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Homeostasis of Human NK Cells Is Not IL-15 Dependent

Herve Lebrec,* Michelle J. Horner,† Kevin S. Gorski,‡ Wayne Tsuji,§ Dong Xia,¶ Wei-Jian Pan,‖ Gary Means,‡ Greg Pietz,‡ Nianyu Li,† Marc Retter,¶ Kathy Shaffer,‖ Neha Patel,‖ Padma K. Narayanan,‖ and Eric A. Butz‡

IL-15 is a proinflammatory cytokine that plays an important role in the development and activation of NK cells and is a potential target for inflammatory disease therapy. Studies conducted in IL-15- and IL-15R knockout mice identified IL-15 as an important cytokine for NK cell homeostasis. Consistent with this information derived from genetically modified mice, we demonstrated that neutralizing IL-15 with a mouse anti-mouse IL-15 mAb (M96) depletes C57BL/6 mouse NK cells. An mAb directed against macaque IL-15 (Hu714MuXHu) was manufactured and demonstrated to block IL-15–induced activation of nonhuman primate (NHP) NK cells in vitro. Neutralization of macaque IL-15 by parenteral administration of Hu714MuXHu reduces (>95%) circulating NK cell counts in NHPs. A blocking mAb directed against human IL-15 (huIL-15; AMG 714) was manufactured. Unexpectedly, when human subjects were treated with the blocking anti–IL-15 Ab AMG 714 in clinical trials, no reductions in circulating NK cell counts were observed despite achieving significantly higher exposures than the levels of Hu714MuXHu needed to cause NK cell count reductions in NHPs in vivo. Both AMG 714 and Hu714MuXHu are able to block huIL-15 activity in a human T cell blast proliferation and IFN-γ production assay. Both Abs block huIL-15–mediated Stat5 activation and CD69 expression in human NK cells. Collectively, these results demonstrate that NK cell homeostasis is obligatorily dependent upon IL-15 in both mice and NHPs, but that IL-15 is dispensable for maintenance of circulating human NK cells. The Journal of Immunology, 2013, 191: 000–000.

Interleukin-15 is a pleiotropic cytokine that synergizes with other inflammatory cytokines and may play a prominent role in a variety of autoimmune diseases, making it an attractive target for therapy. Evidence exists that IL-15 plays a key role in murine NK cell homeostasis: NK cells are absent in IL-15 knockout (IL-15ko) (1) and IL-15Rα knockout mice (2). Treatment of IL-15ko mice with exogenous IL-15 restores normal counts of peripheral NK cells (1), indicating that IL-15 is required for the development and/or survival of NK cells in mice. Subsequent work has shown that IL-15 does play an important role in NK cell development (3–5). However, because mature NK cells do not survive when they are adoptively transferred into IL-15ko mice, it is clear that IL-15 is also required for the maintenance of mouse NK cells (6, 7). Similar adoptive transfer experiments have shown that IL-15 must be presented to the NK cell in trans by another cell (8, 9), normally a dendritic cell (10–12).

Consistent with experiments documenting the role of IL-15 in murine NK homeostasis, treatment of macaques with a signal-blocking human IL-15 (huIL-15) muatein reduces NK cell numbers (13). Similarly, the Jak inhibitor tofacitinib (CP-690,550), which inhibits the signaling of all common γ cytokines, has also been shown to induce a profound reduction in the number of circulating NK cells in macaques (14, 15).

Attempts to elucidate the processes involved in the development and homeostasis of human NK cells have relied on studies performed either in vitro (16) or in SCID mice reconstituted with human cells (9). These studies have generally supported the finding that IL-15 promotes the proliferation and survival of NK cells.

To support the potential development of an anti–IL-15–targeted therapy, we have developed several reagents for preclinical and clinical studies: M96, a neutralizing IgG2a mouse anti-mouse IL-15 mAb; Hu714MuXHu, a chimeric mAb consisting of the variable domains of M111 (a rat anti–huIL-15 mAb) grafted to a human IgG1 backbone; and AMG 714, a fully human IgG1 anti–huIL-15 mAb that prevents IL-15–induced cytokine production by human PBMC and IL-15–induced inflammatory activity in human psoriatic skin explant cultures (17). These three mAbs have a common mechanism of action in that they do not prevent the association of IL-15 with IL-15 receptorα (IL-15Ra), but instead prevent signaling by the complete cytokine–receptor complex (IL-15/IL-15Ra/CD122/CD132).

The use of these Abs has allowed a unique opportunity to examine the IL-15 dependence of NK cells in humans as compared with those of two important preclinical animal species. We confirm that survival of mouse NK cells depends upon IL-15 and find that macaque NK cells share a similar requirement for IL-15. However, we also find that the survival of human NK cells is surprisingly independent of IL-15, suggesting that human NK cell biology may differ significantly from our understanding based largely on experiments in mice.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6–8 wk of age and cared for in accordance with the Guide...
for the Care and Use of Laboratory Animals, 8th edition. Animals were group-housed at an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited (Frederick, MD) facility in nonsterile ventilated microisolator housing on corn cob bedding. All research protocols were approved by the Amgen Washington Institutional Care and Use Committee. Animals had ad libitum access to pelleted feed (Teklad 2920; Harlan Teklad) and water via automatic watering system. Animals were maintained on 12:12-h light/dark cycles in rooms and had access to enrichment opportunities.

Cynomolgus macaques
Male/female cynomolgus macaques (Macaca fascicularis), 3–5 y of age, were cared for in accordance to the Guide for the Care and Use of Laboratory Animals, 8th edition. Animals were housed at an indoor Assessment and Accreditation of Laboratory Animal Care International–accredited facility in species-specific housing. All research protocols were approved by an Institutional Animal Care and Use Committee. Animals were fed Certified Primate Diet #2055C (Harlan Teklad) daily in amounts appropriate for the age and size of the animals and had ad libitum access to water via automatic watering system/water bottle. Animals were maintained on a 12:12-h light/dark cycle in rooms at a temperature range of 19.80–24.4˚C and relative humidity range of 17.3–94.6%. Animals were given fruit, vegetable, or additional supplements as a form of environmental enrichment and given various cage-enrichment devices. Animals were commingled to provide psychological enrichment.

IL-15
Recombinant human and macaque IL-15 were produced at Amgen in CKE5cells (18), Ercherichia coli–derived mouse IL-15 was purchased (R&D Systems, Minneapolis, MN). Native huIL-15 was purified from the supernatants of HK-2 cells (ATCC CRL-2114; American Type Culture Collection).

Human blood
Blood was obtained from healthy donors at Amgen with informed consent. PBMC were isolated for functional assays using BD Vacutainer CPT (Cell Preparation Tubes; BD Biosciences) with sodium heparin. The tubes were centrifuged for 30 min at 20˚C at 1600 g. PBMC were isolated and resuspended in 15-ml conical tubes and centrifuged for 15 min at 300 × g. The cell pellet was resuspended, washed with PBS twice, and cultured thereafter in RPMI 1640 with 10% FBS. NK cells were prepared by negative selection using magnetic beads (NK cell isolation kit; Miltenyi Biotec, Bergish Gladbach, Germany).

Cynomolgus macaque blood
Heparinized blood from adult male cynomolgus macaques was purchased and shipped at room temperature to Amgen.

Lymphocyte activation assays
PHA blast assay. Human and cynomolgus macaques PHA-stimulated lymphoblast cells (PHA blasts) were produced as previously described (19) and then cultured with IL-15 and Abs as indicated. After 48 h, 1 μCi [3H]thymidine deoxyribose was added to each well for 6 h, and the incorporated [3H] was counted. The CTL2-2 proliferation assay was performed as previously described (20) using CellTiterGlo (Promega, Madison, WI) per the manufacturer’s protocol. IFN-γ was measured using a capture ELISA (R&D Systems).

Abs
AMG 714 (Amgen) is a fully human anti–huIL-15 IgG1 mAb derived by Gennab (Copenhagen, Denmark) and formerly identified as HuMaxIL-15 (17), M111 (ATCC HB-12062; American Type Culture Collection) is a mouse anti–huIL-15 IgG1 Ab derived at Amgen that neutralizes both human and macaque IL-15. Hu714MuXHu (Amgen) is a chimeric Ab with a human IgG1 Fc and the M111 L- and H-chain variable regions. M96 (Amgen) is a neutralizing mouse anti-mouse IL-15 IgG2a mAb derived by immunizing IL-15ko mice with mouse IL-15 in CFA. Mab50 (Amgen) is a mouse IgG2a mAb of irrelevant specificity used as an isotype control where indicated.

Flow cytometry
Mouse. Directly conjugated Abs against the mouse lymphocyte Ags CD3, NK1.1, DX5, CD8, CD4, CD44, and B220 (BD Biosciences) were used to define lymphocyte populations: T cells (CD3+), CD3+CD4+, CD3+CD8+, CD3+CD44high, naive Th cells (CD3+CD8+CD45RA+), memory T cells (CD3+CD4+CD45RA+), CD45RO+ T cells (CD3+CD8+CD45RA+), cytotoxic T cells (CD3+CD8+CD45RA-, CD45RO-), and NK cells (CD3–CD16+). 

Cynomolgus macaques. PBLS were analyzed immunophenotypically using the following clones of differentiation (CDs): total T cells (CD3+), CD3+ T cells (CD3+CD4+), naive Th cells (CD3+CD8+CD45RA+), memory Th cells (CD3+CD8+CD45RA-), cytotoxic T cells (CD3+CD8+CD45RA-), memory cytotoxic T cells (CD3+CD8+CD45RA-), B cells (CD3+CD20+), and NK cells (CD3+CD16+).

Human. The Ab panels used for human cells were CD3-DTTC × CD69–PE × propidium iodide × CD56–allophycocyanin and CD45RO–Pacific Blue × CD-FITC × CD69–PE × propidium iodide × CD56–allophycocyanin × CD62L–Cy7 × CD4–APC/Cy7. Fluorescence was quantified using a BD LSR II equipped with three lasers (405, 488, and 635 nm). Data acquisition was performed using vendor-provided software (BD Biosciences) and reanalyzed with FlowJo (Tree Star).

Whole-blood stimulations and fixation
A Chinese hamster ovary (CHO) cell line stably expressing IL-15Ra (clone 5E/C, Amgen) was incubated for 60 min at room temperature with 1 mg/ml recombinant huIL-15 purified from an in-house CHO stable expression system (lot #8050-061; Amgen). IL-15–loaded CHO cells were washed two times in 50 ml PBS plus 0.1% BSA (BSA-Endotoxin Free; catalog number 126579; Calbiotech) to remove unbound IL-15, plated at a density of 800,000 cells/well in 96-well plates (catalog number 353077; BD Biosciences), and then incubated with either AMG 714 or Hu714MuXHu in a 10-point dilution series (1–3.8 ng/ml final concentration) for 30 min at room temperature. The whole-blood stimulation was initiated by the addition of 200 μl whole blood/well from a healthy donor collected in 10-ml sodium heparin glass Vacutainer tubes (catalog number 366480 and 500799; BD Biosciences). After 40 min at 37˚C, the entire stimulation mixture was transferred to a 5-ml deep-well plate (catalog 201238-100; Seahorse Bioscience) and 4 ml 1× BD Phosflow Lyse/Fix Buffer (catalog number 558049; BD Biosciences) warmed to 37˚C was added to simultaneously lyse the RBCs and fix the PBMCs. The Lyse/Fix Buffer–treated samples were incubated for 30 min at 37˚C to allow for complete RBC lysis. Following incubation, the plates were centrifuged at 1000 × g for 15 min at 4˚C to pellet the fixed whole-blood PBMCs and then stored at −70˚C in 1× BD Phosflow Lyse/Fix Buffer (BD Biosciences) until analysis.

The pelleted PBMCs were washed with ice-cold PBS plus 1% FBS three times. Extracellular Ab labeling was conducted for 30 min using a mixture of CD14-FITC (catalog number IM0645U; Beckman Coulter) and NK Simultest: CD14-FITC, CD56-allophycocyanin and CD45RO–Pacific Blue (catalog number 612599; BD Biosciences) diluted in PBS plus 1% FBS. After surface labeling, the cells were washed three times in PBS plus 1% FBS, permeabilized with ice-cold 80% methanol, and incubated on ice for 20 min in the dark. Cells were again washed three times and labeled for 30 min with anti-human p-STAT3 (Y694) Alexa Fluor 647 Ab (catalog number 612599; BD Biosciences) diluted in PBS plus 1% FBS. Cells were washed three times and resuspended in PBS plus 1% FBS for analysis.

M96 ELISA assay
M96 anti-mouse IL-15 binding activity was determined using a capture ELISA assay developed at Amgen. Mice IL-15 (Amgen) was used as the capture agent, and IL-15–binding Abs were detected using an HRP-labeled anti-mouse Ig F(ab′)2 (Pierce) followed by a tetramethylbenzidine (TMB) substrate solution (KPL). Serum M96 concentrations were determined by comparison with an M96 standard curve diluted in mouse serum.

Immunosassay for serum AMG 714 and Hu714MuXHu levels
The assays used for determining AMG 714 and Hu714MuXHu concentrations in nonhuman primates (NHPs) and human serum were proprietary sandwich ELISA methods developed at Amgen. For the AMG 714 assay, mouse anti–AMG 714 #82A (Amgen) was used as capture reagent. For the Hu714MuXHu assay, rat anti–AMG 714 (M111; Amgen) was used as capture reagent. AMG 714 or Hu714MuXHu assay standards and quality control samples were available. Biotin-labeled polyclonal rabbit anti-AMG 714 and rabbit anti-M111 (Fab′)2 polyclonal Ab (biotinylated anti-Hu714MuXHu) were used as detection reagent for AMG 714 and Hu714MuXHu, respectively, in combination with streptavidin–poly-HRP, a TMB substrate solution. The resulting colorimetric reaction readout (OD) was converted into concentrations using the concurrently analyzed standard curve. In the assays, the minimum levels of detection for AMG 714 and Hu714MuXHu were 0.05 and 0.1 μg/ml, respectively.

One-month repeat-dose cynomolgus macaque study design
Male and female cynomolgus monkeys were assigned to five groups of five animals per sex. Each group received doses of the control article, Hu714MuXHu placebo (group 1), or 30 (group 2), 60 (group 3), or 150 (groups 4 and 5) mg Hu714MuXHu/kg of body weight (mg/kg) at a dose

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AMG 714 CHO was administered to 20 subjects with PsO. (150 and 300 mg s.c. every 2 wk for 10 wk of treatment) or placebo was administered in a multiple ascending-dose study, NCT00443326, in which AMG 714 CHO preceded a phase 1b placebo at 40 healthy volunteers in a phase 1a singleascending-dose study, AMG 714 20050193, which preceded a phase 1b placebo was administered to 40 healthy volunteers in a phase 1a singleascending-dose study, AMG 714 HYB, which preceded a phase 1b placebo administered to humans in clinical trials. HuMax-IL-15 and AMG 714 have identical amino acid sequences but HuMax-IL-15 was produced in HYB stock colony. Following the completion of data collection, all animals were returned to the stock colony.

**Single-dose cynomolgus macaque study design**

Male cynomolgus monkeys were assigned to four groups. Each group received dose preparations that delivered 0.1 or 1 mg Hu714MuXHu/kg of body weight (mg/kg) (n = 3 each). 150 mg/kg AMG 714-CHO (n = 4), or 150 mg/kg AMG 714-hybridoma (HYB) (n = 4) by i.v. injection into a saphenous vein. Hu714MuXHu was given at a dose volume of 1 ml/kg, and AMG 714-HYB and AMG 714-CHO were given at a dose volume of 7.5 ml/kg. Following the single dose, animals in groups 1 and 2 (Hu714MuXHu) underwent a 6-wk observation period, and animals in groups 3 (AMG 714-CHO) and 4 (AMG 714-HYB) underwent a 5-wk observation period.

Serum concentrations of Hu714MuXHu were determined at days 1, 3, 5, 8, 14, 21, 28, 35, and 42. Serum concentrations of AMG 714-CHO or AMG 714-HYB were determined at days 1, 3, 5, 8, 14, 21, 28, 35, and 35. Blood samples for clinical pathology and immunophenotyping were collected twice prior to initiation of treatment; predose on day 1 and on days 3, 5, 8, 14, 21, 28, 35, and 42 for animals in groups 1 and 2; and predose on day 1 and on days 3, 5, 8, 14, 21, 28, and 35 for animals in groups 3 and 4. Following the completion of data collection, all animals were returned to the stock colony.

**AMG 714 clinical studies**

HuMax-IL-15 (AMG 714 HYB) and AMG 714 (AMG 714 CHO), fully human mAbs that specifically bind and inhibit IL-15, have been administered to humans in clinical trials. HuMax-IL-15 and AMG 714 have identical amino acid sequences but HuMax-IL-15 was produced in HYB cells and AMG 714 was produced in CHO cells. Subjects with rheumatoid arthritis (RA) received AMG 714 HYB in two clinical trials. Healthy volunteers and subjects with psoriasis (PsO), received AMG 714 CHO in two clinical trials.

Thirty patients with RA received single s.c. doses of AMG 714 HYB (0.15, 0.5, 1.0, 2.0, 4.0, or 8.0 mg/kg) followed by open-label extension for all subjects (except those in the 0.15-mg/kg dose group) with 4.0 mg/kg s.c. weekly for 4 wk in a phase 1–2 Genmab trial (21).

A total of 180 patients with active RA (six or more swollen and six or more tender joints and C-reactive protein ≥ 28 mm/h) despite treatment with one or more conventional disease-modifying antirheumatic drugs entered the trial. A total of 122 patients were randomized to one of four dose levels of AMG 714 HYB (40, 80, 160, and 280 mg), and 58 were randomized to placebo administered every other week by s.c. infusion for 12 wk in NCT00433875.

AMG 714 CHO (30, 100, 300, and 700 mg s.c. and 100 mg i.v.) or placebo was administered to 40 healthy volunteers in a phase 1a single ascending-dose study, AMG 714 20050193, which preceded a phase 1b multiple ascending-dose study, NCT00443326, in which AMG 714 CHO (150 and 300 mg s.c. every 2 wk for 10 wk of treatment) or placebo was administered to 20 subjects with PsO.

**Results**

In vivo neutralization of IL-15 results in loss of NK cells in the mouse

To examine the effect of neutralizing IL-15 in mice with an intact immune system, a neutralizing Ab was generated by immunizing IL-15ko mice with recombinant mouse IL-15 formulated in adjuvant. The resulting M96 mAb bound to mouse IL-15 (Fig. 1A) and was able to neutralize mouse IL-15–induced proliferation of CTLL2 cells (Fig. 1B). When C57Bl6 mice were treated with the M96 mAb, there was a rapid and profound reduction in the number of DX5+NK1.1+ NK cells in the spleen (Fig. 1C), with approximately half of the NK cells eliminated by day 1 and >90% of the NK cells eliminated by day 7. Thereafter, the NK cells returned to normal levels over a period of 2 to 3 wk as the mAb was cleared. The number of blood and splenic B cells (B220+) and CD4+ T cells (CD3+CD4+) was not affected by M96, though there was some reduction in the number of memory phenotype CD8+ T cells (CD3+CD8+CD44hi) at higher doses of the Ab (Supplemental Fig. 1).

**Figure 1.** The M96 Ab binds and blocks the activity of mouse IL-15 and induces a rapid and transient loss of NK cells in vivo. (A) M96 (open triangles) but not control mouse IgG (filled squares) binds to mouse IL-15 in a capture ELISA. Binding was detected using an HRP-labeled anti-mouse Ig F(ab')2 followed by a TAM TRB substrate solution. Absorbance units are indicated. (B) CTLL2 cells were stimulated with 2.5 ng/ml murine IL-15 in the presence of the indicated concentration of M96 anti-mouse IL-15 or isotype control Ab. After 18–24 h, the proliferation of the cells in culture was measured using CellTiterGlo per the manufacturer’s directions. (C) Female C57Bl6 mice were treated with a single i.p. dose of 10 mg of M96 anti-IL-15 mAb or PBS control on day 0. At the times indicated, groups of three treated and control mice were euthanized, and the number of CD3+DX5+NK1.1+ NK cells was determined. The numbers of NK cells present in the spleen (filled symbols/solid line) are indicated as a percent of the numbers of NK cells present in the control animals. Serum levels of M96 mAb were also determined and are indicated by the open symbols/dotted line. Error bars indicate SE. The experiment was repeated two times with similar results. aIL-15, Anti–IL-15; RLU, relative light units.
Blocking IL-15 in NHPs causes a profound reduction in circulating NK cell counts after repeated dosing

When cynomolgus monkeys received Hu714MuXHu at 30, 60, and 150 mg/kg s.c. or at 150 mg/kg i.v. once weekly (on days 1, 8, 15, 22, and 29), peripheral blood NK (CD3^−/CD16^+) cell counts decreased rapidly (within 2 wk) and significantly (absolute counts on day 15 reaching 4–14.2% of predose [day 26] counts). This effect was observed in both males and females at all dose levels (Table I). This effect was maintained as long as circulating Hu714MuXHu serum levels remained above a threshold of ∼1000 ng/ml. The effect was reversible as demonstrated by NK cell counts measured on study day 309 being similar to predose values and values from animals that received vehicle (Fig. 2).

Hu714MuXHu and AMG 714 can both decrease NK cell counts in NHPs after a single dose

The purpose of this study was to determine a threshold dose of Hu714MuXHu for NK cell depletion (dose levels explored were 5, 30, 60, and 150 mg/kg, a dose previously known to affect NK cells in macaques) and, more importantly, to evaluate the ability of AMG 714 to affect NK cell counts in cynomolgus monkeys because NK cell depletion had not been observed in clinical trials. The dose level of AMG 714 (150 mg/kg) was justified by the relatively poor anticynomolgus IL-15 antagonistic activity of AMG 714 as compared with Hu714MuXHu. Although both molecules are equipotent against huIL-15 (anti–IL-15 IC_{50} of 70 and 77 ng/ml in a PHA blast

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**Table I. Circulating NK cell counts and associated Hu714MuXHu serum concentrations in cynomolgus macaques**

<table>
<thead>
<tr>
<th>Route</th>
<th>Dose Level (mg/kg)</th>
<th>PD −16</th>
<th>PD −6</th>
<th>Day 15^a (n = 5/sex)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total Lymphocytes (×10^3/μl)^b</td>
<td>NK Cells (×10^3/μl)</td>
<td>Total Lymphocytes (×10^3/μl)</td>
</tr>
<tr>
<td>i.v.</td>
<td>0</td>
<td>7.46/9.27</td>
<td>1.25/0.63</td>
<td>6.07/3.92</td>
</tr>
<tr>
<td>i.v.</td>
<td>30</td>
<td>8.32/8.17</td>
<td>0.81/0.81</td>
<td>5.38/3.73</td>
</tr>
<tr>
<td>i.v.</td>
<td>60</td>
<td>8.07/11.29</td>
<td>0.78/1.03</td>
<td>4.6/4.7</td>
</tr>
<tr>
<td>i.v.</td>
<td>150</td>
<td>7.39/17.1</td>
<td>0.84/1.09</td>
<td>4.63/3.32</td>
</tr>
<tr>
<td>s.c.</td>
<td>150</td>
<td>9.78/7.75</td>
<td>1.02/0.87</td>
<td>5.76/4.26</td>
</tr>
</tbody>
</table>

Data are male/female unless otherwise indicated.
^aPredose day 15; 7 d postdose.
^bCD3^−/CD16^+.
^cCorresponding values on day 1 were: BLLOQ, 878, 1700, 3350, and 1560 μg/ml, respectively; on day 22: BLLOQ, 1490, 2760, 5740, and 3640 μg/ml, respectively. Values are C_{max} for s.c. group and C_{0} for i.v. groups.
^p < 0.05.
BLLOQ, Below lower limit of quantification; PD, predosing period.

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**FIGURE 2.** IL-15 blockade reduces circulating NHP NK cells. Male (M; (A)) and female (F; (B)) cynomolgus monkeys received doses of either the control article (0 mg/kg) or 30, 60, or 150 mg of Hu714MuXHu/kg of body weight (mg/kg) at a dose volume of 5 ml/kg once weekly for at least 4 wk (five doses, study days 1, 8, 15, 22, and 29). Immunophenotyping of NK (CD3^−/CD16^+) cells was conducted twice during the predosing period, predose on dosing days 15 and 30, and during the recovery period on days 57, 85, 113, 141, 169, 197, 225, 253, 281, and 309 (days 161, 169, 197, 225, 253, 281, and 337 not shown).

**FIGURE 3.** AMG 714 depletes macaque NK cells. Male cynomolgus monkeys received dose preparations that delivered 0.1 or 1 mg of Hu714MuXHu/kg of body weight (mg/kg), 150 mg/kg AMG 714-CHO, or 150 mg/kg AMG 714-HYB by i.v. injection. Following the single dose, animals receiving Hu714MuXHu underwent a 6-wk observation period, and animals receiving AMG 714-CHO and AMG 714-HYB underwent a 5-wk observation period. NK cell counts were determined at days 1, 3, 5, 8, 14, 21, 28, 35, and 42 of dosing.
proliferation assay for AMG 714 and Hu714MuXHu, respectively), AMG 714 is poorly active against cynomolgus IL-15 (anti–IL-15 IC50 of 1700 and 97 ng/ml in a PHA blast proliferation assay for AMG 714 and Hu714MuXHu, respectively; data not shown).

The toxicokinetic evaluation indicated that after a single i.v. injection, Hu714MuXHu and AMG 714 were distributed primarily in the systemic circulation with low systemic clearance and long t1/2. Dose-normalized Hu714MuXHu and AMG 714 toxicokinetic exposures in cynomolgus monkeys were comparable.

Hematology findings considered likely related to administration of the test articles included mildly decreased absolute lymphocyte count for animals given either 0.1 or 1 mg/kg i.v. dose level of Hu714MuXHu and for animals given 150 mg/kg of AMG 714. The mean absolute lymphocyte count was lowest when absolute NK cell counts were at or near their nadirs.

Immunophenotyping results indicated that administration of Hu714MuXHu at a dose of 0.1 or 1.0 mg/kg produced a marked and relatively prolonged decrease in NK cell values (Fig. 3). Decreases were noted beginning on days 3 or 5. Values returned to predose levels on day 42 in the 0.1 mg/kg group only. Administration of AMG 714 (150 mg/kg) produced a transient but notable decrease in NK cell values, beginning on days 3 or 5; all animals receiving AMG 714 exhibited evidence of recovery by day 28. It was noteworthy that the effect was less pronounced and the return to baseline values occurred more quickly with AMG 714 than when cynomolgus monkeys received a single dose of 0.1 or 1.0 mg/kg of Hu714MuXHu. This study demonstrated that the effect observed with surrogate molecule Hu714MuXHu on NK cells is also present in monkeys after administration of the anti–huIL-15 AMG 714 molecules. The difference in dose levels necessary to achieve comparable effects likely reflects, at least in part, the difference in inhibitory potency of these different molecules for cynomolgus IL-15.

Blocking IL-15 does not cause a decrease in circulating human NK cells

Clinical trials described above enrolled healthy volunteers, subjects with RA, and subjects with PsO. The safety profiles of AMG 714-HYB and AMG 714-CHO were reasonable without imbalance in occurrence of infections, with a single serious infection associated with AMG 714-HYB, and without observed malignancies. Clinical effect was observed in subjects with RA with impact on acute-phase reactants. Clinical benefit in PsO was not demonstrated.

Blood was drawn from treatment subjects 1 d before treatment with AMG 714 and at intervals after treatment. NK cells were determined by flow cytometry at each time point using CD3, CD56, and CD16 as markers. To date, our clinical experience extends to 192 AMG 714–treated subjects, and, despite the fact that clinical and biochemical effects were seen in human subjects treated with AMG 714-HYB (Supplemental Fig. 2), no demonstrable change has been detected in NK cell numbers in clinical trials with single dosing of healthy subjects (AMG 714 phase 1a results shown in Fig. 4) or repeated doses in RA or PsO trials (every other week for up to 12 wk; Supplemental Fig. 3). This was true for total CD3

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\text{FIGURE 4. } \text{IL-15 blockade does not affect human NK cell counts. Healthy volunteers received a single dose of AMG 714 (} n = 6/\text{group} \text{) or placebo (} n = 10 \text{) as indicated above each panel in phase 1a study AMG71420050193. At the indicated times, blood was drawn, and the number of NK cells was determined by flow cytometry for CD3, CD56, and CD16. Shown are the values for CD56^{bright} \text{ NK (CD3}^+ \text{CD56}^+ \text{CD16}^+ \text{in open triangles), CD56}^+ \text{ NK (CD3}^+ \text{CD56}^+ \text{CD16}^+ \text{ in gray squares), and total NK (black circles) plotted as percent of initial values for each subject (with SDs). There were no consistent dose-dependent changes in any of the NK cell populations.}
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\text{FIGURE 5. } \text{AMG 714 does not deplete human NK cells at high AMG 714 serum levels. (A) A comparison of exposure levels achieved in humans receiving AMG 714 in whom no NK cell loss was observed and macaques receiving an NK-depleting dose of Hu714MuXHu. AMG 714-CHO was measured in serum of six healthy volunteers receiving a single s.c. dose of 700 mg AMG 714-CHO (filled triangles) and three cynomolgus monkeys receiving a single i.v. dose of 0.1 mg/kg dose of Hu714MuXHu (open squares). (B) A comparison of Ab levels in cynomolgus macaques following treatment with a single dose of AMG 714-CHO (150 mg/kg; } n = 4 \text{, filled triangles, } \sim 65\% \text{ NK depletion), AMG 714-HYB (150 mg/kg; } n = 4 \text{, open triangles, } \sim 65\% \text{ NK depletion), Hu 714MuXHu at 1.0 mg/kg } (n = 3 \text{; filled squares; } \sim 85\% \text{ NK depletion), or 0.1 mg/kg } (n = 3 \text{; open squares; } \sim 80\% \text{ NK depletion; same data as in (A)). Data are plotted as mean } \pm \text{ SD. Percent NK cell losses indicated represents the maximum depletion observed in Fig. 4.}
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CD56+ NK cells and for the CD56hi (mostly CD16-) and CD56low (mostly CD16+) subpopulations.

**AMG 714 serum levels in human subjects exceeded the Hu714MuXHu exposure levels associated with decreased NK cell counts in NHPs.**

To rule out the possibility that the observed cross-species differences on NK counts were the result of different levels of exposure to AMG 714 or Hu714MuXHu, circulating levels of AMG 714 in human subjects were compared with circulating levels of Hu714MuXHu in NHPs. As illustrated in Fig. 5, a single dose of 700 mg AMG 714 (or ~10 mg/kg) administered s.c. to healthy subjects was associated with circulating AMG 714 of up to 86.8 μg/ml in an individual on day 7. That dose level was not associated with changes in peripheral NK cell counts. In contrast, a single dose of 0.1 mg/kg Hu714MuXHu administered i.v. to NHPs was associated with maximum circulating levels of Hu714MuXHu of 1.65 μg/ml. It was also demonstrated that AMG 714, when administered at the dose level of 150 mg/kg in NHPs (a dose associated with decreased peripheral NK cell counts), was associated with AMG 714 circulating levels reaching ~2720 μg/ml. The high circulating levels of AMG 714 necessary to affect NK cell counts in NHPs are consistent with the above described low potency of AMG 714 against cynomolgus IL-15.

**AMG 714 and Hu714MuXHu similarly inhibit IL-15 activities on primary human NK cells in vitro.**

Studies in mice have suggested that trans-presented IL-15 may be of particular importance to NK cell homeostasis (10–12). To test the ability of AMG 714 to block trans-presented IL-15, CHO cells were transfected with huIL-15Ra (CHO–IL-15Ra). These cells were then pulsed with recombinant huIL-15, extensively washed, and added to preparations of resting PHA blast T cells. Trans-presentation of IL-15 resulted in T cell proliferation and IFN-γ secretion that were inhibited by AMG 714 and Hu714MuXHu but not by a control human IgG1 (Fig. 6), indicating that both Abs were indeed able to block trans-presented IL-15.

To verify that both Abs were similarly able to inhibit NK cell responses to IL-15, we examined the ability of the Abs to block IL-15 signals in the NK population. In whole blood treated for 1 h with IL-15 and then stained for NK markers and p-STAT5, both AMG 714 and Hu714MuXHu were able to prevent the phosphorylation of STAT5 in response to IL-15–pulsed CHO–IL-15Ra cells where control IgG1 did not (Fig. 7A). Similar results were seen in response to CHO cells cotransfected with both IL-15Ra and huIL-15 (data not shown). Furthermore, although CD69 expression was induced in isolated primary human NK cells treated for 3 d with

**FIGURE 6.** Anti–huIL-15 mAbs AMG 714 and Hu714MuXHu block huIL-15 presentation in trans. CHO–IL-15Ra cells treated with 50 μg/ml mitomycin C, pulsed with 200 ng/ml huIL-15 for 30 min, and then washed thoroughly. In 96-well plates, 2.5 × 10⁴ CHO–IL15Ra cells were paired with 5 × 10⁵ human PHA blasts and the indicated concentration of mAbs. Top panel, After 48 h, the cells were pulsed with 1 μCi [³H]thymidine deoxyribose (Tdr) for 6 h, and the incorporated [³H] was counted by liquid scintillation (TopCount, Perkin-Elmer). The open square indicates the proliferation observed when CHO-IL15Ra cells treated with mitomycin but no IL-15 were paired with the PHA blasts. SEs for triplicate samples are shown. Bottom panel, IFN-γ production in the cultures was determined by ELISA.

**FIGURE 7.** AMG 714 blocks trans-presented IL-15 signaling in primary NK cells. (A) Whole blood was incubated with IL-15Ra-CHO cells. After 1 h at 37˚C in the presence of the indicated concentrations of AMG714, Hu714MuXHu, or control IgG1, the cells were stained for cell-surface markers, fixed, permeabilized, and stained for p-STAT5 (Y694). p-STAT5 staining for NK (CD56⁺CD3⁻) cells. Similar data were obtained with PBMC from another donor in this experiment and in several other experiments. (B) Human NK cells were treated with 30 ng/ml of IL-15 with various concentrations of AMG714 or Hu714MuXHu. After 3 d, cells were harvested and stained for flow cytometry as described in Materials and Methods. Percentages of CD69 expression were calculated by comparing the median fluorescence intensity of each individual sample to samples treated with IL-15 only.
30 ng/ml of soluble IL-15, this expression was prevented by both AMG 714 and Hu714MuXHu (Fig. 7B).

Discussion

Based upon previous work performed in our laboratories and others’ with IL-15 and IL-15RaKO mice, we expected that IL-15 blockade by neutralizing mAbs would sharply reduce the number of NK cells by depriving them of a necessary homeostatic cytokine. This proved to be the case when we treated mice (Fig. 1) or macaques (Fig. 2) with anti–IL-15 mAbs. However, when human subjects were treated with the anti–IL-15 mAb AMG 714, there was no apparent loss of total NK cells or in CD56<sup>hi</sup> or CD56<sup>low</sup> NK subsets (Fig. 4), even though the maximal exposure of the human subjects to AMG 714 was much higher than the minimum levels of Hu714MuXHu required to significantly reduce the number of circulating NK cells in monkeys (Fig. 5).

Several lines of experimentation suggest that this lack of effect on NK cells does not result from a failure to adequately reach AMG 714 serum concentrations sufficient to neutralize IL-15 in vivo and that AMG 714–treated RA patients showed effects consistent with neutralization of IL-15’s proinflammatory activities (objective reductions in C-reactive protein and erythrocyte sedimentation rate with a greater proportion of patients treated with AMG 714 than placebo achieving American College of Rheumatology 20 responses at weeks 12 and 16 [data not shown]). Moreover, AMG 714 recovered from patient serum was fully active in preventing IL-15–induced cytokine production in ex vivo assays, ruling out the possibility that the mAb was inactivated in vivo (data not shown).

When used at sufficiently high doses, AMG 714, which is much less active against macaque IL-15 than Hu714MuXHu, was able to deplete macaque NK cells (Fig. 3), supporting the hypothesis of a species difference in IL-15 requirement for NK homeostasis. AMG 714 and Hu714MuXHu were able to block trans-presentation of huIL-15 to primary human T cells (Fig. 6) and NK cells (Fig. 7).

Our data with the M96 anti-mouse IL-15 mAb are consistent with our own and others’ earlier work showing that mouse NK cells are dependent upon IL-15 for survival, and our experiments in cynomolgus macaques suggest that the situation is similar in monkeys. However, neither healthy human subjects nor subjects with RA or PsO who received AMG 714 evidenced a drop in NK cell numbers. This was true for the duration of the Ab treatment with single-dose administration or with repeated dosing weekly for up to a month and every other week for up to 12 wk. Because it would appear that we have effectively blocked IL-15 signaling with AMG 714 in these subjects and not seen any effects on circulating NK cell numbers, we conclude that IL-15 is not strictly required for the survival of human NK cells in vivo.

There are conditions under which some mouse NK cells can survive or proliferate in the absence of IL-15. Consistent with this, mice lacking IL-15 or its receptor do contain a very small number of NK cells (1, 2). These cells, and the few NK in Rag<sup>−/−</sup>IL2Rγ<sup>−/−</sup> mice, can proliferate in response to murine CMV in an IL-12–dependent manner (22). That they survive at all in the absence of IL-15 signals indicates that they are different from the much larger dominant NK cell populations, and it is likely that their expansion under these conditions represents an acute response to a pathogen, rather than a sustained homeostatic mechanism. In the AMG 714 clinical trials, we saw no overt evidence of infection (reported as adverse events, elevated WBC counts, fever, or changes in T cell numbers).

Mouse CD127<sup>+</sup> NK cells are a minority population of NK cells (23), among which there is a CD25<sup>+</sup> population that can expand in response to T cell–derived IL-2 in the absence of regulatory T cells and develop into mature NK cells (24). However, although it is possible that some of the subjects with RA or PsO who received AMG 714 may have had chronic ongoing immune responses, this was not true of the healthy control subjects that received the Ab and have no evidence of regulatory T cell deficits. In addition, the macaques that received either AMG 714 or Hu714MuXHu are not gnotobiotic animals, have a diverse flora, and can be exposed to opportunistic pathogens and yet were not protected from NK loss in the absence of IL-15.

IL-15 plays a unique and important role in NK cell homeostasis in mice and macaques but appears not to play that same role for human NK cells. The question then arises: what alternative signals or cytokine (s) might play a role in their homeostasis? Interestingly, treatment of monkeys and human patients with the Jak1,3 inhibitor tofacitinib (CP-690,550) induces a reduction in the number of circulating NK cells (14, 15, 25). Tofacitinib inhibits Jak3 and thus type 1 and 2 IFNs and multiple common γ-chain cytokine signaling, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 signaling (26). Therefore, it may be that another cytokine, or combination of cytokines, acting through the common γ-chain (CD132) contributes to NK homeostasis in humans. We contend that the homeostasis of human NK cells does not have the same obligate dependence on IL-15 that mouse and macaque NK cells do and that human NK cell biology differs importantly from that of these model species. Future work to identify the critical factor(s) that provide support for human NK cells will likely reveal a unique pathway for therapeutic intervention.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figures
Legends:

S1. M96 depletes NK cells and some memory phenotype CD8+ T cells. Groups of 3 female C57B6 mice were treated with the indicated doses of M96 anti-IL-15 mAb or PBS control. Seven days later the spleens were harvested, single cell suspensions made, and analyzed by flow cytometry for NK cells (CD3-NK1.1+: filled diamonds), CD4 T cells (CD3+CD4+: circles), total CD8 T cells (CD3+CD8+: open triangles), memory phenotype CD8 T cells (CD3+CD8+CD44high: filled triangles) and B cells (CD3-B220+: open squares). Data are shown as percent difference from PBS treated control mice. A similar pattern of NK depletion was also seen in blood, lymph nodes, bone marrow and liver (data not shown). The experiment was repeated with similar results.

S2. In vivo biological activity of AMG-714 in a phase 2 rheumatoid arthritis clinical trial. A) Subjects receiving 280 mg AMG 714 showed an increase in ACR20 responses over placebo at week 14 (54% vs 38%, combined enrollment cohorts). The increase was not statistically significant (p = 0.10 CMH test). However, a statistically significant difference in ACR20 response in the 280-mg group compared with placebo was observed at weeks 12 (64% vs 34%; p = 0.003, Fisher’s exact test) and 16 (66% vs 38%; p = 0.003, Fisher’s exact test).

Among secondary endpoints, acute phase reactants decreased significantly compared with placebo beginning at week 4 C-reactive protein (B, p < 0.0001) and erythrocyte sedimentation rate (C, p = 0.005), Wilcoxon rank-sum test) and remained significantly improved throughout the study. Notably, C-reactive protein concentrations in the 280-mg group decreased rapidly from baseline (60% lower at week 4) and were approximately 50% to 67% lower than placebo concentrations throughout the study.

S3. Human NK cell numbers are not reduced during sustained treatment with AMG 714. Patients with active RA were treated every other week for 12 weeks with the indicated doses of AMG 714 HYB (arrows) in study #NCT00433875 (the first dose was doubled as a loading dose). For a subset of 13 or 14 patients per dosing group (68 total) baseline NK cell numbers were determined prior to the first treatment and thereafter at weeks 4, 8,
Human NK are not IL-15-dependent 12, 16 and 24. Total NK cell numbers (CD3-CD16/CD56+) are depicted for each of the dose groups. Standard deviation for NK percentages for each group at each timepoint averaged 46%, with a range of 32-70).
Supplementary Figure 1

Human NK are not IL-15-dependent
Supplementary Figure 2

A. % Achieving ACR20

B. CRP (mg/dL)

C. ESR (mm/hr)
Supplemental Figure 3

Human NK are not IL-15-dependent