INTRODUCTION

Cytokines of the γ-chain family have the potential to be used in different clinical settings, such as AIDS and cancer immunotherapy, due to their ability to regulate the homeostasis of the immune system and to boost host responses against pathogens and tumor antigens. Interleukin-15 (IL-15) supports the development, proliferation, survival and trafficking of several lymphocyte subsets, including NK, CD8+ and γδ T cells. It has also a non-redundant role in the establishment and maintenance of CD8+ T-cell memory.1–3 The efficient secretion of IL-15 requires co-expression of the IL-15-binding protein named IL-15 receptor alpha (IL-15Ra) in the same cell.4–6 Co-production of the two molecules leads to intracellular association of IL-15 and IL-15Ra in the endoplasmic reticulum, stabilization of both molecules and efficient transport to the cell surface.6,7 This complex anchored to the cell surface is trans-presented to cells expressing the β/γ subunits comprising the low-affinity IL-2 receptor and IL-15 receptor,12 or is secreted in a soluble form upon cleavage of the transmembrane domain of the IL-15Ra.13 Indeed, the circulating form of IL-15 in biological fluids is in complex with soluble IL-15Ra (sIL-15Ra) in both mice and humans.14 Several studies in mice showed that the soluble heterodimeric IL-15:sIL-15Ra (hetIL-15) has superior pharmacokinetics and a 10- to 100-fold increase in agonistic activity over single-chain IL-15 in vivo.10,13,15–18

For therapeutic purposes, hetIL-15 can be provided in vivo as recombinant protein or by gene delivery. Systemic delivery of protein may cause significant toxicity, as it has also been reported for single-chain IL-15 (refs 19, 20) and other cytokines, such as IL-2 (refs. 21, 22) or IL-12.23 Toxicity may be reduced or eliminated through the delivery of cytokine genes, which are expressed for short periods of time. Among gene therapy approaches, the use of naked DNA is promising because of its simplicity, flexibility and possibility of repeated applications owing to the absence of immunity against the vector (for review see Ferraro et al.24). Successful gene therapy depends largely on the optimization of gene expression and on the method of delivery (for recent reviews see Kutzler and Weiner,25 Felber et al.26). We have previously described methods to develop efficient expression vectors for IL-15, combining mRNA optimization (RNA/codon optimization) of the IL-15-coding sequences and substitution of the signal peptide with other efficient secretory signals to optimize trafficking of the molecules.27 We also produced vectors expressing IL-15 in combination with either membrane-associated or soluble IL-15Ra, which resulted in stable, secreted heterodimeric IL-15:sIL-15Ra, with a final improvement of more than 1000-fold in the systemic level of bioactive IL-15 compared with vectors expressing the wild-type IL-15 cDNA.19 Substantial improvement in gene delivery using plasmid DNAs has been achieved by intramuscular injection followed by in vivo electroporation (EP).28–30 In vivo EP of naked DNAs results in increased DNA uptake and in enhanced gene expression by the cells at the injection site. Intramuscular injection followed by in vivo EP (IM/EP) has been widely used as delivery method to improve the expression and immunogenicity of human/simian immunodeficiency virus (HIV/SIV) DNA vaccines in macaques31–33 and humans.36–38 In addition, DNA EP was used to deliver cytokine genes, including IL-12 and IL-15, as vaccine adjuvants in preventive and therapeutic SIV DNA immunization in macaques.39–42 Cytokine gene delivery by in vivo EP has also
been successfully employed as cancer treatment in several preclinical and clinical studies. Intratumoral delivery of single-chain IL-15-expressing DNA by EP resulted in the complete regression of established B16 melanoma tumors in mice. In melanoma patients, the delivery of the IL-12-expressing DNA by EP has been shown to be safe, with lower toxicity in comparison to the systemic delivery of the recombinant protein.

In the present work, optimized DNA vectors encoding human heterodimeric IL-15 were delivered in rhesus macaques by the IM/EP method. Elevated levels of IL-15 were detected in the plasma and were associated with an increased proliferation of NK and T cells, with no adverse effects. These results demonstrate that intramuscular administration of optimized IL-15 vectors in non-human primates results in systemic bioactive levels of heterodimeric IL-15, suggesting possible applications in vaccination regimens and immunotherapy protocols.

RESULTS

Generation of optimized vectors expressing human IL-15 heterodimers

The optimization of DNA vectors expressing the heterodimeric IL-15 is essential for the efficient gene delivery of the cytokine in vivo. We have previously shown that combining two approaches, namely mRNA optimization (RNA/codon optimization) of the coding sequence and substitution of signal peptide with the granulocyte macrophage-colony stimulating factor secretory signal, results in synergistically improved expression and secretion of bioactive single-chain IL-15. As we have shown that the IL-15 circulating in the body is the heterodimeric form in association with the so-called IL-15Ra, we generated dual gene expression vectors producing the heterodimeric cytokine IL-15:IL-15Ra to assure expression of both chains upon in vivo DNA delivery. We produced and tested two plasmids encoding for heterodimeric IL-15:IL-15Ra cytokine, that is, IL-15:IL-15Ra (hetIL-15, plasmid AG153) and IL-15:IL-15RaFc (hetIL-15-FC, plasmid AG256). The hetIL-15 DNA produces the human soluble heterodimeric IL-15:IL-15Ra comprising a truncated IL-15Ra that lacks the transmembrane and cytoplasmic regions (Figure 1a, left panel). The hetIL-15-FC is a fusion protein comprising the human soluble heterodimeric IL-15, in which the sIL-15Ra chain is fused to the Fc region of human IgG1 (Figure 1a, right panel). The fusion to the Fc fragment results in a more stable form of IL-15 heterodimer and may also have the ability to act as a cell-associated cytokine in vivo, mimicking IL-15 trans-presentation.

![Figure 1](image-url)

**Figure 1.** Generation of optimized vectors expressing human IL-15 heterodimers. (a) Maps of the dual promoter plasmids encoding two forms of the heterodimeric IL-15 cytokine, hetIL-15 (AG153; left panel) and hetIL-15-FC (AG256; right panel). The IL-15Rx and IL-15 genes are under the control of the human cytomegalovirus (hCMV) and the simian CMV (siCMV) promoter, respectively. The polyadenylation signals from bovine growth hormone (BGH) and the simian virus 40 (SV40) were used for the IL-15Rx and the IL-15 subunit, respectively. The plasmids contain the kanamycin resistance gene for selection in bacteria. Human HEK293 cells were transiently transfected with hetIL-15 DNA-expressing IL-15:sIL-15Rα cytokine, that is, IL-15:IL-15Ra cytokine, that is, IL-15:IL-15Ra (hetIL-15, plasmid AG153) and IL-15:IL-15RaFc (hetIL-15-FC, plasmid AG256). The hetIL-15 DNA produces the human soluble heterodimeric IL-15:IL-15Ra comprising a truncated IL-15Ra that lacks the transmembrane and cytoplasmic regions (Figure 1a, left panel). The hetIL-15-FC is a fusion protein comprising the human soluble heterodimeric IL-15, in which the sIL-15Ra chain is fused to the Fc region of human IgG1 (Figure 1a, right panel). The fusion to the Fc fragment results in a more stable form of IL-15 heterodimer and may also have the ability to act as a cell-associated cytokine in vivo, mimicking IL-15 trans-presentation.
We studied the expression of these vectors after transient transfection in human HEK293 cells (Figure 1b). Co-expression of either sIL-15Ra or sIL-15RaFc with single-chain IL-15 resulted in similar levels of cytokine production measured by ELISA. More than 90% of total IL-15 was found in the cell supernatant, demonstrating efficient and comparable secretion of the cytokine upon its intracellular association with either sIL-15Ra or sIL-15RaFc. The secreted forms of IL-15 and sIL-15Ra were also visualized by western immunoblot (Figure 1c). sIL-15Ra encoded by hetIL-15 DNA migrated as a broad band of ~50 kDa (lane 1, exposure 60°), whereas sIL-15RaFc fusion protein encoded by hetIL-15-FC DNA migrated at ~75 kDa (lane 2, exposure 60°). In both cases, broad bands were indicative of extensive glycosylation, as previously reported. 13 Human IL-15 is also glycosylated and the western immunoblot analysis revealed three forms of IL-15 upon transient transfection of hetIL-15 DNA (lane 1, exposure 270°) or hetIL-15-FC DNA (lane 2, exposure 270°), namely, unglycosylated ~13 kDa, partially glycosylated ~15 kDa and fully glycosylated ~17 kDa proteins. Western immunoblot analysis performed under non-reducing condition (SDS without β-mercaptoethanol) revealed the formation of disulfide bond between the Fc regions of two hetIL-15-FC molecules (Figure 1d, lane 2, band ~200 kDa), showing that hetIL-15Fc is secreted as dimers, as expected. The formation of IL-15:sIL-15Ra complexes was verified on native gels. No single-chain IL-15, sIL-15Ra or sIL-15RaFc was detected in the supernatants of HEK293 cells transfected with either DNA, suggesting secretion of the heterodimeric forms (Figure 1e).

Detection of IL-15 in the plasma of macaques upon intramuscular delivery followed by in vivo EP of DNAs expressing heterodimeric IL-15

To test whether systemic levels of IL-15 could be achieved in vivo, the plasmids encoding the two heterodimeric forms of IL-15 were administered into rhesus macaques by IM/EP. We measured circulating human IL-15 in the plasma of two macaques (M701 and M511) injected with hetIL-15 DNA, and in two macaques (M084 and M917) injected with hetIL-15-FC DNA. Each macaque received a dose of 8 mg of DNA every 3 days. The plasma levels of the heterodimeric IL-15 were measured at the indicated time points using a commercially available human IL-15 ELISA. Endogenous levels of IL-15 before treatment ranged between 12 and 24 pg ml−1 in these macaques (Figure 2a). We have previously reported that a single administration of heterodimeric IL-15-expressing DNA via IM/EP results in IL-15 plasma levels that peak at day 3 or 4 after the injection, followed by a decline to baseline levels within 1 week. 23 Plasma IL-15 levels increased approximately twofold over baseline 3 days after the first injection, and continued to increase upon subsequent injections. Peak levels of ~100 pg ml−1 were reached between day 7 and 12 (range = 85–175 pg ml−1). In three of the four animals, no further increase in plasma IL-15 was observed after the fourth and fifth DNA injection. Plasma IL-15 levels were still higher than the baseline values at the time of the last measurement (day 15, 3 days after the last DNA injection), with the exception of animal M701 (Figure 2a). Interestingly, the plasmids encoding the two different heterodimeric forms of IL-15 produced similar IL-15 plasma levels overtime and similar area-under-the-curve values (Figure 2b).

Hematologic profile of rhesus macaques treated with DNA-encoded heterodimeric IL-15

The cytokine DNA injections were well tolerated with no adverse effects. The hematologic profile of the four animals was monitored at days 0, 2, 7 and 14 after the start of the treatment and no abnormalities were detected. Lymphocyte, monocyte, eosinophil, basophil and neutrophil counts were within the normal range reported for rhesus macaques throughout the study. The lymphocyte and neutrophil profiles are reported in Table 1. Similarly, no abnormalities were detected in the platelet count, hematocrit or hemoglobin content. Serum chemistry analysis was also performed at the same time points (Table 2). A transient increase in aspartate aminotransferase and creatinine phosphokinase was observed in all animals, whereas only one animal (M511) had a small increase in alanine aminotransferase. However, measurements in historical control macaques suggest that blood

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**Table 1.** Absolute lymphocyte and neutrophil counts during IL-15 treatment

| Macaque | Cell count after initiation of IL-15 treatment (cells per μl blood) |
|---------|------------------------------------------------------------------|
|         | Day 0 | Day 2 | Day 7 | Day 14 |
| Lymphocytes (cells per μl blood) | |
| M701    | 3312  | 2360  | 1980  | 2856  |
| M511    | 4225  | 4161  | 2964  | 4628  |
| M084    | 2001  | 3116  | 2024  | 2552  |
| M917    | 3300  | 2592  | 1834  | 2146  |
| Neutrophils (cells per μl blood) | |
| M701    | 3384  | 3127  | 2205  | 2240  |
| M511    | 2015  | 2701  | 4368  | 3827  |
| M084    | 4002  | 4182  | 6072  | 2204  |
| M917    | 3750  | 2322  | 3111  | 4958  |

Lymphocytes and neutrophil absolute counts were reported as cells per μl blood overtime, before and during the heterodimeric IL-15 treatment for the individual animals. Abbreviation: IL, interleukin.

Figure 2. Plasma IL-15 levels after intramuscular delivery of IL-15 heterodimer DNAs followed by in vivo electroporation in rhesus macaques. (a) Two groups of macaques (N = 2) were subjected to intramuscular injection followed by in vivo EP using hetIL-15 DNA or hetIL-15-FC DNA vectors at days 0, 3, 6, 9 and 12, as marked by arrows, using a dose of 8 mg DNA per injection. Plasma IL-15 levels were measured overtime before, during and after the IL-15 treatment by ELISA and reported for the animals receiving hetIL-15 DNA (left panel) or hetIL-15-FC DNA (right panel). (b) Area-under-the-curve (AUC) of IL-15 plasma levels over the 15 days was determined for the individual animals.
samples taken from sedated animals could have abnormally high creatinine phosphokinase values because of increased tonic muscular activity sometimes associated with the use of ketamine as anesthetic drug. Transient elevation of creatinine phosphokinase has been also previously noted following EP to the muscle. The majority of the values returned to normal range at day 14 of the study (Table 2).

We also examined the effect of the IL-15 treatment on the virological status of the animals. The macaques were infected with SIVsmE660 (M917, M701) or SHIV (M511, M084) and controlled viremia from 18 months to 7 years before enrollment in this study. The plasma viral loads were determined in all the animals before and after IL-15 treatment (Table 3). Macaques M917 and M511 had undetectable viral loads before treatment and no changes were observed throughout the study. Macaques M701 and M084 had low pre-treatment viremia (1.8 x 10^3 and 4 x 10^4, respectively), which could contribute to the increased baseline lymphocyte proliferation observed in peripheral blood (see below). No significant changes in viral load were observed during the study in these animals either (Table 3), suggesting that IL-15 treatment does not impact viremia significantly, which is in agreement with previous observations.

The development of anti-IL-15 antibodies upon delivery of human IL-15 into rhesus macaques has been previously described. To determine whether administration of DNAs encoding human heterodimeric IL-15 induced an immune response against the human molecules, macaque plasma samples were screened for the presence of antibodies against human IL-15 or human IL-15Ra by western immunoblot analysis. The presence of antibodies was evaluated using plasma samples collected before the treatment, at day 14 after the initiation of the treatment cycle and at 7 months after termination of the study. All the samples scored negative (data not shown), suggesting that no immune responses against human IL-15 heterodimers were induced under this treatment protocol.

Collectively, these data demonstrate that it is feasible to obtain safe supraphysiological plasma levels of heterodimeric IL-15 in rhesus macaques upon IM/EP delivery of optimized-plasmid DNAs.

Elevated systemic levels of heterodimeric IL-15 result in proliferation of NK and T cells in peripheral blood in rhesus macaques

IL-15 promotes the survival and stimulates the proliferation of several leukocyte populations, including NK cells, canonical CD8+ and γδ T lymphocytes. Therefore, we evaluated the effect of heterodimeric IL-15 on the proliferation of lymphocytes in peripheral blood. Peripheral blood mononuclear cells isolated prior, during and after the IL-15 DNA administrations were stained for Ki-67, a nuclear marker expressed by proliferating cells. At baseline, ~5–10% of γδ T cells (Figure 3a), ~5–15% of CD8+ T cells (Figure 3b) and ~10–20% of CD4+ T cells (Figure 3c) were Ki-67+, with the exception of macaque M084. This animal had an abnormally elevated level of proliferating T cells (see below).

Administration of DNAs expressing heterodimeric IL-15 stimulated the proliferation of γδ and CD8+ T cells in peripheral blood. The percentage of proliferating γδ T lymphocytes peaked between days 9 and 12, when 30–60% of the cells were Ki-67+. In the two animals that received hetIL-15 DNA, the proportion of proliferating γδ T cells was still elevated over the baseline after termination of the treatment (day 15; Figure 3a, left panel). Similarly, CD8+ T cells showed increased proliferation in response to IL-15 heterodimer starting at approximately day 9, and, by day 12, ~20–35% of CD8+ T cells were Ki-67+ (Figure 3b, left panel) and remained elevated up to day 15. No significant changes in the proportion of proliferating total CD4+ T cells were observed throughout the study in any of the IL-15-treated animals (Figure 3c, left panel). Macaque M084 showed significantly higher baseline levels of proliferating T cells (~30% of γδ T and CD8+ T cells, and ~50% of CD4+ T lymphocytes were Ki-67+; Figures 3a–c, diamonds), most likely as a consequence of the immune activation secondary to chronic viremia (Table 3). In this animal, IL-15 DNA administrations resulted in a modest peak increase (~2-fold) in the percentage of both γδ and CD8+ T proliferating cells, following kinetics similar to the other macaques. We also investigated the proliferation of NK cells, defined as CD3+CD16+, in response to heterodimeric IL-15. At baseline, the percentage of NK cells expressing Ki-67 was ~10–30% in the different animals. The proportion of proliferating NK cells peaked between days 9 and 12, when 50–80% of NK cells were Ki-67+ and remained elevated at day 15 (Figure 3d, left panel). In contrast, no significant changes in the percentage of proliferating lymphocytes were observed between day 0 and day

### Table 2. Enzyme levels in blood during IL-15 treatment

| Macaque | Enzyme | Enzyme units |
|---------|--------|--------------|
| M701 | AST (normal range 14–30 U l⁻¹) | 28 * 51* 72* 81* |
| M511 | AST (normal range 20–40 U l⁻¹) | 24 * 221* 76* 33* |
| M504 | AST (normal range 20–40 U l⁻¹) | 23 * 44* 56* 27 |
| M917 | ALT (normal range 0–82 U l⁻¹) | 32 * 44* 68* 30 |
| M701 | ALT (normal range 0–82 U l⁻¹) | 30 * 50 70 60 |
| M511 | ALT (normal range 0–82 U l⁻¹) | 28 * 296* 123* 63 |
| M084 | ALT (normal range 0–82 U l⁻¹) | 18 * 39 39 23 |
| M701 | CPK (normal range 0–136 U l⁻¹) | 135 * 58* 159* 542* |
| M511 | CPK (normal range 0–136 U l⁻¹) | 324 * 1502 1538 244 |
| M504 | CPK (normal range 0–136 U l⁻¹) | 482* 418 2728 288 |
| M917 | CPK (normal range 0–136 U l⁻¹) | 944* 694 1964 351 |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatinine phosphokinase; IL, interleukin. *Values outside the normal range. AST, ALT and CPK were reported as U l⁻¹ in blood overtime, before and during the heterodimeric IL-15 treatment for the individual animals. Normal range for rhesus macaques are also provided.

### Table 3. Viral load levels after initiation of IL-15 treatment

| Macaque | Gender | Age at study onset (years) | Infected by | Time infected before study onset (years) | Viral load (RNA copies per ml plasma) |
|---------|--------|--------------------------|------------|----------------------------------------|--------------------------------------|
| M701    | Male   | 8                        | SIVsmeE660 | 1.5                                    | 18 809                               |
| M511    | Male   | 10                       | SHIV163P3  | 5                                      | < 50                                 |
| M504    | Male   | 13                       | SHIV 89.6P | 7                                      | 40 283                               |
| M917    | Female | 9                        | SIVsmeE660 | 1.5                                    | < 50                                 |

Viral load levels were reported as RNA copies per ml plasma overtime, before and during the heterodimeric IL-15 treatment for the individual animals. Abbreviation: IL, interleukin.
14 in the macaques treated with sham DNA by IM/EP (Figures 3a–d, right panels). These data show that the cell proliferation observed in the animals treated with heterodimeric IL-15 DNA was due to the specific effects of the IL-15 treatment.

We further analyzed the phenotype of the proliferating NK cells to determine whether a specific subset preferentially responded to heterodimeric IL-15. In particular, we studied expression of CD8, as it has been reported that human NK cells expressing the CD8 antigen have more potent cytotoxic function compared with their CD8− counterpart. In all animals, the majority (>85%) of CD3+ CD16+ NK cells expressed CD8 at baseline (Figure 4a). CD8+ NK cells preferentially expanded in response to IL-15 heterodimer, and by day 9, we found an approximately two- to fourfold increase in the proportion of proliferating CD8+ NK cells, whereas no significant increase in the frequency of Ki67+ cells was observed in the CD8− counterparts (Figure 4a). As reflection of the great

![Figure 3.](image-url)
proliferation of NK cells in response to systemic levels of heterodimeric IL-15 after repeated DNA injections (Figure 3d), the absolute NK cell count in blood increased by two- to sixfold (Figure 4b, top panel). Further analysis revealed that, indeed, the CD8+ NK cell subset accounted for this increase (Figure 4b, middle panel), whereas IL-15 had little effect on CD8− NK cells (bottom panel). Only a marginal increase in the absolute count of NK cells was observed in macaque M917, possibly as a result of a more rapid movement of cells to the tissues.

In conclusion, we noted a hierarchical proliferative response in peripheral blood of rhesus macaques treated with IL-15 DNA, with NKγδ4γδ4CD84CD4+ T cells, suggesting that the different lymphocytes have different threshold of response to systemic IL-15 levels. In agreement with the pharmacokinetics, no differences in biological effects were observed after delivery of either hetIL-15 or hetIL-15-FC DNAs.

Effects of IL-15 on different memory T-cell subsets
We also investigated the effects of both forms of the heterodimeric IL-15 on subsets of CD8+ and CD4+ T cells. Analysis of the surface markers CD45RA, CD28, CD95, CCR7 and CD127 allowed distinguishing among different memory T-cell subsets. For both CD8+ and CD4+ lymphocytes, naïve T cells (TNa) were defined as CD95−CD28−CD45RA−CCR7+, central memory (TCM) as CD95−CD28−CD45RA−CCR7+, transitional memory (T TM) as CD95−CD28−CD45RA−CCR7+ and effector memory (TEM) as CD95CD28−, as previously reported and shown in representative flow plots (Figures 5a and 6a). In the case of CD8+ T cells, a population defined as T-stem memory cells (T SCM) has been described as CD95−CD28−CD45RA−CCR7+CD127+ (Figure 5a).49,50 This population comprising the least differentiated memory T cells is capable of self-renewal generating TCM and it is strictly dependent on IL-15.49,50 Before treatment, few CD8+ TNa cells were Ki-67+ and their frequency did not change significantly as a consequence of the heterodimeric IL-15 treatment (Figure 5b). In contrast, the proportion of CD8+ T SCM, T CM, T TM and TEM fractions that expressed Ki-67 increased approximately two- to threefold during the course of the study, consistent with a proliferative response induced by the hetIL-15 treatment. The frequency of proliferating memory CD8+ T cells peaked between days 12 and 15, and all the memory compartments were affected similarly by the treatment (Figure 5b). Despite this elevated proliferation, no increase in the absolute count of CD8+ T cells in blood was observed in any of the animals (data not shown), suggesting either a rapid mobilization to the tissues or proliferation-linked apoptosis, as previously reported.19,51,52 The gating strategy used to identify the different CD4 T memory cells is depicted in Figure 6a. Heterodimeric IL-15 also induced proliferation of TEM CD4+ in all animals with the exception of M084, and of TEM CD4+ in two of the four animals, but to a lesser extent than CD8+ T cells (~1.5- to 2-fold; Figure 6b). A modest variation in the frequency of proliferating cells in the T CM CD4+ compartment was only observed in animal...
M511, whereas no changes were observed in the CD4+ TN subset in any of the animals (Figure 6b). Taken together, these data suggest that heterodimeric IL-15 induces the preferential proliferation and accumulation of lymphocytes characterized by the expression of CD8 antigen, either NK or T cells. In addition, heterodimeric IL-15 targets both CD8+ and CD4+ T cells, mainly those with a memory/effector phenotype.

**DISCUSSION**

Several investigators have tested the effect of single-chain IL-15, provided as recombinant protein, in non-human primates. Among these studies, recombinant *Escherichia coli*-derived single-chain human IL-15 has been tested in preclinical studies in macaques upon intravenous injection, subcutaneous injection and continuous intravenous infusion. The subcutaneous and continuous intravenous protocols are currently being evaluated in phase I clinical studies in patients with refractory metastatic melanoma and metastatic renal cell carcinoma (NCT01572493 and NCT01727076). Administration of single-chain IL-15 resulted in an increase in the absolute number and proliferation of peripheral NK and memory CD8+ T cells. Increased proliferation and migration to peripheral tissues of effector memory CD4+ T cells was also reported, although no significant effects on CD4+Foxp3+ Treg cells were observed.ْ
T-regulatory cells were observed.\textsuperscript{19,53,54} It has been shown that high-dose IL-15 is able to expand Tregs in vitro, although these cells were characterized by weak suppressive activity.\textsuperscript{55,56} Single-chain IL-15 delivery to macaques did not result in a preferential proliferation of Tregs as compared with the general CD4\(^+\) T-cell population.\textsuperscript{19} Although administration of the single-chain IL-15 was overall well tolerated, transient neutropenia and hypotension were observed,\textsuperscript{19,20} effects possibly related to the spike levels of IL-15 in serum.

Several reports suggested that gene therapy approaches could reduce or eliminate the toxicity associated with cytokine treatment. However, the major limitation of these preclinical and clinical studies was the undocumented efficiency of gene expression. This report explores the delivery, efficiency and safety of heterodimeric IL-15:sIL-15R\(\alpha\) gene therapy in rhesus macaques. Although single-chain IL-15 was shown to be bioactive in non-human primates,\textsuperscript{19,36} the use of heterodimeric IL-15 may provide additional advantages, as the heterodimeric IL-15:sIL-15R\(\alpha\) is a natural IL-15 form circulating in the blood,\textsuperscript{14} and is bioactive and more stable in vivo compared with monomeric IL-15.\textsuperscript{10,13} The physiological IL-15 plasma levels in rhesus macaques (~10–20 pg ml\(^{-1}\)) were elevated 5- to 10-fold upon repeated delivery of DNA encoding heterodimeric IL-15:sIL-15R\(\alpha\) without any sign of toxicity. Elevated IL-15 levels were observed for more than 10 days, which suggests continuous production of IL-15 from the injected DNA vectors. Importantly, IL-15 delivered as DNA did not induce the high systemic cytokine spikes, likely responsible for the toxicity associated with that treatment, commonly observed after administration of recombinant proteins. Muscle is a physiologic location for IL-15 production\textsuperscript{57} and the IL-15 gene therapy did not cause any adverse effects in macaques. The achieved systemic levels of IL-15 were biologically relevant and had the expected effects on target populations. Heterodimeric IL-15 induced the proliferation of NK, \(\gamma\delta\) T cells, all memory CD8\(^+\) T cells, including stem cells memory, and effector memory CD4\(^+\) T cells in peripheral blood. Higher proliferation resulted in increased blood NK absolute cell counts. These results demonstrate that delivery of IL-15 as DNA or as recombinant protein induces similar effects in macaques. Interestingly, the systemic IL-15 peak levels achieved by DNA injections were similar to the ones obtained in macaques treated via the subcutaneous route with 1 \(\mu\)g kg\(^{-1}\) of purified human IL-15 heterodimer (unpublished data), but persisted for a longer
period of time, indicative of continuous production of IL-15 from the injected DNA vectors in vivo. Therefore, heterodimeric IL-15 gene therapy using naked DNA delivered by IM/EP offers a versatile and effective way to obtain systemic-acting level of IL-15, without toxicity.

The use of SIV-infected macaques has also allowed us to evaluate the effects of IL-15 gene therapy on viral replication during chronic infection, in the absence of antiretroviral treatment. It has been reported that IL-15 administration during acute SIV infection resulted in increased viral set point and accelerated disease progression.10,19 However, other reports suggested that IL-15 treatment during chronic infection does not contribute to viral replication.9,19 In agreement with these findings, no differences were observed in the virological status of the animals before and after IL-15 DNA treatment, suggesting that IL-15 gene delivery is safe in the context of chronic HIV/SIV infection. In addition, heterodimeric IL-15 was able to promote the expansion of T cells, including effector memory CD4+ in infected animals.

In the present report, we have also compared the bioactivity of two different forms of heterodimeric IL-15. Heterodimeric IL-15: sIL-15Rα was shown to be superior to single-chain IL-15 in anti-cancer activity in several mouse studies.10,17,18,60,61 In macaques, both forms of the heterodimeric IL-15 expressed after DNA injection resulted in similar plasma levels and similar bioactivity. This is in contrast to the reported mouse data, where DNA injection resulted in increased plasma half-life. 15 The reason for this discrepancy could be either impaired entry into the blood stream of human IL-15: sIL-15RαFc from the site of production or, most likely, the high binding affinity of human IgG to the murine neonatal Fc receptor form.62,63 These results suggest important differences in the properties and biological activity of the two IL-15 heterodimers observed in mice and macaques.

In conclusion, improvements in vector design and delivery methods indicate that it is feasible to use IL-15 heterodimer-encoding DNA to produce systemic levels of bioactive cytokine, with minimal toxicity. This study provides a strong rationale for the evaluation of IL-15 heterodimer gene therapy for clinical applications, as an alternative to the recombinant protein administration.

MATERIALS AND METHODS

Generation of IL-15 heterodimer-encoding DNAs

Optimized dual promoter plasmids expressing the two chains of the human heterodimeric cytokine IL-15: sIL-15Rα (hetIL-15) have been previously described.10 Briefly, the backbone vector used for the generation of the IL-15 heterodimer-expressing constructs, pDP, contains two expression cassettes, consisting of the human cytomegalovirus promoter linked to the bovine growth hormone polyadenylation signal; the simian cytomegalovirus promoter linked to the simian virus 40 (SV40) polyadenylation signal in a counter-clockwise orientation; and the kanamycin-resistance gene. The hetIL-15 DNA (AG153) used in this study contains RNA-optimized IL-15 under the control of simian cytomegalovirus promoter, and RNA-optimized sIL-15Rα without the transmembrane and cytoplasmic tail (amino acids 1–205) under the control of human cytomegalovirus promoter; the vector produces a soluble form of human heterodimeric IL-15. A similar dual promoter plasmid, in which the C-terminus of the sIL-15Rα region is genetically fused to the Fc region of human IgG1 immunoglobulin, was also generated (hetIL-15-FC, AG256).

In vitro transfection and IL-15 heterodimer measurement

Expression levels of human IL-15 heterodimer from hetIL-15 and hetIL-15-FC DNAs were tested by transient transfection of human HEK293 cells. Cells were plated in 60-mm tissue culture dishes at the density of 1 x 10⁶ cells in complete Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and allowed to adhere overnight. The next day, the cells were transfected with 100 ng of IL-15 heterodimer-expressing DNAs, using the calcium phosphate co-precipitation technique. Cotransfection of 50 ng of the green fluorescent protein expression vector pFRED143 served as internal control. After 48 h, culture supernatant and cells, lysed in a 0.5% Triton buffer, were harvested. Human IL-15 levels were measured by colorimetric ELISA (Quantikine Human IL-15 immunoassay; R&D Systems, Minneapolis, MN, USA) or by western immunoblot (using the polyclonal goat anti-human IL-15Rα antibody AF247 (R&D Systems)). For western immunoblot assays, aliquots (1/200) from the extracellular fractions were resolved on 12% TGX Bio-Rad gels (Bio-Rad, Hercules, CA, USA) under reducing conditions (treated with SDS and β-mercaptoethanol) or non-reducing conditions (treated with SDS without β-mercaptoethanol). TGX Bio-Rad gels (4–15%) were used to analyze proteins under native conditions (no β-mercaptoethanol, no SDS). After transfer, the membranes were probed with a mixture of anti-IL-15 and anti-IL-15Rα antibodies (1:1000), followed by the anti-goat HRP secondary antibody (Calbiochem, Billerica, MA, USA). The protein bands were visualized on immunoblots by Enhanced Chemi-Luminescence (GE HealthCare, Pittsburgh, PA, USA) and imaged using autoradiography or the ChemiDoc XRS+ system (Bio-Rad). Green fluorescent protein levels in cell extracts were measured using a SpectraMax Gemini EM fluorimeter (Molecular Devices, Sunnyvale, CA, USA).

IL-15 DNA injection in rhesus macaques

Indian rhesus macaques (Macaca mulatta) were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International, at the Advanced BioScience Laboratories, Inc. The animals received 8 mg of plasmid DNA expressing different forms of the heterodimeric IL-15 by IM/EP using the ELGEN electroporation device (Inovio Pharmaceuticals, Inc.). The plasmid DNA was delivered at four sites (left and right inner thighs and left and right upper arms), using 0.5 ml per site of an endotoxin-free plasmid DNA preparation (Qiagen, Vale, CA, USA) at the concentration of 4 mg ml⁻¹. Macaques received IL-15-expressing plasmid DNA every 3 days for a total of five treatments. Animals M701 and M917 had been infected with SIV E660 for 18 months before initiation of this study. Animals M511 had been infected with SHIV 162P3 for 5 years. Animal MO84 had been infected with SHIV 89.6P for 7 years. Viral loads were measured before and after the IL-15 treatment.

Plasma IL-15 measurements in rhesus macaques

Rhesus macaques were bled at different time points prior, during and after IL-15 heterodimer DNA administration (day 0, 2, 5, 6, 7, 9, 12, 14 and 15). IL-15 plasma levels were evaluated using a chemiluminescent immunoassay (Quantiglo, Q1500B; R&D Systems), according to manufacturer’s instructions. This assay cross-reacts with rhesus macaque IL-15, allowing the determination of endogenous plasma IL-15 levels prior to treatment.

Analysis of lymphocyte subsets in peripheral blood mononuclear cell and BAL

Peripheral blood mononuclear cells were isolated by Ficol density gradient centrifugation and cryopreserved in liquid nitrogen until analysis. Immunophenotypic analysis was performed using the following directly conjugated anti-human antibodies: APC-Cy7 CD3 (clone SP34-2), V500 CD4 (clone L200), PE-CD95 (clone DX2), PECy7-CD16 (clone 3G8) and PE-yt CD4 (clone B1) were obtained from BD Biosciences (San Diego, CA, USA); APC-CRCR7 (R&D Systems; clone 150053); AF405-CD8 (Caltag, Buckingham, UK; clone 385), PerCpCy5.5-CD28 (Biolegend, San Diego, CA, USA; clone CD28.2), AF700-CD45RA (Abd Serotec, Raleigh, NC, USA; clone F8-11-13) and PECy7-CD127 (Biolegend; clone A019D5) were also used in these analysis. Cell proliferation was monitored by staining with AFT00- or FITC-Ki-67 Ab in cells permeabilized with the Fixp3 Staining Buffer Set (eBioscience, San Diego, CA, USA). All the samples were acquired in a LSR II Flow Cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar, Inc., Ashland, OR, USA).

Determination of the immunogenicity of human IL-15 and IL-15Rα in macaques

Monkey plasma samples were analyzed for the development of antibodies against human IL-15 and human IL-15Rα using western immunoblot. hetIL-15 protein (150 ng, dissolved in a buffer containing SDS and β-mercaptoethanol) was resolved on 12% TGX Bio-Rad polyacrylamide gel electrophoresis and transferred to nitrocellulose (Millipore, Billerica, MA, USA) using the Trans-Blot Turbo System (Bio-Rad). Horseradish peroxidase-conjugated goat anti-human IL-15Rα and human IL-15 antibodies (1:1000) were used as primary antibodies. Immunoreactivity was revealed with the chemiluminescent substrate ECL Western blotting detection reagents (Amersham, Arlington Heights, IL, USA).
gels and transferred onto nitrocellulose membranes (Life Technologies, Grand Island, NY, USA), which were probed with plasma (1:1000 dilution) from DNA-treated animals followed by anti-monkey IgG-HRP labeled (1:10,000 dilution). The presence of antibodies reacting against human IL-15 or human IL-15Rα was evaluated using plasma samples collected before the treatment, on day 14 after the initiation of the treatment cycle and 7 months after termination of the study.

Statistical analysis
Statistical analysis was performed using Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

CONFLICT OF INTEREST
CB, AV, GNP and BKF are inventors on US Government-owned patents and patent applications related to DNA vaccines, IL-15 and gene expression optimization. NYS is a full-time employee of Inovio Pharmaceuticals and as such receives compensation in the form of salary and stock options. This does not alter our adherence to all the policies on sharing data and materials.

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