STAT3 inhibition enhances the therapeutic efficacy of immunogenic chemotherapy by stimulating type 1 interferon production by cancer cells

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Précis: STAT3 inhibitors may improve the therapeutic benefits of anthracyclines through augmenting cancer cell-autonomous type I IFN response.

Running Title: STAT3 depletion enhances tumor cell immunogenicity

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Abstract

STAT3 is an oncogenic transcription factor with potent immunosuppressive functions. We found that pharmacological inhibition of STAT3 or its selective knockout in cancer cells improved the tumor growth-inhibitory efficacy of anthracycline-based chemotherapies. This combined effect of STAT3 inhibition/depletion and anthracyclines was only found in tumors growing on immunocompetent (not in immunodeficient) mice. As compared to Stat3-sufficient control tumors, Stat3−/− cancer cells exhibited an increased infiltration by dendritic cells and cytotoxic T lymphocytes post-chemotherapy. Anthracyclines are known to induce several stress pathways that enhance the immunogenicity of dying and dead cancer cells, thereby stimulating a dendritic cell-dependent and T lymphocyte-mediated anticancer immune response. Among these therapy-relevant stress pathways, Stat3−/− cancer cells manifested one significant improvement, namely an increase in the expression of multiple type-1 interferon-responsive genes including that of the chemokines Cxcl9 and Cxcl10. This enhanced type-1 interferon response could be suppressed by reintroducing wild type Stat3 (but not a transactivation-deficient mutant Stat3Y705F) into the tumor cells. This maneuver also abolished the improved chemotherapeutic response of Stat3−/− cancers. Finally, the neutralization of the common type-1 interferon receptor or that of the chemokine receptor CXCR3 (which binds CXCL9 and CXCL10) abolished the difference in the chemotherapeutic response between Stat3−/− and control tumors. Altogether, these results suggest that STAT3 inhibitors may improve the outcome of chemotherapy by enhancing the type-1 interferon response of cancer cells.
Introduction

Anthracycline-based chemotherapies can induce immunogenic stress and death in tumor cells, thereby stimulating a protective anticancer immune response (1). This effect is linked to the capacity of anthracyclines to elicit a series of immunostimulatory stress pathways. Thus, doxorubicin, daunorubicin, epirubicin or mitoxantrone induce autophagic stress (which facilitates the lysosomal secretion of ATP, which then acts on purinergic receptors to attract myeloid cells into the tumor bed) (2), endoplasmic reticulum stress (which stimulates the translocation of the ‘eat-me’ signal calreticulin to the surface of the plasma membrane, where it facilitates the transfer of tumor antigens to dendritic cells) (3), the release of HMGB1 from necrotic cells (which stimulates toll-like receptor 4 on dendritic cells, allowing their maturation) (4), and the stimulation of a type-1 interferon response (which results in the autocrine activation of interferon α/β receptors [IFNAR] for the production of CXCL9 and CXCL10, which in turn stimulates T lymphocyte infiltration into the tumor) (1,5). This compendium of signals and pathway explains the unique capacity of anthracyclines to induce anticancer immune responses, both in preclinical models (6,7) and in breast cancer patients (5,8).

The transcription factor STAT3 is considered as a bona fide oncogene because its activation participates in tumorigenesis by virtue of its capacity to inhibit apoptosis and to stimulate unrestrained advancement through the cell cycle, aerobic glycolysis, metastasis (9-11). Beyond its capacity to stimulate oncogenesis at the cell-autonomous level, STAT3 has profound effects on the tumor microenvironment by stimulating angiogenesis and inflammation and by mediating immunosuppression (10,12). Hence STAT3 activation within tumor cells may contribute to tumor progression. STAT3 activation occurs in multiple immune cell types as well. In TH17 cells, STAT3 activation stimulates the expression of the ectonucleotidases CD39 and CD73, hence augmenting their immunosuppressive actions (13). In Tregs, STAT3 functionally and physically interacts with FOXP3 (14), which might explain why STAT3 enhances the function of FOXP3+ Tregs (15). STAT3 can be activated in distinct myeloid cell types including tumor-associated macrophages and myeloid derived suppress cells, stimulating their immunosuppressive function (16,17). In dendritic cells, depletion of STAT3 improves their capacity to present tumor antigens and to stimulate protective anticancer immune responses (18). As a result, both inhibition of STAT3 in cancer cells and in hematopoietic cells (particularly myeloid populations) has been...
attempted to reduce cell autonomous aspects of cancer and to improve anticancer immune responses, respectively (18-20). Deletion of the Stat3 gene by homologous recombination has yielded proof-of-concept that inhibition of STAT3 can improve cancer immunosurveillance (18-21). Moreover, it has been attempted to inhibit STAT3 by RNA interference locally within the tumor (22) or by injecting small inhibitors that interfere with the transactivation of STAT3 target genes (23,24). Phase-I trials involving small STAT3 inhibitors have been initiated in cancer patients (25-27).

Here, we investigated the possibility of combining anthracyclines with STAT3 inhibition for the treatment of neoplasia. We found that targeted deletion of Stat3 in cancer cells increases their therapeutic response to chemotherapy though a mechanism that is entirely immune-dependent and that specifically involves an increased type-1 interferon response.

**Materials & Methods**

**Cell culture and plasmids.** MCA205 fibrosarcoma cells (H2b) were induced by 3-methylcholanthrene in C57BL/6 mice (28) and were regularly checked for histocompatibility. Authenticated CT26 colorectal carcinoma (H2d) cells were obtained from American Type Culture Collection, immediately amplified to constitute liquid nitrogen stocks and (upon thawing) never passaged for more than 1 month before use in experimental determinations. All cell lines were negative for known mouse pathogens, including mycoplasma. Tumor cells were cultured in DMEM (Dulbecco's Modified Eagle Medium containing L-glutamine, high glucose), supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 1 mM non-essential amino acids and 100 U/ml penicillin and streptomycin. All culture products were from Life Technologies (Saint Aubin, France). Mouse cytokine IL-6 was purchased from Miltenyi Biotec. These parental cell lines were used to generate permanent Stat3−/− subclones using Zinc Finger Nuclease Technology (ZFN). Pre-validated CompoZr™ ZFN plasmids were purchased from Sigma-Aldrich (Ref: CKOZFN42165-1KT, Saint Quentin Fallavier, France). Plasmids pCS2-YFPC/mSTAT3wt and pCS2-YFPC/mSTAT3Y705F were kindly provided by Pr. Pravin B. Sehgal, New York Medical College, Valhalla, New York, USA. Ifnar−/− MCA205 was generated with ZFN technology as described before (5).
Stat3−/− tumor cell line generation and transfection. To generate Stat3-deficient MCA205 and CT26 cells, pre-validated CompoZr™ ZFN plasmids were incorporated into parental cell lines with FuGENE HD regent (Promega, Madison, WI, USA), together with the pcDNA™3.1 (+) vector (Life Technology) which conferred the co-transfected cells a temporary neomycin resistance. Forty-eight hours after transfection, G418 (Geneticin® 0.5 mg/ml, Life Technology) was applied for two days to select transfectants. The co-transfection strategy was applied due to the low transfection efficiency of parental cell lines. After a recovery period of 4 days, part of the transfectants was subjected to mismatch-specific nuclease assay with Surveyor mutation detection kit (Transgenomics) to verify the ZFN activity according to the manufacturer’s protocol. In brief, genomic DNA was extracted with Kapa DNA extraction kit (Kapa Biosystems). Genomic region surrounding the Stat3 ZFN target site was amplified with PCR using these primers: forward: 5’-GGAGAATGGGTTTGCAGTGT-3’; reverse: 5’-ACCTTTGGGAAAAAGGGAAA-3’. The PCR products were incubated in boiling water and slowly cooled to room temperature for re-annealing. Re-annealed PCR products were treated with SURVEYOR nuclease and SURVEYOR enhancer S in 42 °C for 30 min and analyzed on 2% agarose gel with SYBR® gold nucleic acid gel stain (Life Technology). Transfectants bearing DNA mismatches were used to perform single-cell sorting into 96 well plates, using BD FACSria™ III sorter in the CICC platform, Centre de Recherche des Cordeliers (CRC), Paris, France. All clones formed were screened with immunoblots for the expression level of STAT3 protein. To introduce wild type or mutated Stat3 into Stat3−/− clones, pCS2-YFPC/mSTAT3wt and pCS2-YFPC/mSTAT3Y705F plasmids were transfected separately to the Stat3−/− MCA205. YFP positive clones were enriched with BD FACSria™ III sorter.

Tumor models. Naïve female wild type C57BL/6 and BALB/c mice (aged between 6-8 weeks), which are MHC-haplotype compatible with MCA205 and CT26 cell lines, respectively, were purchased from Harlan, Gannat, France. Female nude mice were bred in the animal facility of Institut Gustave Roussy, Villejuif, France. Wild type or corresponding Stat3−/− tumor cells were inoculated subcutaneously (s.c. 5 × 10⁵ cells in 100 μl per mouse) in the thigh of host mice. Chemotherapy was administered when tumor size reaches 20-45 mm² (normally 6 to 8 days after tumor cell injection), either by one single intratumoral (i.t.) injection of Doxorubicin (DOXO, 2.9 mg/Kg in 50 μL PBS), or by intraperitoneal (i.p.) injection of mitoxantrone (MTX, 5.2 mg/Kg in
200 μL PBS). In some experiments, mice also received the STAT3 inhibitor Stattic i.t. (10 mg/Kg or vehicle control, one injection together with DOXO). When appropriate, mice were treated with blocking antibodies (10 mg/Kg per injection) against CXCR3 (InVivoMAb, Clone: CXCR3-173, BioXcell) or IFNAR (InVivoMAb, Clone: MAR1-5A3, BioXcell) intravenously on days 0, 2, 4, 7 after chemotherapy. Tumor diameters (the longest diameter and the diameter in its orthogonal direction) were measured 2 to 3 times per week with calipers and recorded as tumor surface (mm²). DOXO and MTX were from Sigma-Aldrich. Stattic was from Tocris Bioscience.

**ATP release quantification.** Tumor cell lines were seeded at $3 \times 10^4$ cells/well in 24 well plate on day 1. On day 2, the medium in all wells was replaced with pre-warmed fresh medium (300 μl/well), with or without 2 μM MTX. Twenty-four hours after, supernatants were collected, spun at 500×g to remove cell debris and used freshly to dose ATP release with ENLITEN® ATP Assay System (Promega, FF2000, Madison, WI, USA). Chemiluminescence signals were measured with a VICTOR™ X multilabel reader (PerkinElmer). Alternatively, cells were seeded at $6 \times 10^4$ cells/well in 12-well plates on day 1. On day 2, the medium in all wells were replaced with pre-warmed fresh medium (800 μl/well), with or without 2 μM MTX. Twenty-four hours after, trypsinized cells and their original supernatant were collected and combined in Facon tubes, spun at 1500 rpm, 10 min. The pellets were resuspended directly in Krebs-Ringer buffer (125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 0.7 mM KH₂PO₄, 2 mM CaCl₂, 6 mM D-glucose and 25 mM HEPES, pH 7.4) containing 2 μM quinacrine, incubated at 37°C for 30 min. After one wash with Krebs-Ringer buffer, cell pellets were resuspended in PBS containing 2 μg/ml DAPI and tested with flow cytometry (MACSQuant Analyzer, Miltenyi). Data were analyzed with Flowjo software (Treestar Inc., Ashland, OR, USA). All chemicals were from Sigma Aldrich. DAPI was from Molecular Probes®, Life Technologies.

**Cell death, calreticulin exposure and HMGB1 release.** Cell lines were seeded and treated with MTX (the same conditions as for the quinacrine assay) for indicated durations. Early or late apoptosis were analyzed with DAPI and Annexin V staining (BD Pharmingen™, Le Pont-De-Claix, France) following the manufacturer's instructions. Calreticulin exposure was determined by surface staining using rabbit monoclonal antibody (used at dilution 1:500, clone EPR3924, Ref:AJ1124a, ABGENT), followed by staining with Alexa488 conjugated goat anti-rat IgG (H+L)
secondary antibody (used at dilution 1:500, Life Technologies). For the HMGB1 release assay, cell lines were seeded and treated with MTX in the same condition as the ENLITEN® ATP Assay. Supernatants were collected at 24h, spun to remove cell debris, and then used freshly in ELISA-based quantifications (IBL International GMBH, Ref ST51011). Plates were read with a VICTOR™ X multilabel reader (PerkinElmer).

**RNA extraction, reverse transcription and qRT-PCR.** Cells were seeded in 12 well plates (same conditions as for the quinacrine assay) and treated with 2 μM MTX or 1000 U/ml Mouse IFNα (Miltenyi) for 16h. Trypsinized cells and their original supernatant were collected and spun at 300 × g for 10 min. The cell pellets were lysed directed with 350 μL RLT buffer (QIAGEN, Courtaboeuf, France) containing 2-mercaptoethanol (β-ME). Total RNA were extracted from cell lysates using the RNeasy Mini Kit (QIAGEN) and 3 μg of RNA were used in reverse transcription to cDNA with SuperScript® III First-Strand System (Life Technologies). Type I IFN-related gene expression was quantified with pre-validated TaqMan® Gene Expression Assays, using Universal Master Mix II (with UNG) on the StepOnePlus™ Real-Time PCR system (all from Applied Biosystems, Life Technologies).

**Tissue sections and immunofluorescent staining.** Tumors were harvested on days 2 and 7 after chemotherapy (MTX, i.p.) and used to analyze DC and T cell infiltration respectively. Tumors were fixed at room temperature with 10% neutral buffered formalin (CellStorTM pots, from CellPath, Newtown, UK) for 4h and transferred into 30% saccharose in PBS at 4°C for 24h. The remaining liquid on the tumors were wiped off before tumor were embedded in Tissue-Tek® O.C.T. Compound (Sakura, Villeneuve d’Ascq, France). Samples were frozen at -80°C immediately and transferred to -20°C before cutting them by means of a Leica CM 3050S Cryostat. Consecutive tissue sections (5 μm) were performed using poly-L-lysine-coated slides. Immunofluorescence staining were performed as described (30), with the following antibodies: CD11c-Alexa Fluor® 488 (N418), CD86-Alexa Fluor® 647 (GL-1), CD8a-Alexa Fluor® 488 (53-6.7) and CD3-Alexa Fluor® 547 (17A2) from BioLegend; cleaved Caspase-3 (Asp175) from Cell Signaling Technology; Alexa Fluor® 568 conjugated goat anti-rabbit IgG (H+L) secondary antibody from Life technologies.
Western blot. Cells collected from 6 well plates were lysed with 100 μL lysis buffer/well (containing complete protease inhibitor cocktail, Roche) on ice for 30 min. Cell lysates were spun at 12000×g for 20 min to obtain clear supernatant, which were further supplemented with NuPAGE® LDS Sample Buffer and Sample Reducing Agent before boiling for 5 min. Protein migration was performed with NuPAGE® Novex® 4-12% Bis-Tris Protein Gels under 100V constant voltage. For detecting LC3B, a pre-run step (protein non-loaded gel under 80V, 30 min) was required. Proteins on the gel were transblotted onto nitrocellulose membranes under 100V constant voltage for 90 min on ice. The membranes were blocked with 5% non-fat milk in 1× TBS containing 0.1% Tween®-20 (1×TBST) and incubated with primary antibodies: for LC3B (ab51520, Abcam) and STAT3 (REF:610189, BD Biosciences), overnight, 1:500 dilution; for β-actin conjugated with HRP (ab49900, Abcam), 1h at room temperature, 1:10000 dilution. HRP conjugated secondary antibodies (SouthernBiotech) were used at 1:5000. After repeated washing with 1×TBST, bands were visualized with ECL Western Blotting Detection Reagent (Amersham, GE healthcare) using ImageQuant LAS 4000 (GE healthcare). All reagents were from Life technologies if not specified.

Quantification of LC3 dots. Wild type or Stat3−/− MCA205 cells were infected with lentivirus encoding LC3-GFP (EMD Millipore), GFP+ cells were enriched by means of a BD FACSAria™ III sorter. Cells were seeded in BD high-content imaging plates. After the indicated treatment, images were collected with the BD Pathway 855 high-content bioimager (BD bioscience) and GFP-LC3-labeled puncta were analyzed.

Statistical analyses. All results were presented as means ± SEM of n = 3 to 6 parallel assessments. Similar results were obtained from at least twice independent experiments. Statistical differences were determined using unpaired, two-tailed student’s t test or Mann-Whitney U test. Statistical analyses and histogram generation were performed by software GraphPad Prism 5 (San Diego, CA, USA).

Results

Immune-dependent amelioration of chemotherapy by tumor cell-autonomous inhibition of STAT3. In response to the anthracycline DOXO, MCA205 fibroarcomas implanted in syngeneic
C57BL/6 mice reduced their growth. Local injection of the STAT3 inhibitor Stattic (29) failed to affect tumor progression on its own, yet enhanced the tumor growth-reducing activity of DOXO (Fig. 1A). This combination effect entirely depended on a T lymphocyte-mediate anticancer immune response, because MCA205 fibrosarcomas implanted in athymic nu/nu mice failed to reduce their growth in response to either DOXO alone or the combination of DOXO plus Stattic (Fig. 1B).

We used zinc finger nuclease technology to generate several independent clones of MCA205 fibrosarcomas and CT26 colorectal carcinoma cells that lacked Stat3 expression (Fig. 2A). Stat3−/− cancer cells showed a reduced apoptotic response to the anthracycline MTX in vitro (Fig. 2B,C). Nonetheless, if implanted into immunocompetent C57BL/6 mice, Stat3+/− MCA205 fibrosarcomas exhibited a largely improved chemotherapeutic response to DOXO or MTX in vivo (Fig. 2D). Similarly, Stat3−/− CT26 cells growing on immunocompetent syngeneic BALB/c mice exhibited a more vigorous reduction in tumor growth upon anthracycline-based chemotherapy than did Stat3-sufficient WT control cells (Fig. 2E). The improved chemotherapeutic response of Stat3+/− cancer cells was not observed in the context of a T cell-deficient immune system, in nu/nu mice (Fig. 2F), supporting the contention that depletion of Stat3 at the level of the tumors improves the efficacy of anthracyclines in an entirely immune-dependent fashion.

**Enhanced type 1 interferon response by Stat3−/− tumor cells responding to anthracyclines.**

To induce immunogenic cell death, anthracyclines must stimulate a series of stress and death pathways, including autophagy-linked ATP release, nuclear exodus of HMGB1 resulting from secondary necrosis, exposure of calreticulin upon endoplasmic reticulum stress, and the production of chemokines (such as CXCL10) subsequent to an type-1 interferon response (1,5). Anthracyclines could induce STAT3 phosphorylation in MCA205 cells in vitro, and this effect was lost in Ifnar1-deficient cells, indicating that anthracyclines can activate STAT3 in an IFNAR1-dependent fashion (Supplemental Fig. 1). Although Stat3 depletion can stimulate autophagy in some cell lines (30), we found no indication that Stat3−/− MCA205 cells would undergo more autophagy, either in baseline conditions or upon stimulation (Supplemental Fig. 2). Accordingly, Stat3−/− MCA205 cells released normal amounts of ATP in response to MTX, as indicated by the measurement of extracellular ATP (Fig. 3A) or that of residual ATP content in
the cells post-stimulation (Fig. 3B,C). Similarly, Stat3<sup>−/−</sup> MCA205 cells cultured with MTX released normal amounts of HMGB1 (Fig. 3D). Calreticulin exposure could be induced by MTX in WT and in Stat3<sup>−/−</sup> MCA205 cells, although the latter exhibited a reduced calreticulin exposure (Fig. 3E), correlating with their reduced apoptotic response (Fig. 2B,C; see above). In sharp contrast, Stat3<sup>−/−</sup> MCA205 cells exhibited a marked increase in the expression of genes linked to the type-1 interferon response, as determined by qRT-PCR analyses. This applies to the type-1 interferon gene Ifnb1 itself, the transcription factor Irf7, the antiviral genes Oas2, Mx1, Mx2, the gene coding for the pattern recognition receptor Dhx58, a number of other type-1 interferon-induced genes (such as Ifi205, Ifit2 and Rsad2), as well as to the chemokine-encoding genes Cxcl9 and Cxcl10, which both code for CXCR3 ligands (Fig. 3F). Interestingly, Stat3<sup>−/−</sup> cells activated some but not all of these type-1 interferon-responsive genes close-to-normally when directly stimulated with purified IFNα in vitro (Fig. 3G), suggesting a dual impact of STAT3, first on the capacity of anthracyclines to induce type 1 interferons and then the capacity of type 1 interferons to stimulate the expression of downstream target genes. Irrespective of this speculation, it appears that the only known hallmark of anthracycline-induced immunogenicity that is positive affected by Stat3 depletion is the type 1 interferon response.

Enhanced local immune response of Stat3<sup>−/−</sup> tumor cells responding to anthracyclines. In response to anthracyclines, cancers growing on immunocompetent mice exhibit a major alteration in their immune infiltrate. Within 48 hours post-chemotherapy, the frequency of activated dendritic cells with a CD11c<sup>+</sup>CD86<sup>+</sup> phenotype increases, in particular in the vicinity of apoptotic cancer cells staining positively for activated, proteolytically mature caspase-3 (Casp3a), as determined by multi-color immunofluorescence, followed by microscopic quantitation (31,32) (Fig. 4A). In spite of the fact that Stat3<sup>−/−</sup> MCA205 are less prone to undergo apoptosis in response to anthracyclines in ex-vivo culture (Fig. 2C,D), the frequency of Casp3a<sup>+</sup> cells were higher in Stat3<sup>−/−</sup> MCA205 tumors than in WT tumors after chemotherapy with MTX (Fig. 4A,B). Similarly, the frequency of CD11c<sup>+</sup> cells and, more so, that of CD11c<sup>+</sup> CD86<sup>+</sup> mature dendritic cells were higher in Stat3<sup>−/−</sup> cancers than in Stat3-sufficient WT controls, 48 hours post-chemotherapy (Fig. 4C,D). Detection of T cells (which are CD3<sup>+</sup>), in particular cytotoxic T lymphocytes (which are CD3<sup>+</sup> CD8<sup>+</sup>) by in situ immunofluorescence staining (Fig. 4E) revealed similar signs of an increased anticancer immune responses in response to the combination of
STAT3 inhibition and chemotherapy. After anthracycline-based chemotherapy, Stat3-/- cancers manifested a more dense infiltration by T cells (Fig. 4F) and, more specifically, cytotoxic T lymphocytes (Fig. 4G) than did control tumors. Altogether, these results confirm the idea that the combination of anthracyline plus Stat3 depletion can stimulate a particularly vigorous immune-mediated chemotherapeutic response.

Cause-effect relationship between the Stat3-regulated type 1 interferon response and the improved efficacy of anthracyclines. If STAT3 inhibited the local immune response by virtue of its function as a transcription factor, transfection of Stat3-/- cancer cells with Stat3 should reverse the phenotype, while re-introduction of a non-phosphorylatable (Y705F) mutant of Stat3 (in which the tyrosine in position 705 has been exchanged by a phenylalanine, abolishing its dimerization and hence its transactivation capability (33)) should not be able to do so. Accordingly, we found that restoration of Stat3-/- cancer cells with WT Stat3 but not with Stat3Y705F reversed the improved chemotherapeutic response to anthracyclines in vivo (Fig. 5). Reintroducing WT Stat3 also suppressed the exacerbated type-1 interferon response of Stat3-/- cancer cells responding to MTX in vitro, in conditions in which Stat3Y705F failed to do so (Fig. 6A). These results support the possibility that it is indeed the increased type-1 interferon response that accounts for the therapeutic effect of Stat3 depletion combined with chemotherapy.

To further explore this possible cause-effect relationship, we treated mice carrying Stat3-/- MCA205 cancers with antibodies that neutralize either one of the two subunits of the heterodimeric type-1 interferon receptor (IFNAR1), or the CXCR3 chemokine receptor (that mediates T cell infiltration of cancers in response to the chemokines CXCL9 and CXCL10). Both receptor-blocking antibodies reduced the efficacy of chemotherapy against Stat3-/- cancers and actually eliminated the difference in the therapeutic response between WT and Stat3-/- tumors. Altogether, these results suggest that the elevated type-1 interferon response of Stat3-/- cancers explains their improved response to anthracyclines.
Discussion

In this paper, we provide evidence that STAT3 inhibition can synergize with anthracyclines to mediate anticancer effects in preclinical models. Previous studies that were mostly performed in vitro, on cultured human cancer cell lines, suggested that STAT3 activation by anthracyclines may mediate chemoresistance, meaning that inhibition of STAT3 enhances the direct cytotoxic effects of DOXO (34-37). Seemingly corroborating this possibility, we found that anthracycline-based chemotherapy was more efficient against mouse tumor if it was combined with inhibition or deletion of STAT3.

Nonetheless, careful analyses revealed an unexpected mechanism for the synergistic therapeutic interaction between STAT3 inhibition and chemotherapy in preclinical mouse models. STAT3 inhibition did not increase the direct cytotoxic action of anthracyclines on mouse tumor cells in vitro. The synergistic effect was only observed in vivo, on tumors that were growing in fully immunocompetent mice, not in mice lacking thymus-dependent T lymphocytes. Hence, the positive interaction between STAT3 inhibition and anthracycline must be dictated by the immune system. Accordingly, Stat3 depletion and chemotherapy together induced signs of an increased anticancer immune response consisting in a denser infiltration of the tumors by mature dendritic cells and cytotoxic T lymphocytes. Inhibition of STAT3 alone did not cause any sizeable increase in the immune infiltrate. However, after chemotherapy, Stat3+/− cancers exhibited a significantly stronger immune infiltrate than WT tumors. Shortly (48 hours) post-chemotherapy, Stat3−/− tumors also contained more apoptotic cells than Stat3-sufficient control neoplasias. It is tempting to speculate that this enhanced apoptosis reflects the enhanced activity of immune effectors in the tumor bed.

As mentioned in the introduction to this paper, anthracyclines can stimulate anticancer immune responses through their capacity to elicit several immunostimulatory stress pathways that collectively mediate the phenomenon of immunogenic cell death (ICD). A systematic analysis of all known ICD-related pathways led to the conclusion that only one among them, namely the one linked to the induction of type-1 IFN-related genes was over-induced in Stat3−/− cancer cells. Stat3-deficient hepatocellular carcinoma and follicular helper T cells exhibit increased expression
of type-1 interferon-inducible gene (38), in line with a model that STAT3 and type I IFN signaling pathway are mutually repressive (39). In viral infection models, the absence of STAT3 led to hyperactivation of ISGF3 (STAT1:STAT2:IRF9) complex and hence augmented type I IFN response (40,41). However, at present it is not known through which detailed molecular mechanisms, STAT3 inhibition amplifies the induction of type-1 interferon-inducible genes by anthracyclines. Hence, this effect of STAT3 inhibition requires further mechanistic exploration. In line with one previous report (42), this effect is related to the transcriptional activity of STAT3, because a mutant that lacks transactivation potential was unable to repress the type I IFN response. The amplitude of the stimulatory effect of Stat3 deletion on type-1 interferon-inducible genes is clearly higher when MTX rather than purified IFNα is used as a stimulus. This suggests that the initial events triggered by anthracyclines including the stimulation of pattern recognition receptors (such as TLR3) (5) are somehow favored by Stat3 deletion, yet requires further mechanistic exploration. Thus, in response to anthracyclines, Stat3<sup>−/−</sup> cancer cells produced significantly more of the immunostimulatory CXCR3 ligands CXCL9 and CXCL10, while this difference was not detectable upon stimulation with purified IFNα.

The favorable interaction of STAT3 inhibition with anthracycline-based chemotherapy was lost in the absence of thymus-dependent lymphocytes, as well upon the neutralization of the common type-1 interferon receptor or that of the chemokine receptor CXCR3 (which binds CXCL9 and CXCL10). These latter results establish the cause-effect relationship between the exacerbated type-1 interferon-related response and the efficacy of the combined treatment with anthracyclines and STAT3 inhibition. Based on these results, it may be interesting to combine STAT3 inhibitors and anthracyclines (or other immunogenic cell inducers including radiotherapy) in future clinical trials. In such trials, the type-I interferon response should be assessed as a possible predictive biomarker. It may be particularly interesting to use STAT3 inhibitors against tumors that, at difference with those investigated in this paper, are ‘addicted’ to STAT3 and hence require STAT3 for their survival and proliferation. In such a case, the cell-autonomous and the immunological effects of STAT3 may add up in an advantageous fashion.

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References


Figure legends

Figure 1. The STAT3 inhibitor Stattic improves the therapeutic effect of anthracyclines in an immune-dependent fashion. (A-B) Immunocompetent mice (A), or age- and sex-matched athymic nude mice (nu/nu) (B) bearing palpable MCA205 fibrosarcomas (20-45 mm²) were treated by intratumoral injection of PBS or DOXO (50 μL/mouse), alone (left panels) or in combination with Stattic (right panels). Tumor sizes were monitored 2-3 times per week and shown as mean ± SEM at each time point. Each group contains 5-6 mice and these experiments were repeated at least twice yielding comparable results.

Figure 2. Stat3 depletion from tumor cells enhances the antineoplastic effect of anthracyclines in an immune-dependent fashion. (A) Knockout the Stat3 gene with ZFN technology was validated by immunoblots in MCA205 (left) and CT26 (right) cells. (B-C) WT and Stat3⁻/⁻ MCA205 cells were treated with MTX in vitro in 12 well plates. At the indicated time points, cells were collected to assess apoptosis by means of Annexin V-FITC and DAPI staining. Typical dot plots are shown at 24 h (B) and the mean ± SEM (n=3 independent experiments) of the percentages of cells in early apoptosis (Annexin V⁺DAPI⁻) and late apoptosis (Annexin V⁺DAPI⁺) was plotted (C). (D-E) Immunocompetent mice harboring WT or Stat3⁻/⁻ MCA205 (D) or CT26 tumors (E) were treated with either intratumoral injection of DOXO (D, left panel and E), or intraperitoneal injection of MTX (D, right panel) or PBS as vehicle control (D, E). Tumor progression was monitored and depicted as mean ± SEM at each time points. (F) Athymic nu/nu mice were implanted with WT or Stat3⁻/⁻ MCA205 tumors, followed by intratumoral treatment with DOXO. Tumor growth kinetics was illustrated as means ± SEM. These experiments included at least 5 mice/group and results shown are representative for 2-3 independent experiments. For cell death and tumor growth, the unpaired Student's t-test and the unpaired Mann-Whitney U test, respectively, were applied. *, p<0.05, **, p<0.01, ***, p<0.001, ns, not significant.

Figure 3. Impact of Stat3 deficiency on the hallmarks of immunogenic cell death. (A) Supernatants from WT or Stat3⁻/⁻ MCA205 cells under MTX treatment (24h) were collected and used immediately to quantify ATP release with the luciferase bioluminescence assay. (B-C) WT or Stat3⁻/⁻ MCA205 cells were treated with MTX (24h) and the amount of intracellular ATP
vesicles was assessed by staining with quinacrine. DAPI was used to distinguish non-viable cells. The percentages of quinacrine DAPI⁻ and quinacrine DAPI⁺ cells are shown in (B), while the mean fluorescence intensity (MFI) of quinacrine in quinacrine⁺ DAPI⁻ cells is shown in (C). (D) HMGB1 protein levels were measured in supernatants from MCA205 cells (WT versus Stat3⁻⁻) pre-treated with MTX (24h) by ELISA. (E) The exposure of calreticulin on the cell surface was detected by staining with rat monoclonal anti-Calreticulin antibody. Typical dot plots (left panel), and quantitative data are shown (right panel). (F-G) WT or Stat3⁻⁻ MCA205 tumor cells were treated with 2 μM MTX (F) or 1000 U/ml IFNα protein (G) for 16h. Total RNA was extracted, reverse transcribed to cDNA, and relative expression of type I IFN response-related genes were assessed with TaqMan® based qRT-PCR. Ppia was used as endogenous control. Fold changes relative to WT MCA205 PBS condition were calculated by means of the 2⁻⁻ddCT method for each gene. The Y-axis of left panel (G) is log-scaled. All samples were done in triplicates and experiments were repeated at least twice, yielding similar results. *, p<0.05, **, p<0.01, ***, p<0.001, ns, not significant (unpaired Student’s t test).

Figure 4. Enhanced immune cell infiltration after chemotherapy of Stat3⁻⁻ tumors. (A-D) Mice harboring WT or Stat3⁻⁻ tumors received injections (i.p.) of PBS or MTX. Tumor samples were harvested 48h post-treatment and subjected to immunofluorescence staining. Typical images from MTX treated tumors were shown in (A). Apoptosis was detected by staining of cleaved caspase-3 (B), and tumor infiltrating CD11c⁺ cells (C) and mature dendritic cells CD11c⁺ CD86⁺ (D) were quantified as the percentage of all nucleated (Hoechst⁺) cells in the same view field. (E-G) To examine T cell infiltration, tumor samples were harvested 7 days after treatment. Representative images show CD3 and CD8 staining in MTX treated tumors in WT versus Stat3⁻⁻ tumors (E). The bulk of CD3⁺ T cells (F) and CD3⁺ CD8⁺ cytotoxic T lymphocytes (G) were quantified. Each group included 5 tumor samples and each point represents a different view field. Non-consecutive sections from the same tissue were randomly chosen and images were taken both in the center and the periphery of tumors. *, p<0.05, **, p<0.01, ***, p<0.001 (unpaired Student t test).

Figure 5. Re-introduction of WT, but not mutant, Stat3 abolishes the therapeutic advantage of Stat3⁻⁻ tumors. (A-D) WT or mutated (Y705F) forms of Stat3 were stably transfected into
MCA205 Stat3\(^{-/-}\) cells. The responsiveness of Stat3\(^{-/-}\) Tg WT (C) or Stat3\(^{-/-}\) Tg YF (D) to chemotherapy was compared to that of WT and Stat3\(^{-/-}\) tumors, in immunocompetent mice. Red arrows indicate the time when DOXO was administrated. (E) Validation of STAT3 expression upon re-introduction of WT or mutant Stat3 into Stat3\(^{-/-}\) cells. Since WT or mutant STAT3 was fused to GFP, the bands corresponding to transgenic (Tg) STAT3 have a higher molecular weight (104kDa) than the endogenous molecule. (F) Tumor sizes at 16 days post chemotherapy were compared among MTX treated cancers with the indicated genotype. (G) The tumor-growth retarding effect of MTX was compared among different tumors by determining the number of days needed to reach the size of 100 mm\(^2\). All groups included at least 5 mice and one representative experiment out of two is shown. *, p<0.05, **, p<0.01, ns, not significant (unpaired Student t test).

**Figure 6.** Essential role for the overexpression of type I IFN related genes in the improved chemotherapeutic response of Stat3\(^{-/-}\) tumors. (A) Type I IFN-related gene expression with or without MTX treatment (16h) was measured in WT, Stat3\(^{-/-}\), Stat3\(^{-/-}\) Tg WT and Stat3\(^{-/-}\) Tg YF tumor cells. PpiA was used as endogenous control. The relative expression to untreated WT MCA205 cells were calculated by means of the 2\(^{-}\)-ddCT method and shown as fold-change. The Y axis is log-scaled. For gene expression, the unpaired Student's t-test was applied. (B-C) MCA205 tumor cells, WT (B) or Stat3\(^{-/-}\) (C) were inoculated into immunocompetent mice. Once tumor size reached 20-45 mm\(^2\), mice were treated by intratumoral injection of DOXO. Neutralizing antibodies targeting CXCR3 or IFNAR1 was repeatedly injected on the very day of chemotherapy, and 2, 4, 7 days later. Mouse IgG1 (200 \(\mu\)g/injection) was used as an isotype control. All groups included 6 mice. Experiments were repeated twice yielding similar results. For tumor growth, the unpaired Mann-Whitney U test was employed for statistical comparisons. *, p<0.05, **, p<0.01, ***, p<0.001, ns, not significant.
Figure 1

WT mice

nu/nu mice

A

B

Tumor sizes (mm$^2$)

Times (days)

PBS

DOXO

PBS+Stattic

DOXO+Stattic

PBS

DOXO

PBS+Stattic

DOXO+Stattic

Figure 1
Figure 2

A

MCA205

WT

Stat3<sup>-/-</sup>

42kDa

Actin

CT26

WT

Stat3<sup>-/-</sup>

78kDa

B

P<sub>BS</sub>

MTX

WT

Annexin V

WT

Stat3<sup>-/-</sup>

Stat3<sup>-/-</sup>

C

Annexin V<sup>+</sup> DAPI<sup>+</sup>

Annexin V<sup>+</sup> DAPI<sup>-</sup>

WT

Stat3<sup>-/-</sup>

D

WT mice

MCA205

Tumor sizes (mm<sup>2</sup>)

WT PBS

WT DOXO

Stat3<sup>-/-</sup> PBS

Stat3<sup>-/-</sup> DOXO

E

WT mice

CT26

Tumor sizes (mm<sup>2</sup>)

WT PBS

WT DOXO

Stat3<sup>-/-</sup> PBS

Stat3<sup>-/-</sup> DOXO

F

nu/nu mice

MCA205

Tumor sizes (mm<sup>2</sup>)

WT PBS

WT DOXO

Stat3<sup>-/-</sup> PBS

Stat3<sup>-/-</sup> DOXO

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**Figure 3**

A. Extracellular ATP levels measured using the MCA205 MTX assay. (Left) Wild-type (WT) and Stat3−/− cells show no significant difference in ATP extracellular levels. (Right) Corresponding bar graphs demonstrate a similar trend.

B. Quinacrine (Quin) and DAPI (DAPI) staining of cells. (Top) Wild-type (WT) and Stat3−/− cells show no significant difference in the number of Quin+/DAPI− cells. (Bottom) MCA205 MTX analysis reveals a similar trend.

C. MRI quinacrine and DAPI staining of cells. (Top) Wild-type (WT) and Stat3−/− cells show no significant difference in the percentage of Quin+/DAPI− cells. (Bottom) MCA205 MTX analysis reveals a similar trend.

D. MCA205 MTX assay for HMGB1 levels. (Left) Wild-type (WT) and Stat3−/− cells show no significant difference in HMGB1 levels. (Right) Corresponding bar graphs demonstrate a similar trend.

E. Calreticulin and DAPI staining of cells. (Left) Wild-type (WT) and Stat3−/− cells show no significant difference in the percentage of Calreticulin+DAPI− cells. (Right) Corresponding bar graphs demonstrate a similar trend.

F. Gene expression fold change for IFNα target genes. (Left) Wild-type (WT) and Stat3−/− cells show no significant difference in IFNα target gene expression. (Right) Corresponding bar graphs demonstrate a similar trend.

G. Gene expression fold change for additional target genes. (Left) Wild-type (WT) and Stat3−/− cells show no significant difference in additional target gene expression. (Right) Corresponding bar graphs demonstrate a similar trend.
Figure 5

A. MCA205 WT Tumor sizes (mm²)

B. MCA205 Stat3⁻/⁻ Tumor sizes (mm²)

C. MCA205 Stat3⁻/⁻ Tg WT Tumor sizes (mm²)

D. MCA205 Stat3⁻/⁻ Tg YF Tumor sizes (mm²)

E. Western Blot

F. Endpoint tumor size (mm²) with MTX

G. Time to reach 100 mm² with MTX

* ns

MTX

** ns

MTX

WT Stat3 Stat3⁻/⁻ Stat3⁻/⁻ Tg WT Stat3⁻/⁻ Tg YF

Actin

104 kDa

78 kDa

42 kDa
Figure 6

A

Gene expression fold change

WT
Stat3+/+ WT
Stat3−/− WT
WT
Stat3+/+ YF
Stat3−/− YF
WT
Stat3+/+ WT
Stat3−/− YF
WT
Stat3+/+ WT
Stat3−/− YF
WT
Stat3+/+ WT
Stat3−/− YF
WT
Stat3+/+ WT
Stat3−/− YF

B

MCA205 WT

Gene expression fold change

PBS
DOXO
DOXO Ab IFNAR1
DOXO Ab CXCR3

C

MCA205 Stat3−/−

PBS
DOXO
DOXO Ab IFNAR1
DOXO Ab CXCR3


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STAT3 inhibition enhances the therapeutic efficacy of immunogenic chemotherapy by stimulating type 1 interferon production by cancer cells

Heng Yang, Takahiro Yamazaki, Federico Pietrocola, et al.