Myeloid cell-based delivery of IFN-γ reprograms the leukemia microenvironment and induces anti-tumoral immune responses

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Abstract

The immunosuppressive microenvironment surrounding tumor cells represents a key cause of treatment failure. Therefore, immunotherapies aimed at reprogramming the immune system have largely spread in the past years. We employed gene transfer into hematopoietic stem and progenitor cells to selectively express anti-tumoral cytokines/macrophages. We show that interferon-γ (IFN-γ) reduced tumor progression in mouse models of B-cell acute lymphoblastic leukemia (B-ALL) and colorectal carcinoma (MC38). Its activity depended on the immune system’s capacity to respond to IFN-γ and drove the counter-selection of leukemia cells expressing surrogate antigens. Gene-based IFN-γ delivery induced antigen presentation in the myeloid compartment and on leukemia cells, leading to a wave of T cell recruitment and activation, with enhanced clonal expansion of cytotoxic CD8+ T lymphocytes. The activity of IFN-γ was further enhanced by either co-delivery of tumor necrosis factor-α (TNF-α) or by drugs blocking immunosuppressive escape pathways, with the potential to obtain durable responses.

Keywords ex vivo gene therapy; immunotherapy; interferon-gamma; leukemia; Tie2-expressing monocytes

Subject Categories Cancer; Immunology

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Introduction

The tumor microenvironment (TME) has become of central interest to identify novel therapeutic targets. TEMs promote tumor growth and progression through several mechanisms, including polarization of host immunity to prevent anti-cancer immune responses (Binniewies et al., 2018). Thus, recent work has focused on restoring the endogenous capacity of the immune system to recognize and eliminate malignant cells (Riley et al., 2019), particularly with immunotherapy strategies that activate anti-tumoral T cell responses (Ghirelli & Hagemann, 2013; Motz & Coukos, 2013). One component of the immune system exerting several fundamental pro-tumoral functions is represented by tumor-associated macrophages (TAM) (Quatromoni & Eruslanov, 2012; Caux et al., 2016; DeNardo & Ruf-fell, 2019). In particular, a specific subpopulation of pro-tumoral macrophages characterized by the expression of the angiopoietin receptor TIE2 (TIE2-expressing monocytes -TEM) has been identified and associated with proangiogenic as well as immunosuppressive activities (Lewis et al., 2007; Pucci et al., 2009). Such population has been employed in a gene therapy-based approach to achieve reversion of the immunosuppressive TME, as their turnover from bone marrow (BM) progenitors allowed TEMs to be exploited as vehicles delivering interferon-α (IFN-α) following transplantation of genetically-engineered hematopoietic stem and progenitor cells (HSPC) (De Palma et al., 2008). Local expression of IFN-α at the tumor site was associated with a general reprogramming of the immune infiltrate towards a pro-inflammatory rather than anti-inflammatory polarization state. That led to reduced tumor burden through multiple mechanisms, including decreased angiogenesis and metastatic seeding, restored antigen presentation capacity, and...
improved T cell priming and effector functions (Escober et al., 2014, 2018; Catarinella et al., 2016). A phase I/II clinical trial using the IFN-α gene therapy approach started in patients affected by glioblastoma multiforme (NCT03866109). Some of the therapeutic effects, such as enhancement of antigen presentation, are indirectly mediated through induction of IFN-γ expression. IFN-γ is principally expressed by natural killer and T cells engaging in a positive feedback loop with macrophages, which on their side release pro-inflammatory cytokines such as interleukin-12 (IL-12), increase phagocytosis, and upregulate the antigen presentation machinery, thereby favoring the deployment of adaptive immunity (Alspach et al., 2019). IFN-γ is a critical stabilizer of a T-helper 1 phenotype in CD4+ T cells and confers cytolytic capabilities to CD8+ T cells (Alspach et al., 2019). Early studies have shown that IFN-γ-insensitive mice developed tumors more rapidly than wild-type (WT) controls (Kaplan et al., 1998). Since then, IFN-γ signaling, both in tumor cells and in the TME, has emerged as a pivotal effector of anti-tumor immunity during the “elimination” and “equilibrium” phase of cancer immunoediting and correlates positively with clinical responses to immunotherapies (Ivashkiv, 2018; Alspach et al., 2019). With type-I interferons, tumor necrosis factor (TNF-α), and some interleukins (e.g., IL-12), IFN-γ is a top cytokine candidate to stimulate antitumor immunity. We hypothesized that gene-based, tumorspecific IFN-γ delivery exploiting TEMs augmented antitumor immune responses while avoiding side effects associated with systemic administration. In an aggressive leukemia mouse model, we show that IFN-γ gene therapy boosted anti-leukemia immune responses, resulting in the elimination of more immunogenic subclones expressing surrogate antigens and selecting for more primitive disease variants that escape immune control. As chronic IFN-γ exposure induced counterregulatory responses undermining its efficacy, we have shown that combination therapies improve therapeutic efficacy.

Results

Gene therapy-based delivery of IFN-γ and TNF-α

To evaluate the antitumor potential of cytokines in the context of our HSPC gene therapy platform (De Palma et al., 2008; Escober et al., 2014), lineage-negative HSPC from CD45.1 donor mice were transduced with lentiviral vectors expressing either mouse IFN-γ, TNF-α, or a biologically inactive variant of human NGFR under the control of the Tie2e/p and microRNA 126/-130a target sequences (Fig EV1A and B). When transplanted into lethally irradiated CD45.2 recipients, these cells, independently from the transgene, fully reconstituted hematopoiesis of the animals, with the persistence of gene-marking in vivo with vector copy numbers (VCN) ranging from 0.4 to 1 (Fig EV1C and D). Conversely, transplantation of cells, where these cytokines were expressed from a strong myeloid-specific promoter (SP146-gp91), resulted in 100% lethality by day 17 (Appendix Table S1). Instead, no significant hematologic abnormalities were observed compared with controls, except a minor T cell reduction in the TNF-α group, suggesting specificity of gene expression control by the miRNA-regulated Tie2e/p cassette (Fig EV1C and E), confirmed by the modest up-regulation of IFN-γ-responsive genes in the tissues, without altering blood biochemical parameters and with barely detectable IFN-γ levels in the plasma of engrafted mice (Fig EV1F–H). For IFN-γ, an in-depth toxicity study was performed, confirming that transduced cell engraftment was stably maintained without negative impact on BM progenitor cell numbers (Fig EV1I). Necropsy with organ histopathology did not reveal abnormalities, except for an incidental finding of thymoma in a single mouse (full pathology report in Appendix Supplementary Text).

Transplanted animals were then challenged with a B-ALL (line #11), which has previously been generated by inducing overexpression of miR-126 in HSPCs (Nucera et al., 2016) and has extensively been characterized in the context of IFN-α gene therapy (Escober et al., 2018). While TNF-α gene therapy had limited efficacy, IFN-γ gene therapy showed a substantial initial reduction in leukemia development, followed by leukemia progression in most animals (Fig EV2A). Initial leukemia control in the IFN-γ group was associated with an incremental increase in CD8+ T lymphocytes and a significant increase in MHC II+ macrophages in the spleen (Fig EV2B and C). Encouraged by these data, we replicated the efficacy of IFN-γ in reducing tumor burden (Fig 1A and B) and evaluated its effects on BM cells at different time-points after B-ALL administration. Similar to splenic macrophages, IFN-γ strongly upregulated MHC II on BM macrophages (Fig 1C), which were maintained in numbers on 17 days at significantly higher frequencies than control (Fig 1D). Interestingly, IFN-γ induced an initial up-regulation of MHC II on B-ALL cells (Fig 1E). Diversely from the leukemia-associated myeloid compartment, MHC II expression on B-ALL cells was reduced at the late time-point, potentially indicating leukemia-specific escape pathology report in Appendix Supplementary Text).
Figure 1.
mechanisms. Some mice from the IFN-γ group showed increased proportions of BM T cells at day 12, which decreased to a much lower extent at disease progression on day 17 compared to control (Fig 1F). Within CD8 T cells, the central memory subset was more represented in the IFN-γ group (Fig 1G).

The effects of IFN-γ gene therapy were recapitulated with an independently generated B-ALL disease (line #8; Fig EV2D). Of note, some of the treated animals had drastically lower levels of leukemia at the experimental endpoint, with one animal showing barely detectable levels of leukemic cells, both in the peripheral blood and in the BM (Fig EV2E). We, again, observed increased MHC II expression within the BM microenvironment, and a trend towards more macrophages and central memory CD8 T cells (Fig EV2F–J). Furthermore, in a solid tumor model of colorectal carcinoma, tumor size was significantly reduced in the IFN-γ group (Appendix Fig S1A), and so was the tumor weight (Appendix Fig S1B). IFN-γ induced an increase in CD8+ T lymphocytes (Appendix Fig S1C) and a concomitant reduction in CD11b+ myeloid cells within the TME (Appendix Fig S1D).

To approach a more clinically relevant experimental model, we tested the efficacy of IFN-γ gene therapy in a therapeutic setting. Mice challenged with line #11 B-ALL received chemoradiotherapy for disease control and transplant conditioning, and were then infused with gene-modified lineage-negative HSPCs (Fig 1H). Importantly, in this therapeutic setting, IFN-γ gene therapy resulted in significant leukemia growth inhibition compared to control animals (Fig 1I). In replicate experiments, where vincristine chemotherapy and irradiation were given earlier after B-ALL injection (Fig EV3A), most animals were cured from leukemia in both IFN-γ and control groups (Fig EV3B). To model B-ALL relapse, mice surviving the first challenge were then injected with a B-ALL subclone of line #11 (NGFR+/Ovalbumin+). Mice from the IFN-γ gene therapy group showed a significant delay in relapse kinetics (Fig EV3C), which translated into improved clinical condition (Fig EV3D) and prolonged survival (Fig EV3E).

To further confirm clinical translatability, a humanized Tie2.IFN-γ construct was designed, validated for functionality and absence of toxicity on human culture-derived M2 macrophages and CD34+ HSPC, and tested in a therapeutically relevant model of human B-ALL, in combination with CD19 CAR-T cells (Fig EV3F–L).

**IFN-γ effects on leukemia growth are immune-mediated**

Next, we challenged the mice with a B-ALL subline that expresses the dominant-negative variant of IKAROS1 (IKAROS6-IK6) in line #8 (B-ALL #8-IK6) (Nucera et al., 2016). IK6 is found in human B-ALL and promotes B-cell differentiation block, progenitor proliferation, and DNA damage accumulation (Fig EV4A) (Nakase et al., 2000; Tonnelle et al., 2001; Sezaki et al., 2003; Kano et al., 2008; Iacobucci et al., 2012). B-ALL #8-IK6 is expected to be more immunogenic than the parental line, due to the expression of human antigens (IK6, co-expressed with dNGFR marker gene) and the potential accumulation of novel tumor-specific antigens. The leukemia #8-IK6 grew similarly to the parental #8 line in sub-lethally irradiated animals (Nucera et al., 2016). Instead, when BM-reconstituted mice were injected with #8-IK6 (Fig EV4B), more CTRL mice died of B-ALL compared with IFN-γ (4/12 CTRL and 2/13 IFN-γ in two separate experiments; Fig EV4C and D). Interestingly, only IFN-γ-treated mice showed loss of the NGFR antigen co-expressed with IK6 on B-ALL cells (Fig EV4E). All the mice that survived the first challenge became resistant to further challenges from both high doses of #8-IK6 B-ALL, as well as its parental #8 B-ALL, suggesting active immunization against leukemia-associated antigens (Fig EV4D). Taken together, these data indicate that IFN-γ gene therapy facilitates the deployment of immune responses directed against leukemia-associated antigens.

To further confirm the role of the immune system in mediating the effects of IFN-γ gene therapy, we transduced IFN-γ receptor 1 knockout (IFN-γR1 KO) HSPCs with control or IFN-γ-expressing vectors and transplanted them, in parallel to WT CD45.2 HSPCs, into lethally irradiated CD45.1 recipients. After confirmed hematopoietic reconstitution (Fig 2A), mice were challenged with B-ALL (line #11). Even though in vivo VCIN was lower than in previous experiments (0.3–0.4) for mice transplanted with IFN-γ-transduced cells (Fig 2B), WT-transplanted animals receiving IFN-γ gene therapy manifested reduced disease growth. In contrast, this effect was absent when mice were transplanted with IFN-γR1 KO-transduced with the IFN-γ vector. Furthermore, IFN-γR1 KO-transplanted animals showed higher levels of disease compared with WT-transplanted animals (Fig 2C), probably due to lack of response to endogenous IFN-γ. This lack of IFN-γ efficacy was accompanied by the absence of changes within BM populations, including MHC II expression (Fig 2D), CD8+ T lymphocytes (Fig 2E), and memory T cell subpopulations (Fig 2F), which were instead observed in animals transplanted with IFN-γ-transduced WT cells. Therefore, we demonstrated that the anti-tumoral activity of IFN-γ is dependent mainly on the capacity of the immune cells to respond to its stimulation, as the lack of the IFN-γR1 on hematopoietic cells led to loss of efficacy.

### Figure 2. IFN-γR1 knockout HSPCs display loss of IFN-γ anti-tumoral activity.

A. Engraftment of CD45.2 donor cells and lineage composition following transplantation of wild-type (WT) or IFN-γ receptor 1 knockout (KO) HSPCs transduced with either the control Tie2.NGFR (CTRL) or Tie2.IFN-γ (IFN-γ) LV (mean ± SD, each dot represents an individual mouse, WT CTRL = 7 mice, WT IFN-γ = 7 mice, KO CTRL = 7 mice, KO IFN-γ = 7 mice).

B. Vector copy number (VCN) in peripheral blood at 8 weeks post-transplantation (mean ± SD, each dot represents an individual mouse).

C. B-ALL progression measured as absolute number of OFP cells in the peripheral blood (mean ± SD, each dot represents an individual mouse; *P = 0.0286 at day 10; **P = 0.0174 at day 12; ***P = 0.0002; ****P ≤ 0.0001, ordinary two-way ANOVA).

D. MFI of MHC class II-positive macrophages, identified by F4/80 expression, in the BM (mean ± SD, each dot represents an individual mouse; WT IFN-γ vs. WT CTRL: *P = 0.0221, Mann–Whitney test).

E. Percentage of CD8+ T lymphocytes within OFP+ CD45+ BM cells (mean ± SD, each dot represents an individual mouse).

F. Maturation state (CD62L+CD44+ double negative, CD62L+CD44+ effector memory, CD62L+CD44+ naive and CD62L+CD44+ central memory T cells) of CD8+ lymphocytes (mean ± SD, each dot represents an individual mouse; WT IFN-γ vs. WT CTRL: ****P ≤ 0.0001, ordinary one-way ANOVA).

Data information: Statistical analysis of panel (C) is shown in Appendix Table S6.
Figure 2.
Single-cell RNA sequencing unveils an initially broad antitumor response, followed by loss of IFN-γ responses and selection of more aggressive B-ALL variants

Next, we undertook an unbiased transcriptional characterization of the B-ALL immune microenvironment performing 10× single-cell RNA sequencing (scRNAseq) on total BM cells from mice treated or not with IFN-γ gene therapy (controls indicated as blue dots, IFN-γ as yellow dots in Fig 1B). Quality control metrics are summarized in Table EV1. Unsupervised clustering identified many transcriptional states representative of the major cell types present in BM, with SingleR classification revealing a broad representation of the different hematopoietic cell types, including B-ALL, myeloid, and lymphoid clusters (Fig 3A and Table EV2). In detail, the unsupervised and custom analysis revealed subpopulations within the myeloid (Fig 3B and C) and lymphoid (Fig 3D and E) compartments. Moreover, we detected a small cluster of M2-like macrophages expressing a characteristic TEM signature (Fig EV5A and B) (Pucci et al, 2009), as well as a distinct subset of non-classical monocytes (mHB-M2) that have recently been associated with disease progression in B-ALL (Fig EV5C and D) (Wikowski et al, 2020). Notably, the mHB-M2 subset was overrepresented in IFN-γ-treated cells at day 17, indicating a possible mechanism of therapy resistance (Fig EV5D and E).

To understand what cell populations responded to IFN-γ, we interrogated myeloid- or lymphoid-tailored IFN-γ-related gene expression signatures. Dendritic cells, M2-like macrophages, and interferon-stimulated macrophages showed highest IFN-γ signature expression, which increased over baseline in the IFN-γ group. At the same time, little response was observed in non-classical, inflammatory, and proliferating monocytes (Appendix Fig S2A). In addition, gene therapy induced a broad IFN-γ response in most T-/NK cell subsets on day 12, which was lost in a fraction of cells on day 17 (Appendix Fig S2B).

Next, we compared the relative representation of the different subpopulations in the sequenced samples. IFN-γ-treated animals showed increased myeloid progenitors (CMPs + GMPs), dendritic cells and, surprisingly, non-classical monocytes at the expense of proliferating monocytes (Fig 4A), as well as an increase in cytotoxic CD8+ T lymphocytes on day 12 (Fig 4B). To shed light on the mechanisms governing loss of IFN-γ efficacy, we performed gene set enrichment analysis (GSEA) using the hallmark gene set of the Molecular Signatures Database (MSigDB) on the pre-ranked gene list obtained from the comparison of day 17 vs. day 12 (Fig 4C). With the notable exception of M2-like macrophages, which increased their inflammatory profile on day 17, all other cell populations downregulated inflammatory hallmarks (including IFN-α and IFN-γ response) while upregulating gene sets related to energy metabolism, cell cycle, and proliferation (Fig 4C and Tables EV3–EV5). Given that the M2-like macrophage cluster highly resembles TEMs (Fig EV5A and B), it is likely that this population continues to express IFN-γ from our vector construct resulting in autocrine stimulation. However, the small proportion of TEM in the leukemia microenvironment may not be sufficient to prevent B-ALL progression. Notably, we see widespread induction of IL-27, a T cell pro-survival factor (Schneider et al, 2011), in myeloid cells from the IFN-γ group, persisting to day 17 (Fig 4D).

To assess the impact of myeloid-derived IFN-γ release on the T cell compartment, we performed single cell TCR sequencing of BM-infiltrating T cells (Fig 4E and F). On day 12, the most represented TCRs showed a higher clonal abundance within IFN-γ-treated mice than controls. Most T cell clones expressing highly represented TCRs in IFN-γ-treated mice at day 12 map onto the cytotoxic CD8+ cluster (Fig 4F), possibly representing tumor-specific T cells that may help in early leukemia control, but became unable to persist while leukemia progressed, as evidenced by downregulation of transcriptional programs associated with the transcription factor Tox (Table EV5, cytotoxic CD8+). CD4+ T cells and some cytotoxic CD8+ T cells showed expression of T cell exhaustion markers (Fig 4G), but with barely any differences between the CTRL and IFN-γ groups.

Similarly, B-ALL showed transcriptional heterogeneity by unsupervised analysis (Fig 5A), with transient up-regulation of an IFN-γ gene signature, as well as MHC II molecules (Fig 5B and C). This was accompanied by a reduction in the potential neoantigen OFP, which is co-expressed with the miR-126 oncogene (Nucera et al, 2016) (Fig 5D). Moreover, B-ALL cells from IFN-γ-treated animals displayed decreased viability and reduced expression of the proliferator marker Mki67 compared to controls (Fig 5E and F). Based on the MHC II molecule expression module score, we divided B-ALL cells from IFN-γ d12 into an MHC II high subpopulation, displaying enrichment for IFN-responsive gene modules, and an MHC II low subpopulation, with higher expression of proliferative and oxidative phosphorylation pathways (Fig 5G and H). Moreover, at day 17, B-ALL cells from treated animals showed reduced expression of the
Figure 3.

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Figure 4.
IFN-γ receptors and signal transducers (Ifngr1, Ifngr2, and Jak1), accompanied by reduced intracellular signaling via Stat1 and Irf1 (Fig 5I), possibly indicating the development of a resistance mechanism to IFN-γ (Arenas et al, 2018).

**Enhancing IFN-γ anti-tumoral activity by combination therapies**

As the delivery of IFN-γ by gene therapy showed promising effects on the immune microenvironment at an early time-point when the leukemic burden was limited, but loss of efficacy on day 17, we next tested whether combination therapies were able to enhance the effectiveness of IFN-γ gene therapy in controlling the weakly immunogenic, parental B-ALL.

First, we multiplexed our TEM-based gene therapy platform by co-delivering dual combinations of IFN-γ, IFN-α, and TNF-α, all expressed from the Tie2e/p-miRT vector backbone stably integrated into HSPC. Mice transplanted with HSPC co-transduced with IFN-α/IFN-γ, IFN-α/TNF-α or IFN-γ/TNF-α successfully engrafted to similar levels as control LV-transduced cells or cells transduced only with the IFN-α vector, resulting in average in vivo VCNs between 0.3 and 1.0, variably distributed between the individual cytokine components (Fig 6A). Mice were then challenged with B-ALL (line #11). All cytokine groups showed reduced B-ALL growth compared to control (Fig 6B). The IFN-γ/TNF-α combination revealed improved efficacy, with some animals showing very low levels of OPF disease until euthanasia, even at total VCNs below 1 of the combined cytokines (Fig 6B). Efficacy correlated with increased percentages of MHC II+ macrophages for the conditions that included IFN-γ (Fig 6C), while the percentage of CD8+ T cells was exclusively enriched in the most promising combination of IFN-γ with TNF-α (Fig 6D).

As a second approach, given that IFN-γ may activate a series of feedback mechanisms ultimately leading to loss of efficacy (Benci et al, 2019), we evaluated synergy with drugs blocking immunosuppressive escape pathways: (i) checkpoint blockers targeting inhibitory signaling cascades induced after T cell activation; (ii) inhibitors of the indoleamine 2,3-dioxygenase (IDO), upregulated in myeloid cells upon IFN-γ stimulation (see Fig EV1G) that blunts T cell expansion by catabolizing tryptophan in the tumor microenvironment. After hematopoietic reconstitution with IFN-γ- or control-transduced HSPC and B-ALL challenge, we treated the mice with either monoclonal antibodies directed against the inhibitory T cell checkpoints CTLA4 (αCTLA4) or LAG3 (αLAG3), thereby reducing T-cell exhaustion, or with the IDO inhibitor 1-methyltryptophan (1-MT). Checkpoint blockade led to a variable response in both IFN-γ and control animals. Some showed a very low disease burden at the endpoint analysis, and others had disease levels comparable to the respective isotype controls (Fig 6E). Noteworthy, a higher proportion of mice from the IFN-γ group showed a strong response compared to the control group (2/7 vs. 1/5 mice for αCTLA4; 4/7 vs. 1/5 mice for αLAG3, respectively). Mice reconstituted with control HSPC showed little disease inhibition following treatment with the IDO inhibitor (Fig 6E). Instead, mice from the IFN-γ gene therapy group showed a strong, homogeneous response to IDO inhibition, possibly reflecting a synergistic effect (Fig 6E). While all groups receiving gene therapy-based delivery of IFN-γ showed higher percentages of MHC II+ macrophages than the controls (Fig 6F), the increase in CD8+ T cells was especially important in animals receiving the checkpoint blockers, and inversely correlated with disease levels (Fig 6G).

In summary, we showed that the anti-leukemic efficacy of myeloid-based delivery of IFN-γ could be improved by checkpoint blockers and IDO inhibitors, at least in part by amplifying T cell responses, which may be blunted upon chronic IFN-γ exposure.

**Discussion**

We here demonstrate stable genetic engineering of mouse hematopoiesis with an Ifn-γ transgene, whose expression is transcriptionally targeted to a subset of tumor-infiltrating myeloid cells using a previously described and extensively characterized lentiviral vector platform (De Palma et al, 2008; Escobar et al, 2014, 2018). Advantages of this delivery platform include limited systemic cytokine exposure and the need for a single, one-off treatment only, as genetically modified HSPCs guarantee persistence. We show immune-mediated inhibition of tumor growth in two B-ALL models representative of the human disease (Nucera et al, 2016) and a heterotopic colorectal cancer model, even when only 20% of hematopoietic cells carry the genetic modification, as evidenced by the in vivo VCN of 0.2. Our B-ALL model rapidly induces an immunosuppressive microenvironment, including downregulation of MHC II genes, up-regulation of IL-10 (Escobar et al, 2018), and accumulation of non-classical monocytes, which have recently been shown to play a
Figure 5.
pivotal role in B-ALL progression (Witkowski et al., 2020). Similar to IFN-α (Escobar et al., 2018), IFN-γ monotherapy did not cure mice from a weakly immunogenic disease. Compared to IFN-α, IFN-γ led to a strong induction of MHC II molecules, presumably enhancing antigen presentation (Escobar et al., 2018). We noted a loss of response to IFN-γ treatment (leukemic cells included) at the late-timepoint, with only M2-like macrophages (Fig 5A) maintaining an active IFN-γ pathway. This argues against the loss of cells expressing transgenic IFN-γ, expected to be prominently within M2-like macrophages, where Tie2 promoter activity is highest. While the proportion of M2-like macrophages may point to intratumor heterogeneity, i.e., the presence of pre-existing resistant B-ALL subpopulations that are positively selected under treatment. Many of the pathways upregulated in B-ALL cells from CTRL d conditions, extracted from the scRNAseq dataset (**P ≤ 0.0001, Welch’s t-test).

We observed that IFN-γ causes early proliferation impairment, as a direct effect on B-ALL cells, in addition to immune-mediated effects on the more immunogenic subclones (Fidanza et al., 2017). This “equilibrium phase” is followed by an “escape phase” where less immunogenic and more aggressive B-ALL subpopulations are selected. Indeed, we found transcriptional alterations in B-ALL cells over time, indicating activation of signaling pathways associated with immature stem cell phenotype (Dierks et al., 2007; Lin et al., 2010; Dagklis et al., 2015), metabolic switch to oxidative phosphorylation (Aykin-Burns et al., 2009; Schafer et al., 2009; Kamarajugadda et al., 2012; Lagadinou et al., 2013; Jiang et al., 2014; Ghanbari Movahed et al., 2019) and increased proliferation (de Barrios et al., 2020) suggesting a disease-intrinsic mechanism of resistance. Our finding that only part of the B-ALL cells upregulates MHC II in response to IFN-γ may point to intratumor heterogeneity, i.e., the presence of pre-existing resistant B-ALL subpopulations that are positively selected under treatment. Many of the pathways upregulated on day 17 in the B-ALL overlapped with those found in the MHC II low fraction on day 12, supporting this hypothesis. Downregulation of MHC II on acute myeloid leukemia cells has recently been identified as a principal mechanism of relapse after allogeneic transplantation, which was overcome, at least in vitro, by IFN-γ treatment (Toffalori et al., 2019). To best exploit this cytokine therapeutically, it will be relevant to further study the mechanisms and determinants of IFN-γ responsiveness in leukemia.

In conclusion, we have shown how local delivery of IFN-γ represents a safe and efficient strategy to achieve TME reprogramming leading to early leukemia control. In-depth characterization of the healthy BM and leukemic compartment identified IFN-γ’s fundamental role in antitumor and antigen-specific responses, which are lost over time through different mechanisms impinging on T cell activity and augmenting aggressiveness of the disease. This strategy could be applied to other malignancies such as solid tumors, and its effects could be enhanced by further tackling the immune evasion mechanism acquired during disease progression.
Figure 6.
Materials and Methods

Plasmid construction and lentiviral vector production

The Tie2.ILN-γ, Tie2.TNF-α, and Tie2.NGFR lentiviral vectors (LV) were generated by cloning a murine Ifnγ cDNA (CAT#: MR227155, OriGene) or a murine Tnfs cDNA (CAT#: MR212145, OriGene) or dNGFR into Tie2 flp/miRT backbone (Escobar et al., 2014). Likewise, a human IFNγ cDNA was cloned into the human TIE2 flp/miRT backbone (Escobar et al., 2014). The primers used to amplify Ifnγ cDNA and Tnfs cDNA included a stop codon at the end of the coding sequence. Concentrated VSV-G-pseudotyped LV stocks were produced and titrated as described previously (De Palma & Naldini, 2002). 293T cells were regularly tested for mycoplasma contamination.

Vector copy number

For VCN analysis, 5 ng/µl of genomic DNA was used to perform by droplet digital PCR, as previously described (Milani et al., 2017), using primers/probe sets designed on the primer binding site region of LV, or on the Tie2 region and the specific cytokine, and normalized on murine endogenous DNA by a primers/probe set against the murine Sema3a gene (Appendix Table S2). The PCR was performed following manufacturer’s instructions (Bio-Rad) and read with QX200 reader. Analysis was performed with QuantaSoft Analysis Pro Software (Bio-Rad).

Validation of the human IFN-γ construct

All primary cells were obtained from donors that signed informed consent forms approved by the Ospedale San Raffaele Ethics Committee, in accordance with the Declaration of Helsinki, the Department of Health and Human Services Belmont Report and the Good Clinical Practice guidelines of the International Conference on Harmonization. CD14+ monocytes were isolated from buffy coats obtained from healthy donors by positive selection (Miltenyi) and cultured for 9 days in RPMI 1640 (15% FBS, 5% human serum), 10 ng/ml rhM-CSF (PeproTech), and 20 ng/ml rhIL4 (Miltenyi Biotech) from day 5. After 9 days of culture, 200 ng/ml of LPS (Sigma-Aldrich) was added for 24 h, and IFN-γ concentration was measured in cell culture supernatants by standard sandwich ELISA (antibody clones NIB42 and 4S.B3, BD Biosciences). LV transduction of monocytes was accomplished at a multiplicity of infection (MOI) of 10 following a 3-h exposure to Vpx-VLP (https://doi.org/10.3389/fimmu.2020.01260). A primary t(9;22) B-ALL was obtained from the OSR biobank following informed consent and IRB approval. The disease was transduced with a lentivirus co-expressing NGFR and Luciferase, passaged in NSG mice, selected for NGFR positivity, and frozen. The B-ALL was intravenously injected into NSGW41 mice, at a dose of 1.2 × 10⁶ cells per mouse. Disease burden was periodically monitored by bioluminescence imaging. After disease detection, mice received weekly vincristine (0.5 mg/kg i.v.) and five times per week dexamethasone (5 mg/kg, i.p.) injections, as shown in the scheme in Fig EV3. Commercially available leukapheresis was subjected to CD34 selection (CliniMACS, Miltenyi). The positive fraction was transduced with the human TIE2-IFN-γ construct (or mock-transduced) as described (Pettrillo et al., 2018), while the negative fraction was used to produce autologous CAR-T cells directed against the CD19 antigen, as described (Casucci et al., 2018), with exception that a lentivirus was used for transduction. Engineered CD34+ and T cells were characterized in vitro, and cryopreserved aliquots were used for transplantation (1 × 10⁶ CD34+ or T cells per dose, intravenously).

Mice

Female C57Bl/6 Ly45.2 and Ly45.1 mice of 6–8 weeks of age were purchased from Charles River Laboratory. Female C57Bl/6 Ly45.1 mice were used as donors. Female NSGW41 mice were obtained from internal breeding at our mouse facility. At time of treatment, animals were 6–8 weeks old. All animal procedures were performed according to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute (IACUC 600, 836, 936, 1095, 1102) and communicated to the Ministry of Health and local authorities according to Italian law. Mice were housed in the two animal facilities of the San Raffaele Hospital (Dibit 1—Via Olgettina 58, 20132 Milan, Italy; Dibit 2—Via Olgettina 60, 20132 Milan, Italy) in groups of 2–5 per cage, with sterilized food, water ad libitum and controlled 12-h/12-h light/dark cycles.
**IFN-γ toxicity studies**

Organs were collected from mice 12 weeks after transplant. Part of them were fixed in formalin and underwent pathology evaluation at the mouse pathology facility of Ospedale San Raffaele. Hearts, lungs, livers, spleens, and BM were collected and frozen at −80°C. RNA was isolated using the miRNeasy Mini Kit (QIAGEN, 217004) according to manufacturer’s instructions. RNA was converted to cDNA using SuperScript™ IV Vilo™ Master Mix (Thermo Fisher Scientific, 11756050). Digital droplet PCR with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 1725270) was employed to measure expression of selected genes. Primers were obtained from BioRad: Ilf1 MmuEG5073430, Cd86 MmuEG5065771, Hprt MmuEG5073006, Actb MmuEG5193531, Ifnl1 MmuEG5073097, Ido1 MmuEG5083069, Il12a MmuEG5079777, Nos2 MmuEG5084795. Blood chemistry was performed on murine serum samples in the Ospedale San Raffaele Mouse Lab with the ILab Aries machine. IFN-γ ELISA was performed on plasma with the Mouse IFN-gamma Quantikine ELISA Kit MIF00 (R&D Systems) according to the manufacturer’s instructions.

**Hematopoietic stem and progenitor cell transplantation**

Bone marrow was harvested from female 6- to 9-week-old C57Bl/6 mice, and lineage-negative HSPCs were purified by immuno-magnetic isolation (Lineage Cell Depletion Kit mouse, Miltenyi, #130–090–858). HSPC transduction, culture, and transplantation in recipient female 8-week-old C57Bl/6 mice were performed as previously described (Escobar et al., 2014). In the therapeutic setting (Figs 1H and I and EV3A and B), mice injected with B-ALL first received a single i.v. dose of 0.5 mg/kg Vincristine between days 3 and 5 from B-ALL administration, followed by 900 Td total body irradiation and infusion of transduced HSPCs.

**Tumor challenge and combination immunotherapies**

For leukemia challenge, mice were intravenously injected with 5 × 10^4 parental B-ALL, unless differently specified in the experiment (Fig EV4B and C: 1 × 10^5 IK6 B-ALL, 4 × 10^5 IK6 B-ALL, 4.7 × 10^5 line #8 B-ALL; Fig EV3F–I: 1 × 10^5 OVA B-ALL line #11). Combination therapies were administered intraperitoneally, as follows:

For checkpoint blocking experiments: 200 μg anti-CTLA4 (clone 9D9 BioXCell, #BE0164) or isotype control antibody (clone MCP-11 BioXCell, #BE0086) were administered intraperitoneally at day 3 upon leukemia injection, followed by 100 μg every 3–4 days for a total of five infusions; 200 μg anti-LAG 3 (clone C9B7W BioXCell, #BE0174) or isotype control antibody (clone HRPN BioXCell, #BE0088) were administered intraperitoneally at day 3 upon leukemia injection, followed by 100 μg every 3–4 days, for a total of five infusions; 8.3 mg 1-Methyl-β-lactalbumin (1-MT; 860646 Sigma) was administered intraperitoneally at day 3 upon leukemia injection, and every 3 days, for a total of five infusions. For solid tumors challenge, mice were subcutaneously injected in the lower right flank with 5 × 10^5 MC38 cells. Growth of tumor mass was followed every 3–4 days by caliper measurement. Tumor volumes were calculated by: V = 0.5236 × length × width². Cells were tested for mycoplasma contamination.

**Blood counts and flow cytometry**

Blood cell counts and hemoglobin levels were obtained on whole blood using ProCyte Dx Hematology Analyzer (IDEXX). All cytometric analyses were performed on FACSCanto II and LSRFortessa instruments (BD Bioscience) and analyzed with the FlowJo software (v. 10.5.3, Tree Star Inc.). Staining of the different compartments was performed as in Escobar et al, 2018 (Escobar et al., 2018). A list of antibodies and staining conditions is provided in Appendix Table S3. The gating strategy is shown in Appendix Fig S3. Solid tumors were dissociated in Collagenase 200 μg/ml (C5138, Sigma-Aldrich), Dispase II 2 mg/ml (17105041, Thermo Fisher Scientific), and DNase I 100 U/ml (04716728001, Roche) for 45 min at 37°C and stained as previously described (Escobar et al., 2014).

**Single-cell RNA sequencing**

**Cell preparation**

Viable (Annexin V-) BM cells were subjected to 2-way sorting, separating B cells from non-B cells: one way to collect CD19⁺ OFF⁺ cells and the other way to collect CD45⁺ CD19⁻ cells. Granulocytes were excluded via Ly6g to reduce percentages to ≈ 15–30%. CTRL d12: mouse D1 36% OFF⁺ disease, after sorting 31% granulocytes; mouse D2 53% OFF⁺ disease, after sorting 33% granulocytes. IFN-γ d12: (i) mouse C3 21% OFF⁺ disease, after sorting 36% granulocytes; (ii) mouse C5 2.7% OFF⁺ disease, no exclusion of granulocytes (8%). CTRL d17: mouse A3 45% OFF⁺ disease, after sorting 35% granulocytes; mouse A4 51% OFF⁺ disease, after sorting 19% granulocytes. IFN-γ d17: (i) mouse B5 59% OFF⁺ disease, after sorting 11% granulocytes; mouse C1 55% OFF⁺ disease, after sorting 18% granulocytes; (ii) mouse D3 49% OFF⁺ disease, after sorting 16% granulocytes; mouse D5 51% OFF⁺ disease, after sorting 9% granulocytes. After sorting, cells were washed, counted, loaded onto the Chromium Controller (10X Genomics) for single-cell bead encapsulation, and processed for library preparation using the Chromium Single Cell 5’ Library & Gel Bead Kit, Chromium Single Cell V(D)J Enrichment and Mouse T Cell Kit, according to manufacturer’s instructions.

**De-multiplexing, quality control, pre-processing, and unsupervised clustering**

scRNAseq libraries (GEX and VDJ) were demultiplexed and processed by the Cell Ranger Single-Cell Software Suite (version 3.1.0, 10X Genomics) using the GRCh38 reference genome and gene annotations (v3.0.0) provided by the manufacturer. In order to quantify custom elements delivered with the lentiviral constructs present in the B-ALL model and used in the gene therapy protocol, synthetic chromosomes and annotations for each constructs/element were appended and included in the reference files. Default parameters were used in all Cell Ranger steps including demultiplexing (mkfastq), gene expression quantification (quant), and VDJ pipeline. We sequenced six samples, obtaining for each of them both single-cell gene expression data and TCR data. The CTRL d17 was discarded from further analysis due to poor quality. We obtained a number of cells per sample ranging from 1,830 to 23,861, for a total of ≈ 85,000 cells (Table EV1). The median UMI count per cell ranged from 3,974 to 5,753, whereas the average number of reads per cell were comprised between 20,300 and 160,000. Regarding the BCR-TCR repertoire libraries, we obtained a number of cells
imported in Seurat object. The disease annotation was carried on by Pruned labels associated with all main annotations were then characterized by a more detailed classification of the former.

Types labels (including cell subtypes labels), the annotation using for each dataset both main labels (including cell transcripts mapping to mitochondrial genes)

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EMBO Molecular Medicine

The paper explained

Problem
Cancer is the second cause of death in developed countries. The immunosuppressive microenvironment surrounding tumor cells represents a key cause of treatment failure. Therapies aiming at reprogramming and restoring anti-tumoral immune responses have largely employed in recent years. However, most of these immunotherapies impact on one single immune compartment.

Results
IFN-γ polarizes immune responses towards anti-tumoral states, but systemic delivery is associated with major side effects. Gene-based, tumor-specific IFN-γ delivery exploiting tumor-associated macrophages avoids systemic side effects while augmenting antitumor immune responses. Local delivery of IFN-γ reduced tumor progression by inducing antigen presentation in the myeloid compartment and on leukemia cells, leading to T cell recruitment and activation. The efficacy of this strategy could be enhanced by combination with therapies targeting additional components of the immune system.

Impact
We developed a strategy to safely and locally deliver IFN-γ into the tumor microenvironment and induce antigen presentation and antigen-specific responses. This therapy led to the reactivation of anti-tumoral immunity, counteracting one of the leading causes of immune escape and treatment failure. Moreover, our approach may be applied to virtually all malignancies, including solid tumors, as IFN-γ represents a key player of immunity in most cancer types.

Cell type annotation
Cell type annotation was assessed by using SingleR (v1.1.11), a computational method, based on correlation analysis that leverages reference transcriptomic datasets of pure cell types to infer the cell of origin of each of the single cells independently. We performed annotation by using the Mousernaseq and the Immgen reference dataset (Heng et al., 2008; Benayoun et al., 2019). We performed a double annotation using for each dataset both main labels (including cell types labels) and fine labels (including cell subtypes labels), the latter characterized by a more detailed classification of the former. Pruned labels associated with all main annotations were then imported in Seurat object. The disease annotation was carried on by highlighting cells expressing at least 1 UMI mapping to the TTA custom gene. In order to get more insights and details regarding the different cell type components, we split the full dataset into four main compartments: myeloid cells (monocytes + macrophages), lymphoid cells (NK-T cells), B cells (including B-ALL) and neutrophils. To perform this step, we set up a data analysis workflow that used both annotations (Mousernaseq main and Immgen main) by intersecting specific labels in order to get rid of discordant or poor-quality annotations. In particular, we selected cells by intersecting selected groups of cells (Table EV2). After preliminary analyses on each of the subsets, outlier cell clusters spotted by marker inspection, which did not fit their compartment, were discarded from subsequent analyses. After common and standard processing with Seurat, as already described above, we applied data integration by using harmony (v.1.0, Korsunsky et al., 2019), including the orig.ident variable in the group.by vars parameter. Cluster marker identification was performed at 0.6 and 1.2 resolutions (FindClusters parameter) with the FindAllMarkers function (min.pct = 0.25, min.pct.cells.group = 10), focusing on upregulated genes only. The treatment effect within each cluster identified was assessed with the FindMarkers function, setting parameters according to the comparison to be perform. In particular, we consider as untreated cells only the ones belonging to the sample GEXN1, due to the low number of cells present in the GEXN2 sample. Gene ontology overrepresentation analysis (ORA) was performed with the ClusterProfiler R package (enrichGO function with default parameters) using as input the marker lists collected and as gene universe the list of genes expressed in the dataset under analysis. Gene Set Enrichments Analysis was performed by using the GSEA function in clusterprofiler R
TCR repertoire analysis

The evaluation of the TCR repertoire was assessed both by inspecting the results obtained from the Cell Ranger VDJ pipeline reports and by using the scRepertoire R package (v0.99.1), https://github.com/ncborcherding/scRepertoire). In particular, after the analysis of both absolute and relative clonotype abundance across samples and conditions (treated vs. untreated groups) with scRepertoire, we focused on the mapping of highly abundant clonotypes (in particular, the top10 most abundant for each condition) onto the UMAP embedding of single-cell expression datasets in order to identify the presence of cluster-specific TCR.

Statistical analysis

Values for statistical significance have been calculated using GraphPad Prism. The respective method used is given in the figure legends. Figures show mean±standard deviation, unless otherwise stated.

Data availability

The datasets and computer code produced in this study are available in the following database: RNA-Seq data: Gene Expression—accession number GSE178941 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178941).

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Conflict of interest

B.Ge. is a founder of Genenta Science, a biotech start-up financing the clinical development of IFN-γ gene therapy. Genenta did not fund the research on IFN-γ gene therapy presented in this manuscript, nor does Genenta have ownership rights on the data presented herein.

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