Reprogramming Nurse-like Cells with Interferon γ to Interrupt Chronic Lymphocytic Leukemia Cell Survival*

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Nurse-like cells (NLCs) play a central role in chronic lymphocytic leukemia (CLL) because they promote the survival and proliferation of CLL cells. NLCs are derived from the monocyte lineage and are driven toward their phenotype via contact-dependent and -independent signals from CLL cells. Because of the central role of NLCs in promoting disease, new strategies to eliminate or reprogram them are needed. Successful reprogramming may be of extra benefit because NLCs express Fcγ-receptors (FcγRs) and thus could act as effector cells within the context of antibody therapy. IFNγ is known to promote the polarization of macrophages toward an M1-like state that is no longer tumor-supportive. In an effort to reprogram the phenotype of NLCs, we found that IFNγ up-regulated the M1-related markers CD86 and HLA-DR as well as FcγRIa. This corresponds to enhanced FcγR-mediated cytokine production as well as rituximab-mediated phagocytosis of CLL cells. In addition, IFNγ down-regulated the expression of CD31, resulting in withdrawal of the survival advantage on CLL cells. These results suggest that IFNγ can re-educate NLCs and shift them toward an effector-like state and that therapies promoting local IFNγ production may be effective adjuvants for antibody therapy in CLL.

Given the crucial role NLCs play in CLL cell survival, a number of immune modulators have been screened for their suitability as therapy against them. Burger et al. (3) showed that SDF-1-blocking antibodies reduced the protective effects of NLCs on CLL cells. Morande et al. (5) recently showed that NLCs were susceptible to Aplidin-induced death, suggesting that its anti-tumoral effects were from targeting CLL cells and NLCs simultaneously. Schulz et al. (6) showed that treatment with lenalidomide changed the functional and phenotypic nature of NLCs by interfering with their nurturing properties.

Interferons have been widely accepted as modulators of macrophage plasticity and activation, and it is known that IFNγ is capable of promoting the differentiation of monocytic cells (7). With regard to therapeutic use, Miller et al. (8) have shown that IFNγ is beneficial for treating immune disorders such as systemic sclerosis and that it displays antitumor and antiangiogenic effects both in vitro and in vivo. IFNγ treatment has also been shown to induce antineoplastic immune responses by sensitizing tumor cells to apoptosis via up-regulation of both MHC class I and II molecules and by enhancing antitumor immune activity while decreasing M2 characteristics in immune cells (9, 10). IFNγ has been successfully used in cases of ovarian cancer, multiple myeloma (11), and bladder carcinoma and, recently, in malignant gliomas (12).

Here we examined the effects of IFNγ on the phenotype and function of NLCs. We found that IFNγ significantly increased the expression of the M1-related markers CD86 and HLA-DR as well as the phagocytic receptor FcγRI. Concurrently, the prosurvival ligand CD31 was down-regulated. Consistent with this, IFNγ-treated NLCs showed superior phagocytic ability toward both opsonized sheep RBCs (SRBCs) and rituximab-coated CLL cells as well as withdrawal of support for CLL cell survival. These results show that IFNγ can reprogram NLCs to function as immune effectors and suggest that therapies that enhance IFNγ production locally may be valuable treatments for CLL, particularly when combined with monoclonal antibodies such as rituximab.

Experimental Procedures

Patient Samples—Peripheral blood was collected from CLL patients with informed consent in accordance with the Declaration of Helsinki and under approval from the Institutional Review Board of Ohio State University.

NLC Culture—Peripheral blood mononuclear cells were isolated from CLL patient blood by density gradient centrifugation over Ficoll-Hypaque (Nycomed, Oslo, Norway) and resus-
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Phagocytosis—Phagocytosis assays were performed as described previously (14). Briefly, NLCs were pretreated with IFNγ or PBS for 72 h. SRBCs (Colorado Serum Co., Denver, CO) were fluorescently labeled using PKH26 dye (Sigma) according to the instructions of the manufacturer and then opsonized with anti-SRBC antibody (Sigma) according to the instructions of the manufacturer. SRBCs were mixed with NLCs, and then cells were gently pelleted by slow centrifugation and incubated for 30 min at 37 °C. Non-ingested SRBCs were lysed with RBC lysis buffer (eBioscience, San Diego, CA) at room temperature for 10 min. Cells were washed with PBS and fixed with 4% paraformaldehyde. Ingested RBCs were counted using fluorescence immersion oil microscopy. The phagocytic index was calculated as the total number of SRBCs ingested by 50 NLCs.

CLL Cell Survival Assay—Nurse-like cells were pretreated with 10 ng/ml IFNγ or PBS control for 72 h. Cells were counted and left overnight for adherence in quadruplicate in 96-well tissue culture plates (5 × 10^4/100 μl of medium). CLL cells were added at (5 × 10^5/100 μl) and incubated for 24 h. Non-adherent CLL cells (100 μl) were harvested and stained with Annexin V FITC/propidium iodide (BD Biosciences) using the protocol of the manufacturer. Data are represented as the percentage of total live CLL cells (Annexin V FITC- propidium iodide-).

NLC Phagocytosis of CLL Cells—NLCs were enriched as described previously, incubated with 10 μg/ml rituximab on ice for 2 h, washed with PBS, and then labeled with wheat germ agglutinin conjugated to Alexa Fluor 647 (5.0 μg/ml, Life Technologies) for 10 min at room temperature. Simultaneously, NLCs were harvested, washed, and incubated with wheat germ agglutinin conjugated to Alexa Fluor 488 (5.0 μg/ml, Life Technologies) for 10 min at room temperature. Following washes, co-incubations of NLCs with CLL cells for 60 min at a ratio of 1:5 (NLC:CLL) were done. Cells were then washed and fixed with 1% formaldehyde for 20 min at 37 °C. Cells were placed onto microscope slides with ProLong® Gold mounting solution (Thermo Scientific) and then examined using confocal microscopy.

Statistics—For NLC gene expression studies, paired two-tailed Student’s t tests were used to compare untreated relative copy numbers to IFNγ-treated relative copy numbers. For the phagocytosis results, paired two-tailed Student’s t tests were used to compare the mean phagocytic index control versus IFNγ-treated cells. For the inhibitor experiments, mixed-effect modeling was performed using SAS 9.4 (SAS Inc., Cary, NC). Significance was counted as p [\textless 0.05].

Results

Characterization of NLCs—CLL-patient NLCs were derived as described under “Experimental Procedures,” and their characteristics were verified via flow cytometry (Figs. 1, A–D). Gating based on forward and side scatter was done in accordance with previous work (1–3) (Fig. 1A). The results showed that the cells were CD68^+ and CD14^dim (Fig. 1B), with some CD68^+ cells also showing expression of CD200R (Fig. 1C). All cells were CD80^− (Fig. 1D).
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**IFNγ Enhances NLC Expression of CD86, HLA-DR, and FcγRI—**NLCs have been described as counterparts to tumor-associated macrophages seen within solid tumors (1, 15, 16). This led to the question of whether they could be reprogrammed away from their tumor-supportive phenotype. Because of their similarities with solid-tumor macrophages, the possibility existed that treatment with cytokines such as IFNγ (9) might be effective. To test this, we treated NLCs for 72 h with IFNγ and measured levels of the M1-related markers (17) NOS2 (nitric-oxide synthase 2), HLA-DR, and CD86. Results showed that IFNγ led to variable effects with NOS2, increasing for some donors and decreasing for others (data not shown). Transcripts for the T cell coactivator CD86 were significantly elevated (Fig. 2A), with a corresponding increase in surface expression (Fig. 2, B and C). Likewise, HLA-DR was significantly elevated at the transcriptional (Fig. 2D) as well as cell surface levels (Fig. 2, E and F) by IFNγ.

We also examined FcγRI, which is the high-affinity IgG receptor and thus can play a role in antibody-mediated responses (18). This receptor has been shown to respond to IFNγ in healthy donor monocytes and macrophages (19, 20) as well as in primary acute myeloid leukemia cells (21). Results showed that treatment of NLCs with IFNγ significantly increased FcγRI transcript (Fig. 2G) and protein (Fig. 2, H and I). However, FcγRIIa, FcγRIIb, FcγRIIIa, and γ chain levels were unaffected (data not shown).

We also tested the effect of IFNγ on SDF-1 levels because this is a major protumoral factor produced by NLCs (3). However, no effect of IFNγ on SDF-1 transcript was seen (data not shown). Collectively, these results suggest that IFNγ does not effect a complete shift toward an M1 phenotype in NLCs but that it does up-regulate molecules involved with effector functions.

**IFNγ Enhances Phagocytosis by NLCs—**Because IFNγ increased expression of the high-affinity IgG receptor FcγRI, we tested the effects of IFNγ on phagocytosis. We treated NLCs with IFNγ for 72 h and then measured their ability to ingest fluorescently labeled, opsonized SRBCs. The results showed that IFNγ-treated NLCs ingested significantly more SRBCs than untreated NLCs (Fig. 3A, plotted in B).

Next, we tested whether IFNγ-treated NLCs would be capable of phagocytosing antibody-coated CLL cells. We treated NLCs with IFNγ for 72 h as above, membrane-labeled them with fluorescent dye, and then incubated them for 1 h with membrane-labeled CLL cells that were opsonized with the anti-CD20 antibody rituximab, which is commonly used for the treatment of CLL. We examined phagocytosis via confocal microscopy between untreated (Fig. 3C) and IFNγ-treated (Fig. 3D) NLCs and found that IFNγ significantly increased the number of ingested CLL cells (Fig. 3E).

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**FIGURE 1. Characterization of NLCs.** A–D, NLCs were generated as described under “Experimental Procedures.” NLCs were analyzed using flow cytometry. Graphs show scatter (A), CD14 (B), CD200R (C), and CD80 (D) in the CD68+ population.

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IFNγ down-regulates CD31 and reduces NLC-mediated survival of CLL cells—CD38/CD31 interactions in cooperation with CD100 promote survival of CLL cells, and it has been shown that blocking antibodies against CD31 can disrupt CLL cell survival (22). Hence, we tested the effects of IFNγ treatment on the expression of CD31 in NLCs. The results showed that 72-h treatment with IFNγ led to a significant reduction in CD31 (Fig. 4A).

The above led us to test whether IFNγ-induced NLC polarization would be sufficient to interfere with NLC-dependent CLL cell survival. We treated NLCs for 72 h and then co-cultured them with CLL cells for 24 h. CLL cell survival was measured by Annexin/propidium iodide staining. The results showed that IFNγ treatment significantly reduced the survival of CLL cells within the co-cultures (Fig. 4B) despite not accelerating the death of control CLL cells in single culture (data not shown).
Discussion

In this study, we found that treatment of NLCs with IFN-γ could reprogram them toward a more effector-like phenotype and also in such a way that they no longer supported the survival of CLL cells. IFN-γ also significantly enhanced the phagocytic ability of NLCs against opsonized SRBCs as well as rituximab-coated CLL cells. These findings suggest that IFN-γ could serve to improve the outcome of antibody therapy for CLL. They also support the earlier observation that NLCs resemble M2-like tumor-associated macrophages (15) because the latter have been found to respond to IFN-γ (9).

In addition, these data suggest that SDF-1, although important for CLL cell survival, is by itself not sufficient. IFN-γ did not significantly decrease NLC SDF-1 but did decrease CLL cell survival in NLC/CLL cultures. This is in agreement with Burger et al. (3), who found that supplementing CLL cells with SDF-1 offered some but not full protection against apoptosis. Additional survival stimuli such as CD31/CD38 interactions, along with others yet to be tested, are likely to contribute to CLL cell survival. Quantifying the full effects of IFN-γ on NLCs with regard to their interactions with CLL cells will require further study.

Direct administration of IFN-γ continues to be tested for conditions including macular edema, HIV, and various tumor types (http://www.clinicaltrials.gov). A synthetic version of IFN-γ (Actimmune) was approved for the treatment of chronic granulomatous disease as well as to delay the progression of malignant osteopetrosis (http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm109130.htm). Our results suggest that such IFN-γ administration may be beneficial against CLL as well. Within the context of antibody therapy, IFN-γ would be induced in natural killer cells, which could act locally upon the NLCs. This could be further strengthened by the co-administration of agents such as IL-12 (23), CpG (24), and TLR8 agonists (25). Such co-treatment would be predicted to significantly enhance antibody-mediated clearance of CLL cells and may also inhibit the development of new NLCs. Given the importance of CD20 antibody-based therapy in prolonging survival of CLL patients, this could represent a major advance for this currently incurable disease.

FIGURE 3. IFN-γ enhances phagocytosis by NLCs. NLCs (n = 3 donors) were treated for 72 h with or without 10 ng/ml IFN-γ and used in phagocytosis assays. A, representative microscopy images of untreated (UT, top panels) and IFN-γ-treated (bottom panels) cells. Shown are bright-field (left panels), fluorescence (center panels), and merged (right panels) images. B, phagocytic index of untreated versus IFN-γ-treated NLCs. C—E, NLCs (n = 5 donors) were treated as above and tested for phagocytosis of CLL cells as described under “Experimental Procedures.” Images show untreated (C) and IFN-γ-treated NLCs (D). E, the average number of CLL cells ingested by NLCs. Error bars represent standard deviation. *, p ≤ 0.05.
Author Contributions—J. C. B., S. T., and J. P. B. conceived and designed the study and wrote the manuscript. S. G., K. F., S. E., B. F. R., and L. R. performed the experiments, collected the data, and double-negative cells were counted as live (n = 4 donors).

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References

1. Ysebaert, L., and Fournié, J. J. (2011) Genomic and phenotypic characterization of nurse-like cells that promote drug resistance in chronic lymphocytic leukemia. *Leuk. Lymphoma* **52**, 1404–1406

2. Tsukada, N., Burger, J. A., Zvaifler, N. J., and Kipps, T. J. (2002) Distinctive features of “nurselike” cells that differentiate in the context of chronic lymphocytic leukemia. *Blood* **99**, 1030–1037

3. Burger, J. A., Tsukada, N., Burger, M., Zvaifler, N. J., Dell’Aquila, M., and Kipps, T. J. (2000) Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood* **96**, 2655–2663

4. Marchesi, F., Cirillo, M., Bianchi, A., Gately, M., Olimpieri, O. M., Cerchiaro, E., Renzi, D., Micera, A., Balzamino, B. O., Bonini, S., Onetti Muda, A., and Avvisati, G. (2015) High density of CD68+/CD163+ tumour-associated macrophages (M2-TAM) at diagnosis is significantly correlated to unfavorable prognostic factors and to poor clinical outcomes in patients with diffuse large B-cell lymphoma. *Hematol. Oncol.* **33**, 110–112

5. Morande, P. E., Zanetti, S. R., Borge, M., Nanini, P., Iancic, C., Bezares, R. F., Bitsmans, A., González, M., Rodríguez, A. L., Galmarini, C. M., Gambale, R., and Giordano, M. (2012) The cytotoxic activity of Aplidin in chronic lymphocytic leukemia (CLL) is mediated by a direct effect on leukemic cells and an indirect effect on monocyte-derived cells. *Invest. New Drugs* **30**, 1830–1840

6. Schulz, A., Dürr, C., Zenz, T., Döhner, H., Stilgenbauer, S., Lichter, P., and Seiffert, M. (2013) Lenalidomide reduces survival of chronic lymphocytic leukemia cells in primary cocultures by altering the myeloid microenviroment. *Blood* **121**, 2503–2511

7. Perussia, B., Dayton, E. T., Fanning, V., Thiagarajan, P., Hoxie, J., and Trinchieri, G. (1983) Immune interferon and leukocyte-conditioned medium induce normal and leukemic myeloid cells to differentiate along the monocytoytic pathway. *J. Exp. Med.* **158**, 2058–2080

8. Miller, C. H., Maher, S. G., and Young, H. A. (2009) Clinical use of interferon-γ. *Ann. N.Y. Acad. Sci.* **1182**, 69–79

9. Duluc, D., Corvaisier, M., Blanchard, S., Catala, L., Descamps, P., Gamelin, E., Ponsoda, S., Delneste, Y., Hebar, M., and Jeannin, P. (2009) Interferon-γ reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. *Int. J. Cancer* **125**, 367–373

10. Prasse, A., Germann, M., Pechkovsky, D. V., Markert, A., Verres, T., Stahl, M., Melchers, I., Luttmann, W., Müller-Quernheim, I., and Zissel, G. (2007) IL-10-producing monocytes differentiate to alternative macrophages and are increased in atopic patients. *J. Allergy Clin. Immunol.* **119**, 464–471

11. Bergsagel, D. E., von Wussow, P., Alexanian, R., Avvisati, G., Bataille, R., Barlogie, B., Borden, E., Caligaris-Cappio, F., Deicher, H., and Durie, B. G. (1990) Interferons in the treatment of multiple myeloma. *J. Clin. Oncol.* **8**, 1444–1445

12. Kane, A., and Yang, I. (2010) Interferon-γ in brain tumor immunotherapy. *Neurosurg. Clin. N. Am.* **21**, 77–86

13. Gavrilin, M. A., Boaqui, I. J., Knatz, N. L., Duncan, M. D., Hall, M. W., Gunn, J. S., and Wewers, M. D. (2006) Internalization and phagosome escape required for Franscically to induce human monocyte IL-1β processing and release. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 141–146

14. Shah, P., Fatechand, K., Patel, H., Fang, H., Justiano, S. E., Mo, X., Jarjoura, D., Tridandapani, S., and Butchar, J. P. (2013) Toll-like receptor 2 ligands regulate monocyte Fcy receptor expression and function. *J. Biol. Chem.* **288**, 12345–12352

15. Giannoni, P., Pietra, G., Travaini, G., Quarto, R., Shyti, G., Benelli, R., Ottaglio, L., Minagiri, M. C., Zupo, S., Cutrona, G., Pierri, I., Balleire, E., Parata, A., Calvaruso, M., Tripodo, C., et al. (2014) Chronic lymphocytic leukemia nurse-like cells express hepatocyte growth factor receptor (c-MET) and indoleamine 2,3-dioxygenase and display features of immunosuppressive type 2 skewed macrophages. *Haematologica* **99**, 1078–1087

16. Boissard, F., Fournié, J. J., Laurent, C., Poupot, M., and Ysebaert, L. (2015) Nurse-like cells: chronic lymphocytic leukemia associated macrophages. *Leuk. Lymphoma* **56**, 1570–1572

17. Mose, D. M. (2003) The many faces of macrophage activation. *J. Leukoc. Biol.* **73**, 209–212

18. Graziano, R. F., and Fanger, M. W. (1987) Fc γ RI and Fc γ RII on monocytes and granulocytes are cytotoxic trigger molecules for tumor cells. *J. Immunol.* **139**, 3536–3541

19. Guyre, P. M., Morganelli, P. M., and Miller, R. (1983) Recombinant human interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. *J. Clin. Invest.* **72**, 393–397

20. Perussia, B., Dayton, E. T., Lazarus, R., Fanning, V., and Trinchieri, G. (1983) Immune interferon induces the receptor for monomeric IgG1 on human monocytes and myeloid cells. *J. Exp. Med.* **158**, 1092–1113

21. Notter, M., Ludwig, W. D., Bremer, S., and Thiel, E. (1993) Selective targeting of human lymphokine-activated killer cells by CD3 monoclonal antibody against the interferon-inducible high-affinity Fc γ RI receptor (CD64) on autologous acute myeloid leukemia blast cells. *Blood* **82**, 3113–3124

22. Deaglio, S., Vaisitti, T., Bergui, L., Bonello, L., Horenstein, A. L., Taman, L., Boumsell, L., and Malavasi, F. (2005) CD38 and CD100 lead a
network of surface receptors relaying positive signals for B-CLL growth and survival. Blood 105, 3042–3050

23. Parihar, R., Nadella, P., Lewis, A., Jensen, R., De Hoff, C., Dierssheide, J. E., VanBuskirk, A. M., Magro, C. M., Young, D. C., Shapiro, C. L., and Carson, W. E., 3rd. (2004) A phase I study of interleukin 12 with trastuzumab in patients with human epidermal growth factor receptor-2-overexpressing malignancies: analysis of sustained interferon γ production in a subset of patients. Clin. Cancer Res. 10, 5027–5037

24. Roda, J. M., Parihar, R., and Carson, W. E., 3rd. (2005) CpG-containing oligodeoxynucleotides act through TLR9 to enhance the NK cell cytokine response to antibody-coated tumor cells. J. Immunol. 175, 1619–1627

25. Stephenson, R. M., Lim, C. M., Matthews, M., Dietsch, G., Hershberg, R., and Ferris, R. L. (2013) TLR8 stimulation enhances cetuximab-mediated natural killer cell lysis of head and neck cancer cells and dendritic cell cross-priming of EGFR-specific CD8+ T cells. Cancer Immunol. Immunother. 62, 1347–1357