Lymphoproliferative Disorders

The effect of fludarabine on interferon-γ production by lymphoid cells from healthy donors and patients with B-cell chronic lymphocytic leukemia

Fludarabine treatment in patients with B-cell chronic lymphocytic leukemia (B-CLL) can trigger or exacerbate the development of autoimmune hemolytic anemia (AHA) through a currently ill-defined mechanism. We here show that exposure of peripheral blood lymphocytes from healthy donors and B-CLL patients to fludarabine increases *in vitro* production of interferon-γ, a key cytokine in the pathogenesis of AHA.

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Fludarabine is a strong immunosuppressive nucleoside that is particularly efficient in the treatment of B-cell chronic lymphocytic leukemia (B-CLL).[1] B-CLL is frequently associated with autoimmune hemolytic anemia (AHA) which, in a proportion of patients, is exacerbated after fludarabine therapy through a currently ill-defined mechanism.[2] Recent studies have demonstrated considerable modifications of normal Th1-Th2 balance in AHA, although the respective contribution of cytokines from these two T-cell subsets to the pathogenesis of AHA is still a matter of debate.[3,4] There is evidence that T cells from AHA patients predominantly secrete interferon-γ (IFNγ) in response to red blood cells antigen, but little or no interleukin-4.[3,5] Moreover, IFNγ production seems to account for the exacerbation of autoantibody-mediated diseases induced by a range of viral infections in mice.[6] We recently reported that fludarabine stimulates activator protein 1 (AP-1), a transcription factor involved in IFNγ gene regulation.[7]

We here investigated whether fludarabine could enhance the production of IFNγ by activated lymphoid cells, a mechanism that might be implicated in AHA exacerbation. Peripheral blood lymphocytes (PBL) from healthy donors, who were informed about the objectives of the study and gave their consent, were obtained after monocyte depletion by adherence onto plastic. PBL were exposed to graded doses of fludarabine (2-200 μM) 20 min before polyclonal activation with the addition of PMA (10 ng/mL) and Ionomycin (5 μg/mL). We found that fludarabine increased the release of IFNγ by activated PBL (Figure 1A) and the percentage of $CD^{2+}IFN\gamma^{+}$ cells evaluated by flow cytometry at 24 hs (%$CD^{2+}IFN\gamma^{+}$ cells: 12±2 vs 26±6, mean±SE, n=20, $p<0.05$, PMA + ionomycin vs PMA + ionomycin + fludarabine). Representative dot plots are shown in Figure 1B. A similar trend was observed upon PBL activation with PHA (Figure 1C). Differences in IFNγ-producing cells could not be ascribed to a reduced sensitivity of this subset to fludarabine since apoptotic levels were comparably low between activated cells cultured in the presence or absence of the drug (% apoptosis assessed by fluorescence microscopy:[7] 5±0.2 vs 5.3±0.2, mean±SE, PHA vs PHA + fludarabine).

We further confirmed that fludarabine increases the percentage of $CD^{2+}IFN\gamma^{+}$ cells by using purified $CD2^{+}$ lymphocytes ($pCD2^{+}$) obtained from peripheral blood mononuclear cells which were depleted of B cells and monocytes with specific antibodies and magnetic beads. $pCD2^{+}$ cultures (>98% of $CD2^{+}$ cells), composed of both T cells and NK cells, were cultured with PHA

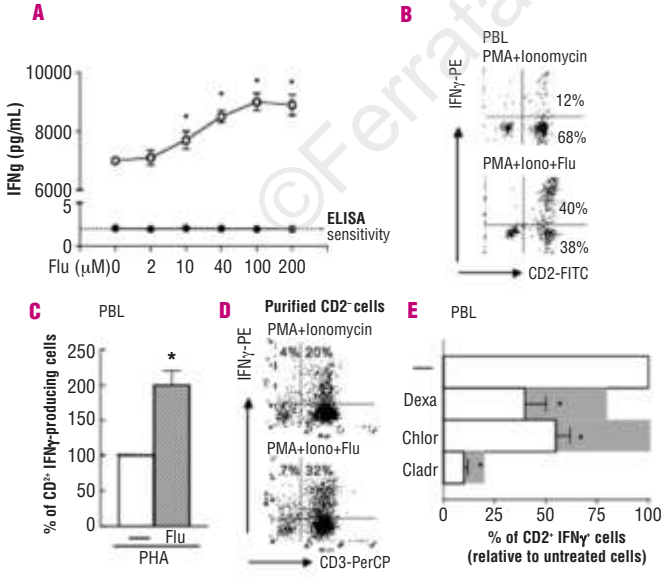

Figure 1. Fludarabine increases IFNγ production by activated lymphocytes from healthy donors. **A.** Peripheral blood lymphocytes (PBL) (2×10^{6} cells/mL) were cultured with fludarabine (2-200 μM) for 20 min before adding PMA (10 ng/mL) and ionomycin (5 μg/mL) (open circle) or medium (solid circle). IFNγ release was evaluated in culture supernatants at 24 h by ELISA. Values are the mean±SEM (n=6). *$p<0.05$ for fludarabine-treated vs. untreated cells: Student's paired t test. **B.** PBL were cultured with fludarabine (40 μM) for 20 min before adding PMA (10 ng/mL) and ionomycin (5 μg/mL). Brefeldin A (10 μg/mL) was added during the last 6 hs of culture in order to inhibit IFNγ release. IFNγ producing cells were detected by flow cytometry at 24h by immuno-fluorescence staining using antibodies against IFNγ (PE-conjugated) and CD2 (FITC-conjugated). The figure shows the dot plots from a representative experiment (n=20) with the percentages of $CD2^{+}$ $IFN\gamma^{+}$ and $CD2^{+}$ $IFN\gamma^{-}$cells. **C.** PBL were cultured with fludarabine (40 μM) 20 min before the addition of PHA (2.5 μg/mL). IFNγ producing cells were detected at 24h as described above. The percentage of $CD2^{+}$ IFNγ producing cells relative to untreated cultures are shown as mean±SEM (n=10). *$p<0.01$ for fludarabine-treated cultures vs untreated cells: Student's paired t test. **D.** Purified $CD2^{+}$ lymphocytes were cultured with fludarabine (40 μM) for 20 min before adding PMA (10 ng/mL) and ionomycin (5 μg/mL). Brefeldin A (10 μg/mL) was added during the last 6 h of culture in order to inhibit IFNγ release. IFNγ-producing cells were detected by flow cytometry at 24h using immuno-fluorescence staining and antibodies against IFNγ (PE-conjugated) and CD3 (PerCP-conjugated). The figure shows the dot plots from a representative experiment (n=4) with the percentages of IFNγ producing cells. **E.** PBL were cultured with dexamethasone (Dexa, 10^{-7} M), chlorambucil (Chlor, 16 μM) or cladribine (Cladr, 35 μM), for 20 min before adding PMA (10 ng/mL) and ionomycin (5 μg/mL). IFNγ-producing cells were detected at 24h as described above. The percentage of $CD2^{+}$ IFNγ producing cells relative to untreated cultures are shown as mean±SEM (n=7). *$p<0.01$ for drug-treated cultures vs untreated cells: Student's paired t test.

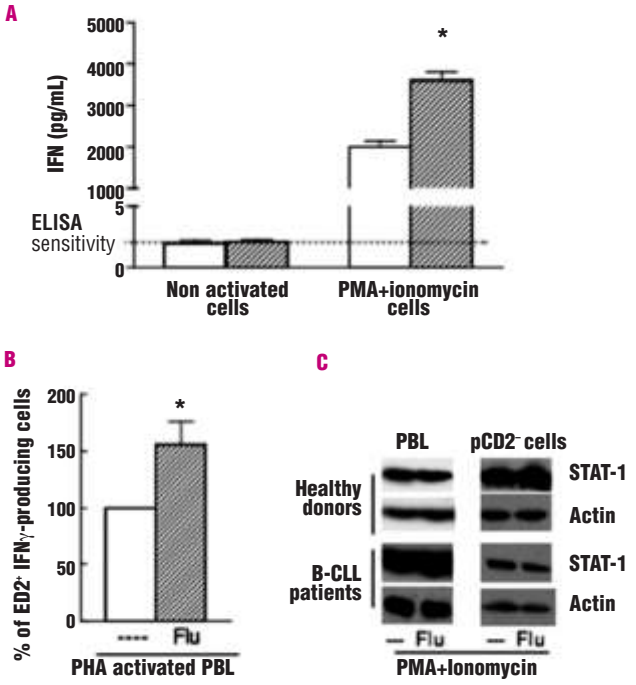

Figure 2. Fludarabine increases IFNγ production by activated lymphocytes from B-CLL patients. **A.** Peripheral blood lymphocytes (PBL) from B-CLL patients (2×10^6 cells/mL) were cultured with (dashed bar) or without (open bar) fludarabine (40 μM) for 20 min before adding PMA (10 ng/mL) and ionomycin (5 μg/mL). Experiments were performed with B-CLL samples containing at least 15% of $CD2^+$ lymphocytes. IFNγ release was evaluated in culture supernatants at 24 h by ELISA. Values are the mean±SEM (n=5). *$p<0.05$ for fludarabine-treated vs. untreated cells: Student's paired t test. **B.** PBL from B-CLL patients were cultured with fludarabine (40 μM) for 20 min before the addition of PHA (2.5 μg/mL). Brefeldin A (10 μg/mL) was added during the last 6 h of culture. IFNγ producing cells were detected as described in Figure 1B. Experiments were performed with B-CLL samples containing at least 15% of $CD2^+$ lymphocytes. The percentage of $CD2^+$ INFμ producing cells relative to untreated cultures are shown as mean±SEM (n=12). *$p<0.01$ for fludarabine-treated cultures vs. untreated cells: Student's paired t test. **C.** PBL (2×10^6) and purified $CD2^+$ lymphocytes from healthy donors and B-CLL patients were cultured with fludarabine (40 μM) for 20 min before adding of PMA (10 ng/mL) and ionomycin (5 μg/mL) or PHA (2.5 μg/mL). STAT-1 protein detection was evaluated at 24 hs by western blot. To this aim, cell pellets were washed with cold saline, immediately resuspended in loading buffer (60 mM Tris pH6.8, 2.3% SDS, 10% glycerol, 0.01% bromophenol blue and 5% β-mercaptoethanol) and boiled at 96°C for 5 minutes. Samples were then separated on a standard 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blotted with antibodies against STAT-1 and actin to compare the total amount of protein in each sample.

and fludarabine as described above. Fludarabine (40 μM) increased IFNγ production by $CD3^+$ and $CD3^-$ lymphocytes (Figure 1D), suggesting that both T cells and NK cells were responsible for IFNγ generation. A similar trend was observed at lower doses of fludarabine (20 or 10 μM) (*not shown*).

Additionally, we found that other pro-apoptotic drugs, such as dexamethasone (10^{-7}M) and chlorambucil (16 μM) decreased the percentage of $CD2^+IFN\gamma^+$ (Figure 1E). Unexpectedly, cladribine (35 μM), a purine analog with structural similarity to fludarabine, also impaired IFNγ production without increasing apoptosis. Given that PBL from B-CLL patients include a reduced percentage of non-leukemic lymphocytes with an array of abnormalities and dysfunctions,[8] it was important to determine whether they could respond in the same way as PBL from healthy donors. Experiments were performed with B-CLL samples containing at least 15% of $CD2^+$ lymphocytes. Fludarabine increased the secretion of IFNγ by activated PBL from B-CLL patients (Figure 2A) and the percentage of $CD2^+$INFγ producing lymphocytes (Figure 2B). Leukemic cells from the samples evaluated did not produce IFNγ (*not shown*). Fludarabine did not affect interleukin-4 production by activated PBL from B-CLL patients (interleukin-4 evaluated by ELISA at 24 hs: 36±6pg vs 28±2pg, mean±SE, n=5, PHA vs PHA+fludarabine).

Since Frank *et al*. previously reported that incubation of peripheral blood mononuclear cells with fludarabine impaired IFNγ production due to sustained depletion of signal transducer and activator of transcription-1 (STAT-1),[9] a key transcription factor required for mitogen activation, we also evaluated STAT-1 protein by western blot. As we expected, fludarabine did not reduce STAT-1 expression in PBL or $pCD2^+$ cultures from normal or B-CLL samples (Figure 2C). Although our observations contrast with those obtained by Frank *et al*., the effect of fludarabine on STAT-1 is unclear. Other authors recently reported, in line with our data, that fludarabine does not affect the STAT-1 pathway.[10] These observations should be taken into account since it has been suggested that fludarabine-induced depletion of STAT-1 protein may explain some of the immunosuppressive effects of the drug.[9]

In conclusion, our results indicate that fludarabine increases *in vitro* INFγ production by activated PBL from healthy donors and B-CLL patients. Given that INFγ has been linked to the pathogenesis of AHA, its enhanced production might be involved in the increased risk of AHA associated with fludarabine treatment in B-CLL patients.

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