CD26 expression and adenosine deaminase activity in regulatory T cells (Treg) and CD4+ T effector cells in patients with head and neck squamous cell carcinoma

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Keywords: cancer, adenosine deaminase (ADA), Treg, Teff, adenosine deaminase; CADO, 2 chloro-adenosine; PGE2, prostaglandin E2; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; CFSE, carboxyfluorescein succinimidyl ester

Adenosine deaminase (ADA) is responsible for the deamination of immunosuppressive adenosine to inosine. In human T lymphocytes, ADA is associated with dipeptidyl peptidase IV (CD26). ADA expression and activity were evaluated in regulatory T cells (Treg) and CD4+ T effector cells (Teff) of patients with head and neck squamous cell cancer (HNSCC). CD4+CD39+ and CD4+CD39− T cells were isolated by single-cell sorting from the peripheral blood of 15 HNSCC patients and 15 healthy donors (NC). CD26/ADA expression in these cells was studied by multicolor flow cytometry, confocal microscopy, RT-PCR and immunohistochemistry in tumor tissues. ADA activity was evaluated by mass spectrometry, suppression of Teff proliferation in CFSE assays and cytokine production by Luminex. CD4+CD39− Treg had low and CD4+CD39+ Teff high CD26/ADA expression and ADA activity in NC or HNSCC. The frequency and suppressor activity of CD39+CD26+ Treg were elevated in patients relative to NC (p < 0.01). However, ADA activity in patients’ CD4+CD39+ Teff decreased (p < 0.05), resulting in extracellular adenosine accumulation. Also, patients’ Teff were more sensitive to inhibitory signals delivered via adenosine receptors, IL-2, IL12 and IFNγ upregulated ADA expression and activity in CD4+CD39− Teff, whereas IL-10, PGE2 and CADO downregulated it. The differentially expressed CD26/ADA can serve as surface markers for functionally-active CD39+CD26+ Treg.

Introduction

The integrity of the immune system in cancer is dependent on a variety of factors, including immunosuppressive and immunomodulating molecules generated in the tumor milieu. These factors interact with cells of the immune system determining patients’ immune competence. Chronic inflammation in diseases such as cancer or viral infections such as HIV-1, is accompanied by tissue damage and hypoxia leading to immune suppression which interferes with innate and/or adaptive immunity.10 Perhaps the most ubiquitous molecular alteration in damaged tissues involves phosphohydrolysis of ATP and ADP, which is catalyzed by the ectonucleotidase CD39.1 Further breakdown of AMP to adenosine by ecto-5’ nucleotidase, CD73, results in a dramatic increase of adenosine concentrations in situ, and in adenosine-mediated downregulation of immune responsiveness.1,11

Adenosine deaminase (ADA) is an enzyme that catabolizes adenosine to metabolites, inosine, thus downregulating biologic effects of adenosine in situ. ADA is present on the cell surface as well as intracellularly, but it does not have its own transmembrane domain and is associated with CD26, a surface glycoprotein with dipeptidyl peptidase IV activity. CD26 serves as a binding protein for extracellular ADA in humans, anchoring it to the cell surface and thus reducing the local levels of adenosine.10 It is thus surface-aligned CD26/ADA complex that by deaminating adenosine prevents its binding to A2A receptors on immune cells. The lack of this signal allows T cells to escape from adenosine-mediated suppression and to promote inflammation.11 Thus, blocking of surface-bound ADA activity enhances exogenous adenosine access to A2A receptors on effector T cells (Teff) and regulates adenosine-mediated suppression in these T cells. It has been well documented that defects in the ADA gene
cause an accumulation of purine metabolites, leading to an inherited form of severe combined immunodeficiency (SCID). Adenosine exerts various biologic effects which are mediated via its four receptors (R): A1, A2A, A2B, and A3. The immunosuppressive effects of adenosine on Teff are mainly performed via A2B signaling. We and others have reported that regulatory T cells (Treg), express CD39 and CD73 but lack CD26 and ADA. We hypothesize that these properties endow Treg with the ability to concentrate pericellular adenosine and use it for immune suppression. Treg maintain peripheral tolerance, we using various cell contact-dependent or contact-independent suppression mechanisms, with adenosine representing a soluble suppressive factor. The Treg frequency and activity are decreased in patients with autoimmune diseases but elevated in cancer patients, where Treg favor tumor development and tumor escape from the host immune system. The role of adenosine in tumor escape has been intensively investigated, and although there is evidence that human tumors can produce adenosine, recent attention has focused on Treg-generated adenosine. Immunosuppressive activities of adenosine and its involvement in neangiogenesis contribute to tumor progression and represent major tumor escape mechanisms.

Extending our initial observations, we show in this study not only that in cancer patients, CD26 and ADA expression is absent in Treg at the protein and mRNA levels, but also that ADA activity is significantly reduced in Teff in cancer patients compared with Teff in NC and that it can be altered upon exposure to different cytokines. Further, expression of CD26 in combination with the absence of CD26 defines a unique, functionally-active subset of Treg detectable by flow cytometry. This Treg subset is increased in the peripheral blood and tumor tissues of patients with HNSCC. Taken together with low ADA expression and activity in Teff of HNSCC patients, the data indicate that adenosine-mediated immune suppression is potentiated in cancer. Downregulation of ADA expression in Teff of HNSCC patients identifies yet another mechanism for increased adenosine levels in the tumor milieu.

**Results**

Adenosine metabolism in Treg and Teff. Recently, we have shown that Treg express CD39, and that CD39 is a reliable marker of human Treg suitable for their isolation. A large majority of CD4 CD39+ cells express FOXP3 (e.g., >72%) and only about 15% weakly express CD26 (Fig. 1A). In contrast, CD26 is overexpressed in most of Teff. CD26 binds ADA at its extracellular domain and, therefore, localizes ADA to the cell surface. We have previously reported that ADA was expressed in Treg but not in Teff. This initial observation is extended here to studies of ADA function in T cells of patients with cancer and of NC. First, by performing confocal microscopy with single-cell sorted CD4 CD39+ Treg and CD4 CD39+ Teff, we confirmed differential expression of CD26/ADA in these cells (Fig. 1B). To determine whether decreased ADA expression in Treg translates into lower ADA activity in converting adenosine to inosine, single cell-sorted CD4 CD39+ and CD4 CD39+ cells were incubated with exogenous adenosine for different time periods. Adenosine levels remaining in the cell supernatants were then determined (Fig. 1C). Teff cells (CD4 CD39+) hydrolyzed more adenosine than CD4 CD39+ Treg. Upon pretreatment of these cells with EHNA, an ADA inhibitor, the ability of Teff to metabolize adenosine was greatly reduced (Fig. 1D). EHNA itself had no effect on adenosine utilization by the cells (data not shown). To confirm that the low levels of adenosine measured in Teff cell cultures were indeed due to increased ADA activity and were independent of adenosine transport, we pretreated Teff using confocal microscopy with single-cell contact-dependent or contact-independent suppression mechanisms, with adenosine representing a soluble suppressive factor. The Treg frequency and activity are decreased in patients with autoimmune diseases but elevated in cancer patients, where Treg favor tumor development and tumor escape from the host immune system.

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functions by reducing adenosine-mediated immunosuppression. To test this hypothesis, we initially compared CD26 expression in CD4+CD39+ Treg cells obtained from NC and patients with HNSCC. The frequency of CD4+CD39+ Treg was found to be significantly reduced in patients vs. NC (Fig. 3A). Sorted CD4+CD39+ Treg cells were next incubated in the presence of exogenous adenosine to measure ADA enzymatic activity. The cells obtained from HNSCC patients deaminated adenosine less efficiently than did NC cells (Fig. 3B), suggesting that Teff in HNSCC had decreased ADA activity. After the addition of EHNA, an ADA inhibitor, the adenosine remaining in the cell supernatants of Teff in both cohorts increased significantly.

Table 1. Gene expression of CD26 and ADA in CD4+CD39+ and CD4+CD39neg T cells subsets

| Individual | CD4+CD39+ | CD4+CD39neg | ADA | CD4+CD39+ | CD4+CD39neg |
|------------|-----------|-------------|-----|-----------|-------------|
| 1          | 1         | 16x         | 1   | 1         | 1.4x         |
| 2          | 1         | 32x         | 1   | 1         | 1.7x         |
| 3          | 1         | 5x          | 1   | 1         | 13x          |

CD39+ and CD39neg cells were single-cell sorted and analyzed for CD26 and ADA expression by RT-PCR. The expression in Treg (CD4+CD39+) was set as 1, and the increased fold expression is shown for CD4+CD39neg Teff. Data were obtained with cells obtained from three individuals.
To determine whether the reduced CD26 and ADA expression in Teff of patients with HNSCC renders these cells more susceptible to adenosine-mediated immunosuppression, CFSE proliferation assays in the presence/absence of CADO, a synthetic analog of adenosine, were performed. Upon incubation of Teff isolated from NC or HNSCC with CADO the ability of these cells to proliferate in response to CD3/TCR signaling was significantly reduced. However, CADO-induced suppression was significantly greater in HNSCC patients' Teff than in NC's Teff (Fig. 3C and D). Thus, Teff in HNSCC were more sensitive to adenosine-mediated immunosuppression.
Surface and intracytoplasmic ADA expression and activity in Teff. The increased susceptibility of HNSCC patients’ Teff to adenosine-mediated immunosuppression could be due to reduced ADA expression in these cells. Figure 4A shows that while equal proportions of Teff in NC and patients express intracytoplasmic ADA, the frequency of Teff expressing surface ADA is lower in patients than NC. Similarly, the MFI of surface ADA was lower in Teff of HNSCC patients (Fig. 4B). However, the MFI for intracytoplasmic ADA was also significantly lower in HNSCC patients’ Teff. Together, the data suggest that low levels of ADA in and on the surface of Teff could be responsible for the observed greater susceptibility of patients’ Teff to adenosine.

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CD4+CD39+ Treg surface could facilitate Treg identification and isolation from PBMC. Our initial data showed that most (86 ± 4%) of CD4+CD39+ Treg were CD26 neg.4 Using sorted cells from PBMC of 15 NC, expression of various Treg-associated markers in CD4+CD39+ or CD4+CD39+CD26neg T cells was studied by flow cytometry. The percentages of FOXP3, GITR and CD25high were significantly higher in the CD4+CD39+CD26neg subset relative to the CD4+CD39+ Treg subset (Fig. 5A). No difference was evident in CTLA4 expression between the two subsets. The data suggest that this combination of surface markers on Treg may be useful for the isolation of Treg populations with a greater purity from PBMC.

The ability of CD4+CD39− and CD4+CD39−CD26− Treg subsets to suppress responder cell (RC) proliferation was also compared. Single cell-sorted CD4+CD39− or CD4+CD39−CD26− suppressor cells (S) obtained from freshly-isolated PBMC of NC

To further explore mechanisms responsible for reduced ADA expression and activity in patients with cancer, Teff were pre-treated with various cytokines or inhibitory factors and tested for ADA surface expression. IL-2, IFNγ and IL-12 increased the percentages of Teff expressing surface ADA, whereas IL-10, CADO and PGE2 decreased the percentages of Teff with ADA surface expression relative to the baseline after 48 h of incubation. These alterations in the frequency of ADA− Teff paralleled increases or decreases in ADA activity measured as consumption of exogenous adenosine by mass spectrometry after treating the cells with the various cytokines or factors (Table 2). These results confirm that ADA surface expression and its activity in Teff are modulated by factors present in the microenvironment.

ADA and CD26 as negative markers for human Treg. In addition to the importance of the CD26/ADA complex for susceptibility of human Teff to adenosine, its absence from the CD4+CD39− Treg surface could facilitate Treg identification and isolation from PBMC. Our initial data showed that most (86 ± 4%) of CD4+CD39− Treg were CD26−. Using sorted cells from PBMC of 15 NC, expression of various Treg-associated markers in CD4+CD39− or CD4+CD39−CD26− T cells was studied by flow cytometry. The percentages of FOXP3, GITR and CD25high were significantly higher in the CD4+CD39−CD26− subset relative to the CD4+CD39− Treg subset (Fig. 5A). No difference was evident in CTLA4 expression between the two subsets. The data suggest that this combination of surface markers on Treg may be useful for the isolation of Treg populations with a greater purity from PBMC.
were co-incubated with autologous CD4+CD25neg RC at different RC/T eff ratios. After a five-day culture, the mean suppressor activity of CD4+CD39neg cells at the 1S:1RC ratio was 41% ± 3, whereas the mean suppressor activity of CD4+CD39neg CD26neg was 61% ± 3 (p < 0.01; Fig. 5B). The suppression of proliferation linearly decreased upon further dilution of S (Fig. 5B). To examine the involvement of the adenosinergic pathway in Treg-mediated suppression, ARL67156, a CD39 inhibitor or ZM241385, a selective A2AR antagonist was added, to some culture wells. As shown in Figure 5C, Treg-mediated suppression was reduced (p < 0.01) using either of these reagents. CD4+CD39-CD26neg Treg not only generate immunosuppressive adenosine in co-cultures with RC but due to the absence of ADA are also able to maintain high adenosine levels in their microenvironment. Pericellular accumulations of adenosine could contribute to the increased levels of suppression mediated by these cells relative to that mediated by CD4+CD39+ cells.

Discussion

ADA is the key enzyme catalyzing the irreversible deamination of extracellular immunosuppressive adenosine into inosine and is, therefore, an important modulator of immune responses. ADA activity is necessary for sustaining Teff proliferation and cytokine production. ADA deficiency results in abnormalities in the development of the immune system (SCID), which are fatal if left untreated. CD26 is a lymphocyte marker that anchors ADA on the T-cell surface. We demonstrate here that Treg, which suppress immune responses, lack ADA as well as ADA-associated CD26 expression at the mRNA and protein levels, and that the lack of surface CD26 in combination with CD39 expression can be considered as a useful biomarker for the definition and isolation of functionally-active human Treg. The identification of reliable surface markers specific for human Treg has been difficult. FOXP3, a transcription factor, considered to be a specific marker for these cells can be also expressed albeit transiently on activated T cells. Further, because of its intracellular location, FOXP3 cannot be used for Treg isolation. The CD4+CD39+CD26+ Treg subset is highly suppressive and capable of not only generating but maintaining high levels of adenosine in their microenvironment. Pericellular accumulations of adenosine could contribute to the increased levels of suppression mediated by these cells relative to that mediated by CD4+CD39+ cells.

Treg present in the tumor microenvironment find themselves in the position to eliminate exogenous adenosine utilizing ADA in order to function. If exogenous adenosine levels are elevated, surface expression of the ADA/CD26 complex on Teff could decrease as a result of its rapid utilization. For example, CD26 cell surface expression is downregulated independently of adenosine receptor expression in tumor cells exposed to adenosine and on lymphocytes in breast cancer. With chronically elevated adenosine levels, as is the case in the tumor microenvironment, such upregulation of ADA in response to hypoxia might exhaust its cellular stores resulting in its lower expression on the surface of Teff, decreased enzymatic activity, increased adenosine levels and thus enhanced suppression of Teff functions. Here, we show for the first time that in cancer patients, the ADA activity in CD4+ Teff is significantly reduced compared with CD4+ Teff in NC. Further, cellular uptake of exogenous adenosine by Teff of cancer patients is reduced relative to that in Teff of NC. Greater sensitivity of patients’ Teff to adenosine-mediated suppression possibly reflects intrinsically increased A2AR activation upon adenosine coupling. While speculative at this time, this scenario provides a partial explanation for why immunosuppressive adenosine is less efficiently cleared and thus more inhibitory for Teff in the tumor microenvironment compared with Teff in NC.

Table 2. Influence of different cytokines on adenosine deaminase activity of Teff cells

| Treatment               | Relative ADA activity (%) (remaining adenosine) |
|-------------------------|-----------------------------------------------|
| Untreated (baseline)    | 100 (1,163 ng/ml)                             |
| IL-2 (50 IU/ml)         | 155 (1,027 ng/ml)                             |
| INFγ (50 IU/ml)         | 150 (1,469 ng/ml)                             |
| IL-12 (50 IU/ml)        | 150 (1,469 ng/ml)                             |
| CADO (5 μM/ml)          | 80 (1,459 ng/ml)                              |
| PGE (1 μM/ml)           | 51 (1,908 ng/ml)                              |
| IL-10 (50 μM/ml)        | 49 (1,938 ng/ml)                              |

**Note:** ADA activity was determined for all samples in duplicate. Remaining adenosine was determined using HPLC analysis. ADA activity was calculated for all samples in duplicate.
In summary, increased adenosine production by CD4+CD39+ Treg and reduced ADA activity in CD4+ Teff of HNSCC patients as shown in this study, contribute to tumor escape by increasing levels of immunosuppressive adenosine. An improvement in immunotherapy for these patients could potentially be achieved by enhancing ADA activity and preventing its downregulation in CD4+ Teff in addition to controlling the generation of immunosuppressive adenosine by Treg and/or tumor cells. These strategies based on pharmacologic interventions with drugs targeting the adenosinergic pathway are available for clinical use today.40,41

Materials and Methods

HNSCC patients and healthy volunteers. Peripheral venous blood samples were obtained from 15 HNSCC patients and 15 age-matched NC. All patients were seen in the Outpatient Clinic of the Department of Otolaryngology at the University of Pittsburgh Medical Center (UPMC) between December 2007 and September 2008. All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh. At the time of blood draw the patients had an active disease prior to any form of therapy.

Collection of peripheral blood mononuclear cells. Blood samples (20–30 mL) were drawn into heparinized tubes and centrifuged on Ficoll-Hypaque gradients (GE Healthcare Bioscience). PBMC were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments.

Separation of Treg. CD4+CD39neg T cells, CD4+CD39+ and CD4+CD39+CD26neg Treg were single cell-sorted from freshly-obtained PBMC of NC and HNSCC patients using a Cytomation MoFlo® high speed sorter after staining of lymphocytes with the relevant antibodies.

Antibodies. The following anti-human monoclonal antibodies (MoAbs) were used for flow-cytometry: anti-CD4-ECD, anti-CD26-PC5, anti-CD25-PE, anti-FOXP3-PE, anti-CD39-PE, anti-GITR, anti-CTLA4-PE and anti-ADA. Anti-CD4+, anti-CD25 Abs and their respective isotypes, were purchased from Beckman Coulter. The anti-FOXP3 (clone PCH101), anti-CD39-PE Abs and secondary PE-labeled goat anti-mouse for ADA staining were purchased from eBioscience. The anti-CTLA4 and anti-GITR-Abs were purchased from R&D Systems, anti-CD26 Ab from BioLegend, and anti-ADA Ab from Abcam. Isotype controls, which served as negative controls, were included with each sample.

Figure 5. The phenotype and suppression mediated by CD4+CD39+ Treg vs. CD4+CD39neg Treg. (A) Freshly sorted PBMC obtained from 15 NC were stained and analyzed by flow cytometry. Expression of conventional Treg markers in the CD4+CD39+ and CD4+CD39neg T cell subsets was determined. Data are means ± SD from 15 independent experiments. (B) Single-cell sorted CD4+CD39neg and CD4+CD39+ cells were CFSE-labeled and stimulated with plate-bound OKT-3 and soluble anti-CD28 in the presence of CD4+CD28+ or CD4+CD39neg suppressor cells and 150 IU/ml of IL-2 for 5 d. The % inhibition of R cell proliferation was determined by flow cytometry and analyzed using the Modfit software. (C) An inhibitor of CD39 activity (ARL67106) or an antagonist of the A2AR (ZM 241385) were added to the suppression assays at the beginning of the co-cultures set up as described in (B). Suppression of CD4+CD39neg cell proliferation mediated by CD4+CD39+ cells or CD4+CD39neg cells at various S/R Cell ratios was determined as described in Materials and Methods. The data are means ± SD from three independent experiments.
appropriate isotype controls were included for each sample. 

Surface and intracellular staining. Freshly isolated cells were stained for flow cytometry as previously described in reference 42.

Briefly, cells were incubated with the antibodies for surface markers titrated using activated as well as non-activated PBMC to determine the optimal staining dilution for each.

Flow cytometry. Flow cytometry was performed using a FACSCalibur flow cytometer equipped with Expo32 software (Beckman Coulter). The acquisition and analysis were restricted to the lymphocyte gate based on characteristic properties of the cells in the forward (FSC) and side scatter (SSC). FSC and SSC were set in a linear scale, and at least 10^5 cells were acquired for analysis, which was performed using the Coulter EXPO32VL.2 analysis program. For additional analyses, gates were restricted to the CD4+CD39- or CD4+CD39-CD26+ subsets.

Immunostaining. Single cell-sorted CD4+CD39- or CD4+CD39+CD26+ cells were cytotoxic-confluent on glass slides and stained using a standard immunoperoxidase-like method. Cells were first fixed using a 1:1 methanol/acetone solution and then dried at room temperature for 4 h. Afterwards, cells were treated with a serum-free protein block (Dako) for 1 h at room temperature, followed by washing with PBS and an overnight incubation at 4°C in the dark with the primary Ab. The following Abs were used: unconjugated anti-human ADA antibody (1:100 dilution, 4°C in the dark with the primary Ab. The following Abs were used: unconjugated anti-human ADA antibody (1:100 dilution, Santa Cruz Biotechnology) or appropriate isotype controls. Slides were then washed and incubated with a donkey anti-mouse-Cy3 (1:500, Jackson Immunoresearch). Next, slides were washed, fixed and evaluated in an inverted Olympus Fluoview 1000 laser scanning confocal microscope under an oil immersion objective (Center for Biology Imaging Core Facility, University of Pittsburgh). For digital image analysis, the software Adobe Photoshop version 7.0 was used.

Mass spectrometry for adenosine. CD4+CD39- or CD4+CD39+ T cells (25,000 cells/well) obtained from NC and HNSCC were incubated with 10 μM exogenous adenosine in either the presence or absence of erythro-9-(2-hydroxy-3-nonyl) adenine (EhNA, 2 μM, Sigma Aldrich), dipyridamole (5 μM, Tocris Bioscience) and/or NBTI (5 μM, Sigma Aldrich) in wells of flat-bottom 96-well plates at the responder cell (RC)/suppressor (S) ratios of 1:1, 2:1, 5:1 and 10:1. Using the same assay format, either ARL67165 (250 μM, Sigma Aldrich), ZM241385 (0.3 μM, Tocris Bioscience) or 2-chloro-adenosine (CADO; 20 μM, Sigma Aldrich) were added to selected wells 30 min prior to the addition of 5 cells. To induce proliferation, RC were stimulated with plate-bound OKT-3 (2 μg/ml) and soluble anti-CD28 mAb (2 μg/ml (Milenyi) in the presence of 150 IU/ml IL-2 for 5 d. All CFSE data were analyzed using the ModFit software provided by Verity Software (Topsham) as previously described in reference 42.

Immunofluorescence. HNSCC tissue samples were embedded in OCT; and 5 μm frozen sections were cut in a cryostat, fixed for 10 min in cold acetone/ethanol (1:1) and dried at room temperature. The following anti-human Abs were used for staining: anti-CD4-FITC, anti-CD3-PE, anti-ADA, anti-POXP3 and anti-CD26 (BD Pharmingen). The secondary Ab was Cy3-labeled donkey anti-rabbit (Jackson ImmunoResearch). To eliminate non-specific staining, tissue sections were incubated with 10% donkey serum for 1 h and then washed in PBS. Sections were incubated with the primary Abs for 1 h in a moist chamber at room temperature. Next, slides were washed and incubated with the secondary Abs under the same conditions. Primary Abs were omitted in all negative controls. Sections were mounted in a mounting medium with DAPI (Vector Laboratories) in order to trace cell nuclei. Slides were evaluated in the Olympus Provis (Olympus) fluorescence microscope under 400x magnification. For digital image analysis the software Adobe Photoshop 6.0 version was used.
LUMINEX. Cytokine levels in supernatants of T effector cell cultures were analyzed by LUMINEX, using a 10-plex Ab bead kit (Biosource/Invitrogen). After 24 h of stimulation with OKT-3 (1 μg/ml) and anti-CD28 (2 μg/ml), supernatants were harvested and stored frozen until analyzed.

Statistical Analysis

All data are presented as means of at least three experiments ± 1 standard deviation (SD). The data were analyzed using the Student’s t-test. p values < 0.05 were considered to be significant.

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