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Innate Immunity Mediated by the Cytokine IL-1 Homologue 4 (IL-1H4/IL-1F7) Induces IL-12-Dependent Adaptive and Profound Antitumor Immunity

Wentao Gao,* Sanjay Kumar,‡ Michael T. Lotze,*†‡ Charles Hanning,‡ Paul D. Robbins,* and Andrea Gambotto*‡†

Recently, several novel members of the IL-1 family have been identified. The possible therapeutic utility and the underlying biologic role of these new members remain unclear. In the present study we analyzed the anti-tumor activity of human IL-1 homologue 4(IL-1H4; renamed IL-7F) by adenovirus-mediated gene transfer (AdIL-1H4) directly into murine tumors. In vitro expression analysis showed that IL-1H4 was a secretory protein. Treatment of an established MCA205 mouse fibrosarcoma by single intratumoral injection of AdIL-1H4 resulted in significant growth suppression. Furthermore, complete inhibition of tumor growth was observed following multiple injections of AdIL-1H4. The anti-tumor activity of IL-1H4 was abrogated in nude and SCID mice and in IL-12−, IFN-γ−, or Fas ligand-deficient mice. In contrast, IL-1H4 was able to confer substantial anti-tumor effects in NKT-deficient mice. These results suggest that IL-1H4 could play an important role in the link between innate and adaptive immunity and may be useful for tumor immunotherapy. The Journal of Immunology, 2003, 170: 107–113.

The IL-1 family members are proinflammatory cytokines that initiate the innate immunity by activating a set of transcription factors, including NF-κB and AP-1 (1–3). The better-studied members of the IL-1 family (IL-1α, IL-1β, IL-1R antagonist (IL-1RA), and IL-18), as well as the fibroblast growth factors are structurally related as β trefoil cytokines (4, 5) that are secreted without signal peptides and do not follow the typical secretion pathways. Recently it has become apparent that the secretion of IL-1β is in the form of rapidly shed microvesicles budding off the plasma membrane (6). That could be true for other members of the IL-1 family as well. Both IL-1α and IL-1β bind the type I IL-1R with subsequent recruitment of a signaling component, the IL-1R accessory protein (IL-1RACP). After the receptor complex forms, a common adapter molecule, My88, binds to the cytosolic portion of the IL-1R, which, in turn, activates IL-1R-associated kinase to phosphorylate TRAF-6. Subsequently IκB kinase (IKK) phosphorylates IκB, resulting in the release and nuclear transport of NF-κB (7–9). Downstream NF-κB then drives a variety of cell processes, including cell survival and secretion of a number of other cytokines associated with an activated cellular phenotype. IL-1RA also binds the type I receptor, but does not recruit IL-1RACP, thus competitively blocking the actions of the agonists IL-1α and IL-1β (10, 11). The type II IL-1R is an additional “decoy” regulator of IL-1 activity. It binds and sequesters the agonist IL-1 without inducing signal transduction (12). IL-18 is a Th1-inducing cytokine, promoting IFN-γ production from T, B, and NK cells, especially in synergy with IL-12 (13–15). IL-18 has a signaling pathway similar to those of IL-1α and IL-1β, but uses its own unique receptor, IL-1R-related protein and a non-binding chain, IL-1RACP-like cell surface molecule, both members of the IL-1R family (16).

Recently, a total of six additional novel IL-1 family members have been identified, which expands the IL-1 family to 10 members. The proposed nomenclature for the IL-1 family are IL-1F1 (IL-1α), IL-1F2 (IL-1β), IL-1F3 (IL-1Ra), IL-1F4 (IL-18), IL-1F5 (IL-1H3, IL-1Hy1, FIL18, IL-1RP3, IL-1L1, and IL-18), IL-1F6 (FIL1e), IL-1F7 (human IL-1 homologue (IL-1H4), FIL1c, IL-1RP1, and IL-1H), IL-1F8 (IL-1H2 and FILη), IL-1F9 (IL-1H1, IL-1RP2, and IL-1e), and IL-1F10 (IL-1Hy2 and FKSG75) (17–24). Preliminary studies showed that these novel members also do not contain a hydrophobic leader sequence. IL-1F5, IL-1F6, IL-1F8, and IL-1F9 encode only the mature protein, while IL-1F7 contains a propeptide sequence like IL-1α, IL-1β, and IL-18. The novel genes show significant sequence homology with IL-1α, IL-1β, IL-1Ra, and IL-18. Furthermore, protein structure modeling suggests the new members are indeed related to IL-1α and IL-1Ra. Interestingly, the novel genes form a cluster with the other IL-1 family members on the long arm of human chromosome 2. The most distantly related members of the family, both IL-18 and its binding protein, are located on the long arm of human chromosome 11 (25). To date, IL-1F9 is constitutively expressed in the placenta, skin, and squamous epithelium of esophagus. However, it could also be induced in vitro in keratinocytes by IFN-γ and TNF-α treatment, and in vivo during a contact hypersensitivity reaction or herpes simplex virus infection. IL-1F6 is expressed in a variety of human tissues, including spleen, lymph node, thymus, tonsil, bone marrow, leukocyte, and fetal brain, as well as several human cell lines (Mo-T, HUT-102, Raji, THP-1, IMTLH, HL60, HPT-4, and T84). It has also been detected in mouse tissues (spleen, placenta, stomach, and tongue) and mouse cell lines (macrophages RAW). IL-1F8 has been detected in human tissue (bone

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3 Abbreviations used in this paper: IL-RA, IL-1R antagonist; IKK, IκB kinase; IL-1F, IL-1 family; IL-1RACP, IL-1R accessory protein; KO, knockout.

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The physiologic roles of these novel proteins remain unclear, but they, like their homologues, are likely involved in the acute, innate inflammatory response. To study the potential therapeutic application of IL-1H4 (IL-1F7) and its underlying biological role, we constructed an adenoviral vector that allows high level expression in murine and human cells. In vitro protein expression showed that IL-1H4 was a secretory protein. In addition, we have demonstrated the ability of adenovirus-mediated gene transfer of IL-1H4 to induce an IL-12- and Fas ligand-dependent anti-tumor response. Complete inhibition of tumor growth was observed following multiple injections of AdIL-1H4 in the most animals. These results suggest that IL-1H4 could play a role in both innate and adaptive immune responses, similar to IL-18. Moreover, IL-1H4 could be useful for cancer gene therapy.

**Materials and Methods**

### Animals and cell lines

Immunocompetent C57BL/6 mice (H-2b), 6–12 wk old, were purchased from Taconic Farms (Germantown, NY). IL-12 p40-deficient C57BL/6 mice were purchased from Hoffmann-La Roche (Nutley, NJ). B6.CB17- Pkrscreptm1Sdy SCID and B6.Cg-Foxn1tm1J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IFN-γ-deficient C57BL/6 (Ifng−/−) and B6Smn-Fasl−/−-deficient mice, which carry homozygous deletions of the IFN-γ and Fas ligand gene, respectively, were also purchased from The Jackson Laboratory. NK-T GKO C57BL/6-decient (C57BL/6-deicient) mice were provided by Dr. T. Nakayama (Chiba University, Chiba, Japan). MCA205 is a methylicholanthrene-induced murine fibrosarcoma cell line (a gift from Dr. S. Rosenberg, National Cancer Institute, Bethesda, MD) and was maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 50 mM 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin. CRE 8 and A549 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM with 10% heat-inactivated FCS, glutamine, and antibiotics (all reagents were from Life Technologies, Gaithersburg, MD).

### Construction of recombinant adenovirus

E1/E2-deleted adenoviral vectors expressing IL-1H4 (AdIL-1H4) were constructed through Cre-lox recombination with reagents provided by Dr. S. Hardy (Somaticx, Alameda, CA). Briefly, a SalI–NovI fragment containing the human IL-H4 cDNA from the plasmid pLL-1H4 was inserted in a modified version of the shuttle plasmid pAdlox (GenBank U62024) and named pAdlox-IL-1H4. E1/E3-substituted recombinant adenovirus was generated by cotransfection of SalI-digested pAdlox-IL-1H4 and Φ5 helper virus DNA into the adenoviral packaging cell line CRE8. Adenoviruses were propagated on CRE8 cells, purified by cesium chloride density gradient centrifugation and subsequent dialysis according to standard protocols, and were stored at −70°C.

### Protein expression

To test IL-1H4 expression in vitro, A549 cells were infected by AdIL-1H4 or AdΔ5 at multiplicity of infection of 100. After infection, DMEM with 1% FBS was used to continue culturing the cells. The cells and conditioned media were harvested 72 h later. The cell pellets were suspended in lysis buffer (15 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, 1 mM DTT, and 10 mM KCl) with proteasome inhibitor cocktail (Novagen, Madison, WI) and lysed by three cycles of freezing/thawing in a dry ice/ethanol bath. The conditioned medium was clarified by filtering through a 0.45-μm pore size filter and then was concentrated 10-fold in a dialysis tube against polyethylene glycol 8000. The condensed medium was dialyzed against lysis buffer. The cell lysates and concentrated conditioned medium were separated on a 15% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The IL-1H4 protein was detected by immunoprobining with primary rabbit anti-IL-1H4 polyclonal Ab (GlaxoSmithKline, King of Prussia, PA) and second HRP-conjugated anti-rabbit IgG and was developed with an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ). Recombinant IL-1H4 was generated as a six-histidine-tagged protein in E. coli as described previously (17).

### Animal experiments

All animals were ear-tagged and randomized before the experiments and were treated and measured in a coded, blinded fashion. On day 0 mice were inoculated intradermally in the shaved left flank with 2 × 106 of MCA205 tumor cells. Each group consisted of five mice. On day 7 (or day 5) tumor-established mice were treated with an intratumoral injection of 107 PFU of AdΔ5 or AdIL-1H4. In the multiple treatment groups, mice received three additional injections of 107 PFU at 3-day intervals (days 10, 13, and 16). Tumor size was measured every 3 days and was expressed as the product of the perpendicular diameters of individual tumors. Results are reported as the mean tumor area (square millimeters) ± SEM.

### Immunohistochemistry

Mice received an intratumoral injection of 1 × 10⁶ PFU of AdIL-1H4 or AdΔ5 7 days after the intradermal inoculation of 2 × 10⁵ of MCA205 cells into the flank. Tumors were removed at 3 or 6 days after the first injection and the second injection. Tumor samples were immediately frozen and embedded in OCT compound (Miles, Elkhart, IN). Serial 5-μm sections were cut using a cryostat, and immunohistochemistry was performed by staining using Abs to CD4, CD8a, CD11b, and CD11c (all from BD Pharmingen, San Diego, CA).

### Statistical analyses

Significant differences in tumor growth were assessed by Student’s t test. The difference between groups was considered statistically significant at p < 0.05.

### Results

**Construction of recombinant adenoviruses encoding IL-1H4 and its expression in vitro**

To examine the biological activity of the IL-1 homologue, IL-1H4, an E1/E3-deleted IL-1H4 recombinant adenovirus was constructed using Cre-lox recombination, termed AdIL-1H4. The AdIL-1H4 virus was propagated on CRE8 cells, purified, and dialyzed by a standard protocol. To assess IL-1H4 expression, the human lung cancer cell line A549 was infected using AdIL-1H4 or AdΔ5 at a multiplicity of infection of 100. Cells and conditioned media were harvested 72 h later and used for Western blotting. Immunoblotting revealed that there were two bands detected in cell lysates, corresponding to the precursor and mature forms of IL-1H4. The recombinant mature form of IL-1H4 migrates slightly slower due to the presence of the six-histadine tag. Although it is also possible that two separate start codons exist within the human IL-1H4 gene, no additional start codons are apparent in the human IL-1H4 gene, as has IL-1F5 (lymph node, thymus, bone marrow, placenta, lung, skeletal muscle, prostate, testis, NK cell, and parathyroid tumor). IL-1F5 is also found in mouse tissue (spleen, kidney, placenta, embryo, stomach, tongue, and skin) and mouse cell lines (macrophages RAW). Transcripts of IL-1F7 were detected in human tissues (lymph node, thymus, bone marrow, placenta, lung, testis, colon tumor, and uterus) and human cell lines (THP-1, U937, A431, IMTLH, KG-1, HL60, HPBMC, HPT-4, and NHDC). IL-1F7 expression could be markedly up-regulated by PMA treatment of PBMC. IL-1F10 is expressed in skin and activated B cells of human tonsil. A receptor binding assay showed that IL-1F10 binds to the sIL-1R, suggesting its role in regulating IL-1R function. Interestingly, IL-1F7 specifically binds the IL-1R with low affinity, but does not bind the putative IL-1RAcP IL-1RaCP, suggesting the possibility of yet another coreceptor waiting to be identified.
Direct injection of AdIL-1H4 into tumor induces regression of an established MCA205 fibrosarcoma in normal C57BL/6 mice

To examine the anti-tumor effects of AdIL-1H4, mice received s.c. inoculation of $2 \times 10^5$ of MCA205 cells into the right flank on day 0, and treatment was administered on day 7. Each group of five or seven mice was treated with intratumoral injection of the control Ad5 vector or AdIL-1H4 ($10^9$ PFU), and tumor size was measured every 3 days. A single injection resulted in significant tumor regression (Ad5 vs AdIL-1H4, $p < 0.01$; Fig. 2A). To investigate whether multiple intratumoral injections had any benefit on tumor regression, additional injections were performed at 3-day intervals. Interestingly, the majority of the treated mice from three separate experiments were tumor free (Fig. 2B). The results of tumor growth in individual mice in a multiple injection experiment are shown in Fig. 2C; four of seven mice were tumor free. To examine whether tumor-mice following IL-1H4 treatment were immune to MCA205 tumor, mice were rechallenged with $5 \times 10^5$ of MCA205 cells on day 45. All mice rejected tumor then, and with multiple subsequent rechallenges, some mice rejected the injected tumor cells quite rapidly, while others grew for a short period, but then were eradicated (Fig. 2D).

Analysis of the mechanism of anti-tumor activity of IL-1H4

IL-18 and IL-12 are synergistic and effective IFN-γ-inducing cytokines (27). Recent studies have indicated that the high level of IFN-γ production can also arise from a synergistic interaction between exogenous IL-18 and endogenous IL-12 (28). IL-18 and

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**FIGURE 1.** Expression and secretion of human IL-1H4 after infection. A549 cells were infected using AdIL-1H4 or Ad5 at a multiplicity of infection of 100. Seventy-two hours later conditioned media and cells were harvested or lysed as described in Materials and Methods. Proteins were separated on SDS-PAGE gel, and Western blot was used to detect IL-1H4 protein expression using purified IL-1H4 polyclonal Ab. Recombinant pro-IL-1H4 and mature IL-1H4 were used as positive controls. Two bands were detected in AdIL-1H4-infected A549 cell lysates. Broad bands were found in AdIL-1H4-conditioned media, indicating that IL-1H4 was probably modified before secretion. The result shown is representative of three different studies performed.

**FIGURE 2.** A. Single injection of AdIL-1H4 into established MCA205 tumor suppressed tumor growth. Groups of five mice (C57BL/6) received $2 \times 10^5$ MCA205 cells by intradermal inoculation into the right flank on day 0. Injections of AdIL-1H4 or Ad5 ($1 \times 10^9$ PFU) into tumor were performed on day 7. Tumor growth in the AdIL-1H4 group was significantly suppressed compared with that in the Ad5 group ($p < 0.001$). The results shown were obtained in three different experiments and are the mean ± SD. B–D. Multiple injections of AdIL-1H4 into established MCA205 tumor substantially retarded tumor growth. Groups of five (or seven) mice (C57BL/6) received $2 \times 10^5$ MCA205 cell by intradermal inoculation into the right flank on day 0. Multiple injections of AdIL-1H4 or Ad5 ($1 \times 10^9$ PFU) into tumor were conducted on days 7, 10, 13, and 16 (indicated by arrowheads). B, Tumors in AdIL-1H4 groups were significantly suppressed compared with those in Ad5 groups ($p < 0.001$). C, The response of an individual mouse to multiple AdIL-1H4 injection is shown. Tumor size is presented as the mean ± SD of the product of perpendicular diameter. Mice free of palpable tumor subsequently received inoculation with $5 \times 10^5$ of MCA205 cells. All rejected this rechallenge with tumor. D, Percentage of mice surviving after MCA205 rechallenge in AdIL-1H4-treated mice.
IL-12 anti-tumor activity is mediated via the Th1 pathway (29). Our preliminary data led us to postulate that IL-1H4 may have mediated its anti-tumor effect via a unique pathway or through a pathway similar to that used by IL-18. To begin to determine the mechanisms underlying the IL-1H4 anti-tumor effect, we examined which cell types are important for the anti-tumor effect as well as which cytokines are regulated.

In the first set of experiments, B6.Cg-Foxn1<sup>nu</sup> nude and B6.CB17-Prkdc<sup>scid</sup>/SzJ SCID mice without functional T and B cells were used. Intratumoral injections of AdIL-1H4 into mice bearing the MCA 205 fibrosarcoma were performed four times at 3-day intervals. No anti-tumor effect was noted in either nude or SCID mice, suggesting that functional T and B cells were required for an anti-tumor effect (Fig. 3, A and B). We also evaluated IL-1H4 effects in IFN-γ-deficient mice (Fig. 4A). No anti-tumor effect was observed in these mice with AdIL-1H4 injection. MCA205 tumor was also established on IL-12 p40/p40-knockout (KO) mice that were then treated with AdIL-1H4 or AdΔ5 (Fig. 4B). Similarly, no anti-tumor effect was observed. Although IL-12 can significantly augment the cytotoxicity mediated by NKT cells, the anti-tumor effect of IL-1H4 in NKT KO mice was not reduced, indicating no apparent role for this cell type (Fig. 4C). Furthermore, Fas ligand has been shown to be essential to mediate the anti-tumor activity of IL-18 (30), whereas IL-12 mediates its anti-tumor effects most prominently through perforin pathways. We tested the effect of adenovirus-mediated IL-1H4 treatment on MCA205 fibrosarcoma established in B6Smn.C3H-Fas<sup>Δld</sup> Fas ligand-deficient mice. Like IL-18 treatment, anti-tumor effects were not observed in mice lacking Fas ligand, suggesting that IL-1H4 has an unusual mix of IL-12- and IL-18-like anti-tumor activities (Fig. 4D), indicating an intermediary role between innate and adaptive immunity. We also (data not shown) tested PBMC following AdIL-1H4 conditional medium stimulation and contrasted this with control AdΔ5 stimulation and could demonstrate no IL-12 production, but approximately equal IFN-γ in both conditions.

**Immunohistochemistry**

To investigate whether AdIL-1H4 protein expression intratumorally induced an immune response or regulated gene expression, we removed the tumors 3 and 6 days following the first and second injections of virus. Immunostaining of tumor samples showed no difference between AdIL-1H4 and AdΔ5 groups in terms of the number of staining cells. Most stained cells were found localized in the periphery of tumors; a few positively stained cells were observed infiltrating areas surrounding the tumor (Fig. 5).

**Discussion**

IL-1H4/IL-1F7 is only expressed at low levels constitutively. This suggests that it may play its greatest role during an acute immune response requiring rapid up-regulation (23). IL-1H4 maps to the long arm of human chromosome 2 in a cluster of other IL-1 genes. Unlike most secreted proteins, IL-1H4 does not contain a hydrophilic leader sequence, but instead contains a significant propeptide sequence similar to that of IL-1α, IL-1β, and IL-18. Although IL-18Rα-chain is a candidate receptor subunit, IL-1H4 does not bind the other requisite IL-18Rαcp, IL-1RacpL, suggesting that high affinity binding must be mediated by at least one other chain (31). IL-1H4 transcripts have been detected in human tissues (lymph nodes, thymus, bone marrow, placenta, lung, testis, colon tumor, and uterus) and human cell lines (THP-1, U937, A431, IMTLH, KG-1, HL60, HPBMIC, HPT-4, and NHDC), and its expression could be up-regulated by PMA treatment of PBMC. To examine the biological activity of IL-1H4, we constructed a recombinant (E1/E3-deleted) adenoviral vector containing the full-length coding sequence for the IL-1H4 gene.

In the first series of experiments the anti-tumor efficacy of a single dose of virus was compared with that of multiple administrations (Table I). Notably, both single and multiple treatments caused significant anti-tumor effects. In particular, multiple treatments resulted in complete eradication of well-established tumor in most animals. Moreover, animals that rejected the tumor and were subsequently rechallenged later at different sites with a higher dose of tumor cells were immune. Consistent with a systemic long term anti-tumor effect of this treatment, this observation led us to a second series of experiments designed to dissect the possible function role of IL-1H4 as an innate mediator of inflammation, promoting the development of systemic cellular immunity.

We first evaluated the effect of adenovirus IL-1H4-mediated delivery in immune-compromised animals. For this purpose we chose B6.CB17-Prkdc<sup>scid</sup>/SzJ SCID and B6.Cg-Foxn1<sup>nu</sup> nude mice, because these mice are characterized, respectively, by the absence of functional T and B cells with retention of apparently normal Ag presentation and NK cell function. In both strains IL-1H4-mediated anti-tumor activity was impaired, consistent with the idea that cellular immunity played a critical role in the anti-tumor activity mediated by IL-1H4.

We then tested whether such treatment could be abolished in the absence of the key cytokines important in promoting an anti-tumor cellular response, specifically IFN-γ and IL-12. No effect of IL-1H4 was observed in IFN-γ KO mice. Most interestingly, when such treatment was applied to tumors borne by IL-12 p40 KO mice, the potent IL-1H4 anti-tumor activity was completely abolished. This is substantially different from that observed in animals treated with IL-18 which is effectively IL-12 independent. Thus, IL-1H4 may promote the development of the anti-tumor response through enhanced IL-12 production. Although IL-12 itself has been
considered the bridge between innate and adaptive immune responses, IL-1H4 appears to be at least one more proximal mediator of this sequence of events linking these important aspects of immunity.

Given that the anti-tumor effect of IL-18 is exerted predominantly through a Fas-dependent pathway (30), we investigated whether this was true for IL-1H4-mediated anti-tumor function as well. B6Smn.C3H-Fasldld-deficient mice, which carry a homozygous mutation of the Fas ligand gene, were tested in the MCA205 fibrosarcoma IL-1H4 treatment model. The IL-1H4 anti-tumor effect was abrogated in this animal strain, consistent with that observed in animals treated with IL-18. Thus, IL-1H4 appears to mediate a mixture of IL-12- and IL-18-like effects when dissected at a mechanistic level, again placing it potentially more proximal in the inflammatory cascade. Although IL-12-mediated anti-tumor activity appears to be NKT dependent, the anti-tumor activity of IL-1H4 appears to be NKT independent. In conclusion, our studies with IL-1H4/IL-1F7 are consistent with a partner molecule with properties similar to IL-18 whose anti-tumor activity depends on IL-12 activity. Our results suggest that IL-1H4 is a potent cytokine for inducing an anti-tumor effect following gene transfer. Thus,
References

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References