

The role of cell type-specific responses in IFN- β therapy of multiple sclerosis

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The mechanism of IFN- β therapy in relapsing-remitting multiple sclerosis (RRMS) is not well understood, but induction of apoptosis in specific leukocyte subsets is likely to be important. Enhanced expression of *TNFSF10* or TNF-related apoptosis-inducing ligand (*TRAIL*) mRNA in unseparated leukocytes has been put forward as a therapeutic response marker, but it is unclear which leukocyte subsets express *TRAIL*. We investigated the basis of *TRAIL* expression in response to IFN- β by studying activation of STATs 1, 3, and 5, p38 MAPK, and NF- κ B in different leukocyte subsets of patients with RRMS. Monocytes, B cells, and T cells showed substantial differences in the activation of p38 and the STATs in response to i.m. injection of IFN- β 1a or stimulation in vitro. Induction of cell-surface *TRAIL*, analyzed in nine leukocyte subsets, was observed only on monocytes and granulocytes and correlated with the activation of p38 and/or NF- κ B in these subsets only, in agreement with previous work in fibroblasts showing that the induction of *TRAIL* in response to IFN- β depends on the activation of p38 and NF- κ B as well as STATs 1 and 2. We propose that, in myeloid cells, the differential activation of p38 and NF- κ B and induction of *TRAIL*, which sensitizes cells to apoptosis, can help to explain differences in responsiveness to IFN- β therapy among patients with RRMS and, furthermore, that such differential patterns of activation and expression may also be important in understanding the therapeutic responses to IFN- α / β in hepatitis and cancer.

humans | phosphoproteins | signal transduction | flow cytometry

IFN- β has been the main treatment for patients with relapsing-remitting multiple sclerosis (MS; RRMS) for many years and, despite the development of other treatments, still remains a widely used first-line disease-modifying drug in MS. IFN- β therapy reduces RRMS relapses by approximately 33% and reduces the development of new brain lesions by approximately 66%. Furthermore, IFN- β slows development of brain atrophy and neurological disability (1, 2). The molecular basis of the therapeutic response to IFN- β is still not well understood, although several immunomodulatory mechanisms have been proposed (3). Furthermore, therapeutic (2) and biological (4, 5) responses to IFN- β vary greatly among individual patients with RRMS, and again the basis is unknown. Inhibition of growth or induction of apoptosis are major therapeutic effects of IFN- α / β in cancer (6) and, likewise, one of the proposed immunoregulatory mechanisms of IFN- β therapy in RRMS is the induction of apoptosis in immune cells involved in pathogenesis (3, 7). IFN- α / β has opposing biological effects on different leukocyte subsets, namely, induction of apoptosis in monocytes and enhanced survival in T and B cells (8, 9). The molecular responses to IFN- β are initiated when it binds to its receptor, leading to activation of the receptor-associated kinases JAK1 and TYK2 and also to the activation of additional kinases (PI3K/Akt, p38, ERK, and JNK), and then to the activation of several transcription factors (TFs), including STATs 1–6, NF- κ B, AP-1, IRF1, IRF4, IRF8, and PU1 (8). Activation of STAT3, STAT5, and NF- κ B are linked to increased cell survival, whereas activation of STAT1 in general, NF- κ B under certain conditions, and p38 especially in hematopoietic cells, lead to apoptosis (6, 9–11). We hypothesized that differential activation of these various signaling proteins could

help to explain cell type-specific induction of apoptosis in response to IFN- α / β (9). In our previous study, by using freshly drawn whole blood from healthy subjects, we observed significant differences in the IFN- β -induced activation of STATs 1, 3, and 5 in primary human T cells, B cells, and monocytes in vitro, with STAT1-dependent gene induction linked to apoptosis in monocytes and STAT3-dependent gene-induction associated with survival in B cells (9). TNF-related apoptosis-inducing ligand (*TRAIL*), a member of the TNF family of type II membrane proteins, induces apoptosis upon binding to death receptors DR4 and DR5 (12). Both *TRAIL* on the cell surface and picomolar concentrations of soluble *TRAIL* rapidly induce apoptosis in a wide variety of transformed cell lines (12). Notably, melanoma cells that are resistant to induction of apoptosis in response to IFN- α / β failed to induce *TNFSF10* (*TRAIL*) mRNA (13). Interestingly, *TRAIL* mRNA is induced in peripheral blood mononuclear cells (PBMCs) of only those patients with RRMS who show a clinical response to IFN- β treatment. However, it is not yet known whether all or only some leukocyte subsets from responders express *TRAIL* mRNA (7) and what the molecular mechanism might be. Of note, induction of *TRAIL* mRNA by IFN- β in fibrosarcoma cells (14, 15) depends on the activation of p38, PI3K/Akt, and NF- κ B, as well as on the formation of ISGF3 (a trimer of tyrosine-phosphorylated STATs 1 and 2 and IRF9), a major TF mediating responses to type I IFNs. Here we have studied cell type-specific activation of STATs 1, 3, and 5, and p38 in leukocyte subsets of RRMS after injection with IFN- β 1a, and how these cell type-specific responses relate to the induction of *TRAIL* on the cell surface. We go on to propose a model to explain differences in individual responsiveness to IFN- β therapy in patients with RRMS.

Results

Differential Activation of STATs and Cell Type-Specific Activation of p38 in Leukocytes of RRMS Patients After Injection with IFN- β 1a or Stimulation in Vitro. We previously found significant differences in the activation of STATs 1, 3, and 5 among leukocyte subsets after stimulation of whole blood of healthy donors in vitro with IFN- β 1a, and linked these differences to differential induction of apoptosis (9). In a control experiment (Fig. S1), the activation of STATs 1, 3, and 5 was similar on two different occasions in monocytes and CD4⁺ and CD8⁺ T cells of one healthy subject after stimulation with IFN- β 1a in vitro. To investigate whether similar differential activation of STATs 1, 3, and 5 also occurs after injection of IFN- β 1a, eight patients with RRMS were initially studied who had previously been treated with IFN- β 1a for various amounts of time. Because the activation of p38 MAPK by IFN- α / β appears to be an important means of generating proapoptotic

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CD4⁺ T cells activated STAT3 compared with monocytes ($P < 0.05$), in agreement with previous results in healthy donors (9). Indeed, when the IFN- β -induced activation of STATs 1, 3, and 5 in monocytes and B and T cells of the eight patients with RRMS (Fig. 2, Right) was compared with activation of STATs in leukocytes of nine healthy donors in response to 500 IU/mL IFN- β in vitro, no significant differences were observed between patients with MS and healthy subjects (Fig. S4). In summary, our data show that, among leukocyte subsets of patients with RRMS, monocytes had the most STAT3-positive and p38-positive cells after IFN- β 1a injection and monocytes had the most STAT1-positive and STAT3-positive cells after stimulation with IFN- β 1a in vitro.

IFN- β 1a-Induced Surface TRAIL Expression ex Vivo and in Vitro Is Seen on Monocytes and Granulocytes only. The activation of p38 by IFN- β is an unusual but important requirement for the induction of a small minority of IFN-stimulated genes, with *CXCL11* (β -R1) and *TRAIL* as major examples (14). TRAIL is proposed to be a marker for responsiveness to IFN- β treatment in MS (7) and is a potent inducer of apoptosis (12). Because we found that none of the CD4⁺ T cells from patients with MS, but monocytes in 88% of the patients, showed p38 activation after injection with IFN- β 1a (Table S1), we examined whether such cell type-specific activation of p38 by IFN- β would also lead to cell type-specific induction of TRAIL on monocytes. To this end, four patients with RRMS were injected with IFN- β 1a and, 18 h later, the induction of TRAIL on the surfaces of CD4⁺ T cells, CD8⁺ T cells, CD25⁺CD4⁺ regulatory T cells, natural killer (NK) cells, NK T cells, B cells, monocytes, plasmacytoid dendritic cells (pDCs), and granulocytes was studied by flow cytometry. Fig. 3 (Upper) shows significant differences ($P = 0.0142$) in the expression of TRAIL on B cells, CD4⁺ and CD8⁺ T cells, granulocytes, and monocytes, with significant induction for only the latter two leukocyte subsets. In addition, NK cells, NK T cells, CD25⁺CD4⁺ regulatory T cells, and pDCs also did not show TRAIL expression after IFN- β 1a injection (only $\leq 1.6\%$ of these subsets were positive; Fig. S5A).

The i.m. injection of IFN- β 1a is reported to result in a concentration of approximately 100 IU/mL in serum (16). We therefore investigated whether in vitro stimulation with a much higher concentration of IFN- β 1a would result in expression of TRAIL on

other leukocyte subsets in addition to monocytes and granulocytes. To this end, blood, collected from the same four patients with RRMS and one additional patient with RRMS before injection with IFN- β 1a, was stimulated in vitro with 1,500 IU/mL IFN- β 1a for 18 h, to mimic the duration of stimulation in the in vivo situation. Fig. 3 illustrates that the same significant differences ($P = 0.0020$) occurred in vitro (Fig. 3, Lower) and in vivo (Fig. 3, Upper), even when a much higher dose of IFN- β 1a was used to stimulate in vitro. Notably, the four patients with RRMS studied for TRAIL induction after injection with IFN- β 1a showed the same expression patterns for TRAIL on monocytes and granulocytes at the individual patient level after in vitro stimulation with IFN- β 1a (Fig. 3). Moreover, B cells, CD4⁺ and CD8⁺ T cells (Fig. 3, Lower), NK cells, NK T cells, CD25⁺CD4⁺ regulatory T cells, and pDCs did not show TRAIL expression after stimulation with IFN- β 1a in vitro (only $\leq 4\%$ of these subsets were positive; Fig. S5B). In summary, of nine leukocyte subsets, we found that only monocytes and granulocytes express TRAIL after IFN- β 1a injection or stimulation in vitro.

Differential Induction of TRAIL on Leukocytes of Patients with RRMS in Response to IFN- β 1a Correlates with Differential Activation of p38 and NF- κ B.

The expression of *TRAIL* mRNA in fibrosarcoma cells in response to IFN- β depends on the formation of ISGF3 and the activation of p38, PI3K/Akt, and NF- κ B (14, 15). To determine whether early activation of specific kinases and TFs could be linked to cell type-specific induction of TRAIL, whole blood samples from four patients with RRMS (nos. 1, 4, 5, and 6) who had been studied for TRAIL protein expression on leukocytes in response to IFN- β in vitro after 18 h (Fig. 3, Lower) were simultaneously studied after 15, 30, and 45 min of stimulation with 1,500 IU/mL of IFN- β 1a in vitro (Fig. 4). Stimulation resulted in STAT1 and STAT2 activation in granulocytes, monocytes, B cells, and CD4⁺ and CD8⁺ T cells in all four samples (Fig. S6) and, therefore, differential formation of ISGF3 cannot be the reason for cell type-specific induction of TRAIL on monocytes and granulocytes. Interestingly, in addition to these cell type-specific effects, we also found differences in individual patients; one of four patients did not show expression of surface TRAIL on monocytes in the ex vivo experiments, and one of five patients did not show TRAIL expression on monocytes in the in vitro experiments (patient 4 in both experiments; Fig. 3). In contrast, TRAIL was expressed on granulocytes in 100% of the patients with RRMS after IFN- β 1a injection and in response to IFN- β 1a in vitro (Fig. 3). Notably, activation of p38 could be linked qualitatively to induction of TRAIL on monocytes (Fig. 4). Patient 4, who showed no p38 activation, also did not show induction of TRAIL, whereas patients 1, 5, and 6, who showed differing amounts of activation of p38, all showed expression of TRAIL on monocytes (Fig. 4). However, activation of p38 was not linked at all to the induction of TRAIL on granulocytes (Fig. 4), suggesting that activation of another signaling protein might be responsible.

There are three NF- κ B binding elements in the promoter of the *TRAIL* gene, and two of those are close to the ISRE element that binds to ISGF3 (14, 15). In addition, in vitro stimulation with IFN- β induces the phosphorylation of the p65 subunit of NF- κ B on an unknown residue in human fibrosarcoma cells (14) and increases NF- κ B (p65/p50) DNA binding in primary human microglia (17). Because optimal activation of NF- κ B requires the phosphorylation of Ser529 and Ser536 in the transactivation domain of p65 (10), we studied the phosphorylation of Ser529 in response to IFN- β . Remarkably, IFN- β -induced activation of p65 correlated quantitatively with the induction of surface TRAIL expression, on both granulocytes and monocytes (Fig. 4). The IFN- β -induced phosphorylation of Ser473 of Akt was also studied, because activated Akt has been shown to mediate Ser529 phosphorylation of p65 through the activation of p38 (18). Furthermore, activation of PI3K/Akt, but not ERK, was linked to induction of *TRAIL* mRNA in fibrosarcoma cells by IFN- β (14, 15). However, the activation of Akt (Fig. 4) or ERK (Fig. S6) did not correlate with surface TRAIL induction on monocytes or

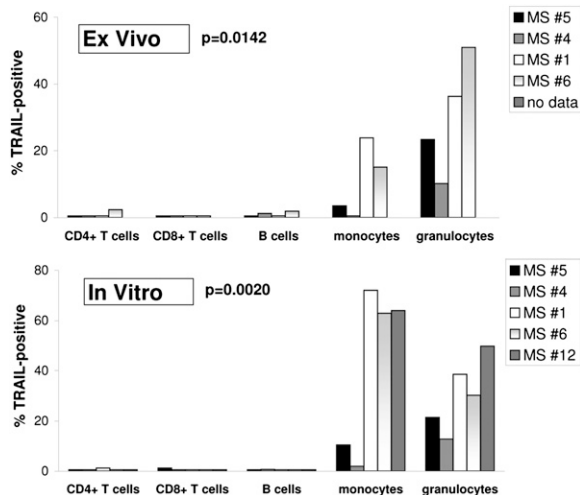


Fig. 3. Differential induction of TRAIL on leukocytes of patients with RRMS after stimulation with IFN- β 1a in vitro and in vivo. The percentage of TRAIL-positive leukocytes was determined for four patients with RRMS (patients 1 and 4–6) 18 h after injection with IFN- β 1a (Upper) or after stimulation of whole blood samples of five patients with RRMS with 1,500 IU/mL IFN- β 1a in vitro for 18 h (Lower). For the latter, blood drawn before injection with IFN- β 1a was treated in vitro. The Friedman test revealed significant differences in TRAIL induction among monocytes, granulocytes, T cells, and B cells ex vivo and in vitro (P values shown).

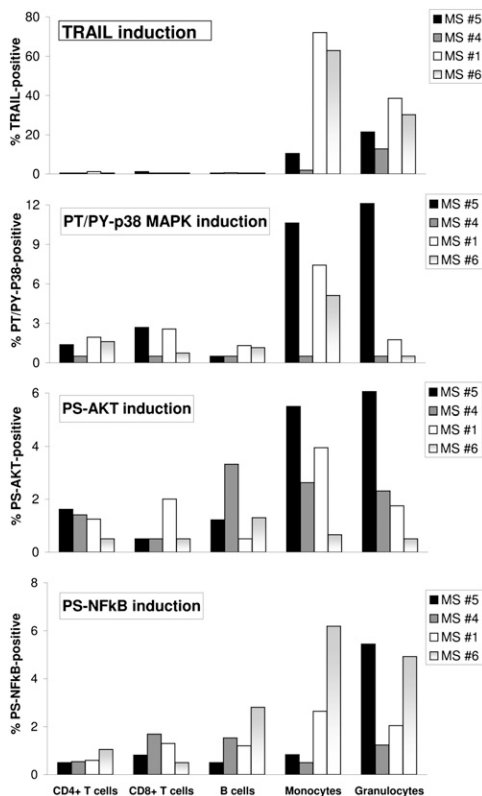


Fig. 4. Differential induction of TRAIL on leukocytes of patients with RRMS in response to IFN- β 1a in vitro correlated with differential activation of p38 and NF- κ B in monocytes and activation of NF- κ B in granulocytes. In whole blood of four patients with RRMS (patients 1 and 4–6), activation of p38, Akt, and NF- κ B and induction of TRAIL protein was determined after short (15, 30, and 45 min) and long (18 h) stimulation with 1,500 IU/mL IFN- β 1a in vitro. Shown for each individual patient are the highest percentages of kinase- and NF- κ B–positive leukocytes observed within the 15- to 45-min timeframe.

granulocytes in a qualitative or quantitative fashion in individual patients. Whole blood samples from the four patients with RRMS were also pretreated with chemical inhibitors of PI3K/Akt and p38 for 30 min and subsequently stimulated with 1,500 IU/mL IFN- β 1a for 18 h (Fig. S7). Inhibition of the IFN- β 1a–induced activation of p38 decreased TRAIL expression only on monocytes (by 60% on average), whereas inhibition of Akt activation had no effect on TRAIL induction on monocytes or granulocytes. In conclusion, the cell type-specific induction of TRAIL on monocytes and granulocytes by IFN- β was correlated with the IFN- β –induced activation of p38 and NF- κ B p65 in monocytes, and to the activation of p65 in granulocytes of patients with RRMS.

Discussion

IFN- β –Induced Cell Type-Specific Signaling in Leukocytes of Patients with RRMS Explains Selective TRAIL Induction. IFN- β is one of the main first-line disease-modifying drugs for patients with RRMS, but the molecular basis of its activity is not understood, and clinical responsiveness varies among patients. Understanding the molecular mechanisms of therapeutic action may allow those who show a poor response to be identified in advance of treatment and may provide insight into MS pathogenesis. It is becoming clear that a complex set of signaling pathways are activated by IFN- β , and we previously proposed that genes stimulated by type I IFNs are dependent on the activation of STATs alone (i.e., “STAT-only genes”), of STATs plus other TFs (i.e., “STAT + TF-genes”), or of other TFs only (i.e., “TF-only genes”) (8). Understanding the complexity of responses to type I IFNs is important for explaining cell type specificity, type I

IFN subtype specificity, and individual responsiveness to treatment (8). With respect to cell type specificity, it was unclear why treatment with IFN- β caused apoptosis in certain cancer cells and monocytes but increased the survival of B and T cells (9). We recently linked increased survival of normal human B cells in response to IFN- β to low numbers of B cells with PY-STAT1 and higher numbers with PY-STAT3 that drive expression of pro-survival genes, whereas IFN- β –induced apoptosis in monocytes is caused by PY-STAT1–dependent proapoptotic genes (9). Here we describe that injection of IFN- β 1a into patients with RRMS led to significant differences in the numbers of monocytes and T and B cells in which STAT3 and p38 become activated. We did not observe any activation of p38 in CD4⁺ T cells, and only in very few B cells, but much higher numbers of monocytes did activate p38 (in 88% of the patients with MS investigated). Because *TRAIL* mRNA induction in fibrosarcoma cells by IFN- β depends on the formation of ISGF3 and the activation of p38, PI3K/Akt, and NF- κ B p65 (14, 15), we hypothesized that cell-surface expression of TRAIL would be induced primarily on monocytes. Induction of surface TRAIL by IFN- β 1a injection has not been previously tested on CD4⁺ T cells, CD8⁺ T cells, CD25⁺CD4⁺ regulatory T cells, NK cells, NK T cells, B cells, monocytes, pDCs, and granulocytes of patients with RRMS. Strikingly, we found significantly increased levels of TRAIL after IFN- β 1a injection only on the surfaces of monocytes and granulocytes, consistent with published data on the induction of *TRAIL* mRNA and release of soluble TRAIL only in normal human neutrophils and monocytes, but not in lymphocytes by IFN- α/β in vitro (19, 20). The selective IFN- β –induced expression of TRAIL observed here on monocytes and granulocytes of patients with MS cannot be explained by a failure to induce the formation of ISGF3 in some subsets, because activation of STAT1 and STAT2 occurred in all leukocyte subsets. Moreover, despite activation of STAT3 in monocytes of patients with MS (Fig. 2), IFN- β did not prevent induction of PY-STAT1–dependent TRAIL. Interestingly, by using chemical inhibitors of p38, we were able to link the selective IFN- β 1a–induced expression of surface TRAIL on monocytes and granulocytes to activation of p38 only in monocytes. Furthermore, in accordance with previous data (14, 15, 18), we found that Ser529 phosphorylation of NF- κ B p65 in granulocytes, and particularly in monocytes, could be connected to cell type-specific induction of TRAIL by IFN- β in patients with MS. However, by using a chemical inhibitor of PI3K, we could not demonstrate in monocytes or granulocytes that TRAIL induction depended on activation of PI3K/Akt by IFN- β , indicating that the observed serine phosphorylation of p65 is not dependent on upstream activation of PI3K/Akt by IFN- β (18) as it is in fibrosarcoma cells (14, 15). In monocytes, it is likely that the activation of p38 by IFN- β caused Ser529 phosphorylation of p65 (18). Perhaps casein kinase 2 is responsible for the activation of NF- κ B in granulocytes in response to IFN- β , because this kinase can also phosphorylate p65 on Ser529 (21). In summary, monocytes and granulocytes are the only two leukocyte subsets in patients with RRMS that express TRAIL, but because only monocytes showed dependency on p38 MAPK activation, IFN- β seems to use different, cell type-specific signaling pathways to induce TRAIL in these two cell types. The reason that *TRAIL*, which is a STAT + TF gene (8), is induced only in granulocytes and monocytes and not in other leukocyte subsets seems to be that the necessary set of TFs is activated by IFN- β in only these two subsets.

Cell Type-Specific Activation of STATs Plus Other TFs as a Model to Explain Individual Responsiveness to IFN- β Treatment. *TRAIL* mRNA in unseparated PBMCs has been proposed as a marker of responsiveness to IFN- β therapy in MS (7), but it is not clear whether the higher *TRAIL* mRNA expression in responders was induced in all or only some of the leukocyte subsets present in PBMCs. Remarkably, a recent study shows exclusive *TRAIL* mRNA induction in monocytes and not in T cells or B cells of clinical responders to IFN- β therapy (22). In our study, TRAIL

expression was found on granulocytes in all patients with RRMS after IFN- β 1a stimulation, both ex vivo and in vitro. In contrast, not all patients with RRMS showed surface TRAIL expression on monocytes in response to IFN- β 1a; we found such expression in only three of four patients after injection with IFN- β 1a, and in four of five patients after in vitro stimulation (Fig. 3). Therefore, 20% to 25% of patients with RRMS did not show expression of TRAIL protein on monocytes, which approximates the expected percentage of nonresponders to IFN- β 1a treatment (2). Based on availability of brain MRI scans, we could only verify that RRMS patient 12 (Fig. 3, Lower) is a responder to IFN- β 1a therapy, and this patient is one of four who did show expression of TRAIL on monocytes in vitro. Although our study was not designed to draw definite conclusions about differences in cell type-specific expression of TRAIL by responders and non-responders, the data are in line with a recent study showing selective expression of TRAIL on monocytes of responders only (22). Therefore, the previously described increase in *TRAIL* mRNA expression in unseparated PBMCs of responders to IFN- β treatment (7) is likely to be caused by selective TRAIL mRNA expression in monocytes only (22). Interestingly, the reason that the latter occurred simultaneously with an increase in secretion of soluble TRAIL in responders and nonresponders (22) might be that all patients with MS show increased TRAIL production on granulocytes, as our preliminary data here suggest. In melanoma cells, sensitivity to induction of apoptosis in response to IFN- α/β depends on the induction of *TRAIL* mRNA (13). In responders, selective induction of *TRAIL* (22) and other proapoptotic genes in monocytes in response to IFN- β may contribute to the immediate induction of apoptosis in monocytes (9) or may prime monocytes to undergo programmed cell death later, when they differentiate into macrophages during migration across the blood-brain barrier (23). Likewise, the induction of TRAIL, observed on the granulocytes of all the patients with RRMS we have studied, may contribute to the observed apoptosis of granulocytes of patients with MS after IFN- β treatment (24). Remarkably, the protective effect of IFN- α/β in experimental autoimmune encephalomyelitis, the animal model for MS, depends entirely on the response of myeloid cells, because the deletion of IFNAR on myeloid cells, but not on other cells, increases disease burden during the effector phase of CNS autoimmunity, through enhanced macrophage invasion, cytokine/

chemokine production, and demyelination (25). Granulocytes and monocytes can both cause breakdown of the blood-brain barrier, and infiltrating macrophages are believed to cause demyelination and axonal loss in MS as well (26–28). Together, our data indicate that myeloid cells constitute a very important target of IFN- β therapy in MS. Therefore, triggering the apoptosis of monocytes and macrophages might be an important mechanism through which IFN- β treatment reduces active lesions in the brains of patients with MS. Notably, monocytes and macrophages play a common pathogenic role in several neuroinflammatory and neurodegenerative diseases, such as neural injury following stroke, Alzheimer's and Parkinson diseases, and HIV-associated dementia (29, 30). Alternative therapeutic strategies that diminish macrophage recruitment into the CNS might be of benefit in all these neurological diseases (26, 31).

The results of our exploratory study of whether cell type-specific signaling occurs after IFN- β injection suggest that the differential activation of p38 and NF- κ B in monocytes and the activation of NF- κ B in granulocytes can help explain the selective expression of TRAIL on these leukocyte subsets in individual patients with RRMS. Based on these data, we hypothesize that individual responsiveness to IFN- β treatment in MS and individual responsiveness to type I IFN therapy in other diseases as well might be explained by cell type-specific induction of certain STAT + TF genes by IFN- α/β . This notion is supported by recent work showing that responsiveness to pegylated IFN- α 2 in chronic hepatitis C infection of the liver can be linked to a cell type-specific increase of ISG15 in Kupffer cells of responders, in contrast to cell type-specific increase of ISG15 in hepatocytes of nonresponders (32). Because *ISG15*, like *TRAIL*, is a STAT + TF gene (8, 33), it remains to be established which STATs or TFs are differentially activated by pegylated IFN- α 2 in Kupffer cells and hepatocytes of responders and nonresponders, respectively, leading to differential induction of ISG15. Kupffer cells are the resident macrophages of the liver, and it is striking that IFN- α/β therapy, both in patients with RRMS and with chronic hepatitis C infection, induces the expression of a STAT + TF gene in myeloid cells of responders only. We therefore speculate that successful therapy with type I IFNs in cancer may rely not only on direct toxic effects on tumor cells, but might also depend on altering the myeloid-derived suppressor cells that otherwise would blunt the antitumor immune response (34). Fig. 5 shows a model

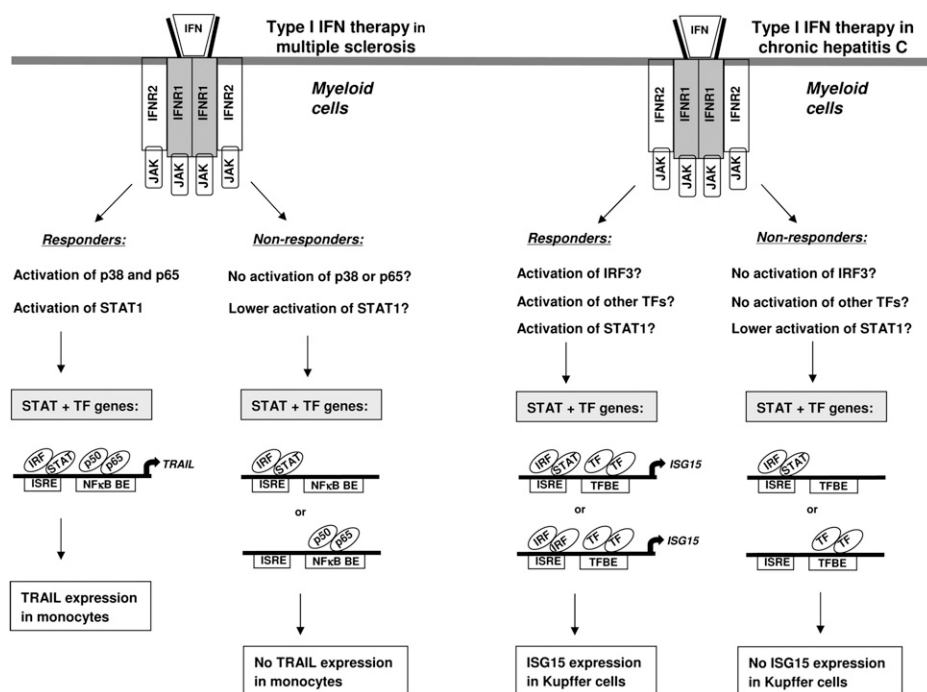


Fig. 5. Model to explain individual responsiveness to IFN- α/β therapy in MS and chronic hepatitis C. We propose that differential activation of TFs only in myeloid cells leads to selective induction of STAT + TF-dependent genes (like *TRAIL* and *ISG15*) in responders to type I IFN therapy, but not in nonresponders. *TRAIL* expression was selectively found in monocytes of responders to IFN- β therapy in MS (22), and here we show that selective activation of p38 and p65 leads to cell type-specific induction of TRAIL on monocytes of patients with MS. The reason that nonresponders do not express TRAIL on monocytes might be a result of failure to activate p38 or p65 (as shown here in MS patient 4; Fig. 4), or much lower activation of STAT1 (35). ISG15 is expressed only in Kupffer cells of responders to pegylated IFN- α 2 therapy in patients with chronic hepatitis C (32). It has to be established whether Kupffer cells of nonresponders have lower activation of STAT1 (8), IRF3 (33), or other TFs, causing no expression of ISG15 in these myeloid cells. NF- κ B BE, NF- κ B binding element; TFBE, TF binding element.

to explain individual responsiveness to IFN- α/β therapy in patients with MS and chronic hepatitis C.

In conclusion, we have demonstrated cell type-specific signaling linked to selective expression of TRAIL in leukocyte subsets of patients with RRMS after IFN- β 1a injection, a previously proposed determinant of clinical responsiveness to IFN- β therapy in MS (7). Therefore, the observed variability in the activation of STATs, a variety of kinases, and NF- κ B by IFN- β 1a in the various patients with RRMS we describe here is likely to underlie the significant patient-to-patient variation in gene expression that has been observed in unseparated PBMCs and whole blood samples of patients after IFN- β injection (4, 5). We are currently determining in a larger study whether differential STAT, kinase, and NF- κ B activation in myeloid cells and other leukocyte subsets in response to IFN- β injection predicts responsiveness to this therapy in MS.

Materials and Methods

Patients, Healthy Controls, and IFN- β Stimulation. Ten healthy controls and 11 patients with RRMS consented to give whole blood. All patients donated blood before injection with IFN- β 1a (Avonex; Biogen Idec), and nine patients with RRMS also donated blood at different times after injection. To enable determination of TRAIL induction by IFN- β , four of the 11 patients with RRMS returned after approximately 3 y. After stimulation with IFN- β , the undiluted whole blood was fixed immediately and erythrocytes were lysed for intracellular detection of p38, PT/PY-p38, PS-Akt, PT/PY-ERK, or

PS-p65 in leukocyte subsets by using flow cytometry (9). Further detailed information is provided in *SI Materials and Methods*.

Detection of Surface TRAIL Protein on Leukocytes by Flow Cytometry. Blood was drawn before and 18 h after IFN- β 1a injection, and surface markers to distinguish leukocyte subsets in whole blood were stained first. Samples were fixed and essentially further processed as described previously (9). To study the dependency of TRAIL induction on activation of PI3K/Akt and p38, whole blood was pretreated with 25 mM Ly294,002 or 20 mM SB203580, respectively (Sigma-Aldrich), for 30 min and subsequently stimulated with 1,500 IU/mL IFN- β 1a. Further detailed information is provided in *SI Materials and Methods*.

Statistical Analysis. InStat 3 software (GraphPad Software) was used for statistical analyses. The nonparametric Friedman test was used first, and subsequent post-hoc analysis was performed by using a Dunn test. Further detailed information is provided in *SI Materials and Methods*.

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