vitro* as an agonistic Ab [36]. On the other hand, 3E10 alone could not enhance IL-6 or IL-13 production by WT BMCMCs (Fig. 2B), although 3E10 as well as DJ8 and 245707, but not 245714, bound to ST2 on the cell surface of BMCMCs (Fig. 2C). We also found that crosslinking of ST2 by 3E10 and anti-rat IgG did not induce IL-6 or TNF production by BMCMCs (data not shown). These observations suggest that DJ8 and 245707, but not 3E10 or 245714, have neutralizing activity for IL-33-mediated mast cell activation, at least *in vitro*. Moreover, these observations indicate that the effect of 3E10 differs between Th2 cells [36] and mast cells.

Effects of anti-IL-33 mAb on cytokine production by BMCMCs

It was shown that ST2-expressing cells were depleted by anti-ST2 polyclonal Ab *in vitro* [52]. Therefore, anti-IL-33 Ab(s) rather than anti-ST2 Ab(s) would be useful for elucidating the role(s) of the IL-33-ST2 pathway *in vitro* and *in vivo*. Accordingly, we next examined the effects of anti-IL-33 mAbs (Nessy-1 and 518017) and polyclonal Ab (AF3626) on cytokine production by BMCMCs in response to rmIL-33. Nessy-1, but not 518017 or AF3626, inhibited IL-33-mediated IL-13 production by BMCMCs (Fig. 3A). However, the inhibitory effect of Nessy-1 was weak in comparison with that of the DJ8 anti-ST2 mAb, as shown in Figure 2A. Therefore, we newly generated anti-IL-33 mAbs (which were confirmed by western blot analysis to recognize rmIL-33; data not shown) and investigated their effects on IL-33-mediated cytokine production by BMCMCs. Ten (1D2, 1F11, 2A2, 2E6, 2C7, 4A3, 4D4, 4G4, 5F1 and 5D11) of 100 tested anti-IL-33 mAbs were able to inhibit IL-33-mediated IL-13 production (Fig. 3B). Like DJ8 (Fig. 2A), some of those mAbs (i.e., 2A2, 2E6 and so on) strongly inhibited IL-33 activity (Fig. 3B).

Effects of anti-IL-33 mAbs on cytokine production by TGC-induced macrophages and BMCMCs

It was recently reported that recombinant IL-33 enhanced LPS-mediated cytokine production by macrophages [43]. Consistent with this, we found that IL-33 augmented IL-6 production by

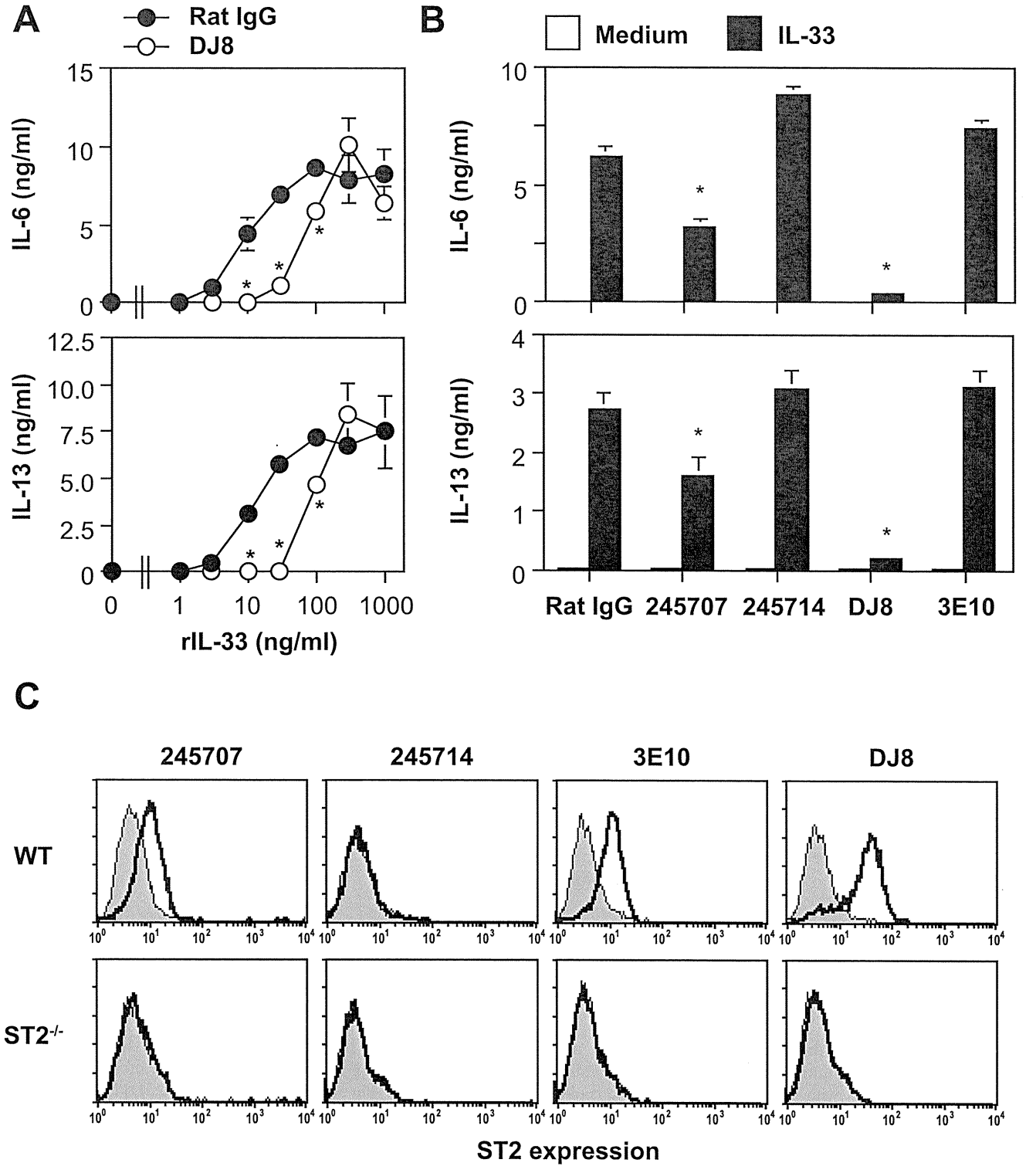

Figure 2. Effects of anti-ST2 mAbs on cytokine production by IL-33-stimulated BMCMCs. B6J-WT BMCMCs were stimulated with 0–1,000 ng/ml (A) or 100 ng/ml (B) rmIL-33 in the presence of 40 μg/ml of several anti-ST2 mAbs or isotype control rat IgG for 24 h. The levels of IL-6 and IL-13 in the culture supernatants were determined by ELISA. Data show the mean + SEM (n = 3). *$p<0.05$ vs. rat IgG+IL-33. The expression of ST2 on the cell surface of BALB-WT and $ST2^{-/-}$ BMCMCs was determined using several distinct anti-ST2 mAb clones. Representative data by flow cytometry are shown (C). Shaded area indicates isotype-matched control IgG staining, and bold line indicates anti-ST2 mAb staining.
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TGC-induced peritoneal macrophages in response to LPS (Fig. 4A). We reported that TGC-induced peritoneal macrophages produced IL-33 in response to LPS [44]. In addition, it is thought that IL-33 is released by necrotic cells after stimulation [53,54]. The proportion of annexin V-negative and propidium iodide (PI)-positive necrotic macrophages, the levels of LDH release in the

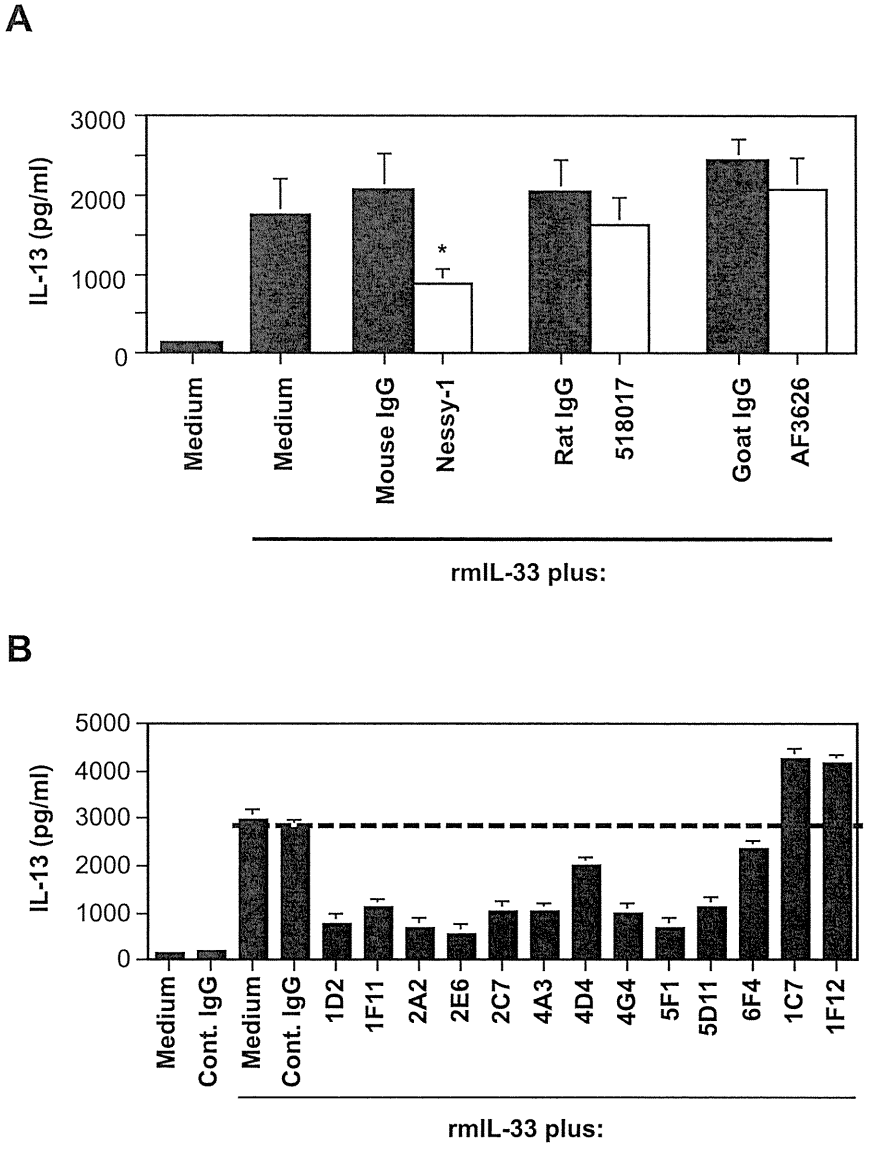

Figure 3. Effects of anti-IL-33 Abs on cytokine production by IL-33-stimulated BMCMCs. B6J-WT BMCMCs were stimulated with 30-ng/ml rmIL-33 in the presence and absence of commercially available anti-IL-33 Abs (A), our newly generated anti-IL-33 mAbs (B) or control IgG (A, B) for 24 h. The levels of IL-13 in the culture supernatants were determined by ELISA. Data show the mean + SEM (n = 3). *$p<0.05$ vs. control IgG+IL-33.
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culture supernatants and the number of IL-33-secreting macrophages were significantly increased at 48 h after LPS stimulation (Fig. 4B–D). Consistent with previous reports [44], we could not detect IL-33 proteins in the culture supernatants and cell lysates by ELISA and western blot analysis, respectively (data not shown). These observations suggest that necrotic macrophage-derived IL-33 may paracrinely promote cytokine production by viable macrophages after LPS stimulation. In support of this, IL-6 production by IL-33$^{-/-}$ macrophages was reduced in comparison with WT macrophages at 24 and 48 h after LPS stimulation (Fig. 4E). To more fully elucidate this, we examined the effects of endogenous IL-33 on cytokine production by LPS-stimulated TGC-induced macrophages in the presence of anti-ST2 mAbs and anti-IL-33 mAbs. The LPS-mediated IL-6 production by TGC-induced macrophages was inhibited by addition of anti-ST2 mAbs DJ8 and 245707, but not 3E10 or 245714 at 48 h, but not 24 h, after LPS stimulation (Fig. 5A). These responses by TGC-induced macrophages were also inhibited by addition of anti-IL-33 mAbs 2C7 and 1F11, but not other mAbs including 5D11, 1D2, 2A2 and 2E6, at 48 h, but not 24 h, after LPS stimulation (Fig. 5B and data not shown). We previously demonstrated that IL-33 mRNA expression was increased in BMCMCs after stimulation with highly cytokinergic IgE [55], FcεRI-crosslinking by IgE and antigens, and PMA+ionomycin, but not LPS [44]. However, the expression level of IL-33 protein by BMCMCs was less than that by TGC-induced macrophages after stimulation [44]. In accordance with this, IL-13 production by BMCMCs was not influenced by addition of any of the anti-IL-33 mAbs at 48 h after IgE stimulation (anti-DNP IgE; SPE-7) (Fig. 5C). These observations suggest that macrophages, rather than mast cells, are potential producers of IL-33, and that macrophage-derived IL-33 can activate macrophages in a paracrine manner after LPS stimulation.

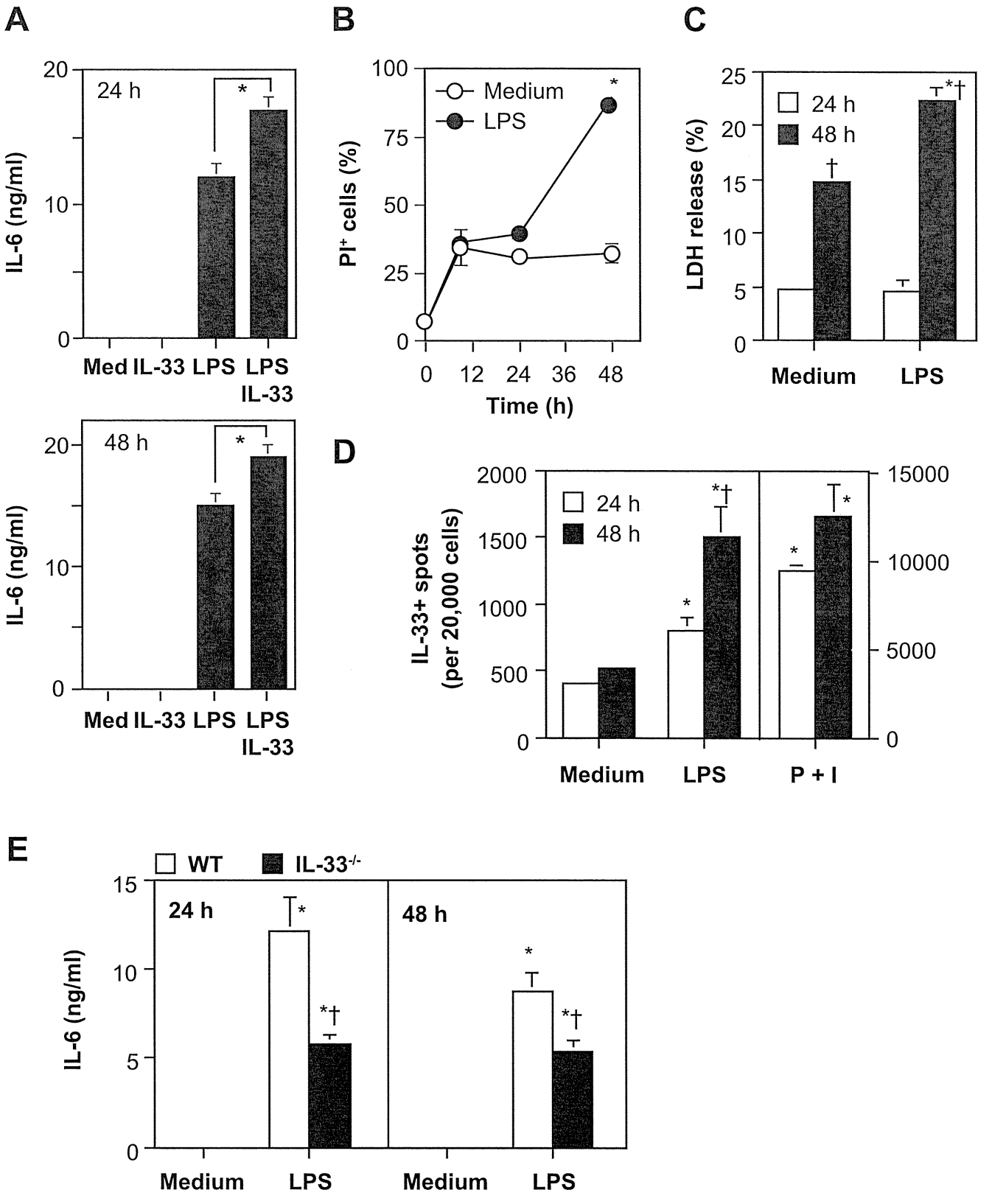

Figure 4. IL-33 enhances LPS-mediated cytokine production by macrophages. TGC-induced peritoneal macrophages derived from B6J-WT mice (A–D) and B6N-WT and -IL-33$^{-/-}$ mice (E) were cultured in the presence and absence of 100 ng/ml LPS, with and without 100 ng/ml IL-33, for 9, 24 and/or 48 h. (A, E) The levels of IL-6 in the culture supernatants by ELISA. (B) The percentage of PI-positive cells by flow cytometry. (C) LDH levels in the culture supernatants. (D) The number of IL-33-secreting cells by ELISPOT. Data show the mean +/± SEM (n = 3 [A] or 4 [B–E]). *$p<0.05$ vs. the indicated group (A) or Medium (B–E), and $^{\dagger}p<0.05$ vs. 24 h (C, D) or WT (E). P+I = PMA+ionomycin.
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