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Staphylococcus aureus enterotoxin A (SEA) stimulates STAT3 activation and IL-17 expression in Cutaneous T-cell lymphoma

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Short Title: Bacterial toxin activates oncogenic signaling.

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We show that Staphylococcal enterotoxins activate oncogenic pathways in CTCL.

This discovery implies a novel role of microbes as drivers of disease progression.

Abstract

Cutaneous T cell lymphoma (CTCL) is characterized by proliferation of malignant T cells in a chronic inflammatory environment. With disease progression, bacteria colonize the compromised skin barrier and half of CTCL patients die from infection rather than from direct organ involvement by the malignancy. Clinical data indicate that bacteria play a direct role in disease progression, but little is known about the mechanisms involved. Here, we demonstrate that bacterial isolates containing staphylococcal enterotoxin-A (SEA) from the affected skin of CTCL patients, as well as recombinant SEA, stimulate activation of STAT3 and up-regulation of IL-17 in immortalized and primary patient-derived malignant and non-malignant T cells. Importantly, SEA induces STAT3 activation and IL-17 expression in malignant T cells when co-cultured with non-malignant T cells indicating an indirect mode of action. In accordance, malignant T cells expressing a SEA non-responsive T cell receptor V beta chain (TCR-Vb) are non-responsive to SEA in mono-culture, but display strong STAT3 activation and IL-17 expression in co-cultures with SEA-responsive, non-malignant T cells. The response is induced via IL-2Rg cytokines and a Janus kinase 3 (JAK3) - dependent pathway in malignant T cells and blocked by Tofacitinib, a clinical-grade JAK3 inhibitor. In conclusion, we demonstrate that SEA induces cell cross-talk-dependent activation of STAT3 and expression of IL-17 in malignant T cells suggesting a mechanism where SEA-producing bacteria promote activation of an established oncogenic pathway previously implicated in carcinogenesis.

Introduction

Cutaneous T-cell lymphoma (CTCL) comprises a group of heterogeneous lymphoproliferative disorders defined by the expansion of malignant skin-homing T cells in a chronic inflammatory environment. Mycosis Fungoides and Sézary syndrome represent the most prevalent forms of CTCL[1,2]. Despite intensive research, the CTCL etiology remains elusive and the pathogenesis is far from understood. Chromosomal instability, abnormal gene expression, gene duplication, and epigenetic deregulation have been implicated in CTCL, but no single underlying genetic or epigenetic event has yet been identified as a likely cause of the disease[3-9]. Persistent activation of Signal Transducer and Activator of Transcription 3 (STAT3)[10] has repeatedly been implicated in CTCL pathogenesis as a potent driver of survival and proliferation of malignant T cells[11-17]. Importantly, Stat3 promotes malignant expression of the proinflammatory cytokine IL-17, including a range of cytokines which have been associated with skin inflammation, immune deregulation, and disease progression[18-23].

It is well established that STAT3 is tyrosine phosphorylated *in vivo* in CTCL skin lesions and in peripheral blood Sézary cells. The level of tyrosine phosphorylation in STAT3 increases in advanced disease[13,24]. Activating mutations are sufficient to turn STAT3 into a full oncogene in experimental animals[10] and activating mutations in JAKs have been described in other hematological malignancies[25-27]. Recently, activating mutations have also been described in a subset (12,5%) of CTCL patients[28,29], but it remains unknown what drives aberrant activation of Jak/STAT signaling in the majority of patients STAT3 activation may become further increased following loss of regulatory control by SOCS3, Protein Inhibitor of Activated STAT3 (PIAS3), and other tyrosine protein phosphatases[19,30] However, presently it remains unclear what drives the dramatic increase and chronic activation of STAT3 in advanced CTCL.

While the etiology of this malignancy remains unclear, recent studies report on a significant geographical and occupational clustering of patient cohorts[31-36]. Thus, cross-analysis of cancer databases in Texas identified several geographic clusters with a 5-20-fold increased CTCL incidence[37]. A potential etiologic agent is unknown, but the environmental factors appear to play an essential role

in CTCL pathogenesis[36,37]. For decades, microbes have been suspected to play a key role in CTCL – both as etiologic agents and as drivers of life threatening complications[22,38-42]. So far, firm evidence for a microbial etiology in CTCL is lacking[43,44], but clinical data indicate that bacteria may play an important role in progression and mortality in advanced disease[39,40,45]. Whereas *Staphylococcus aureus* is a common commensal organism in healthy individuals, it is a major source of morbidity in CTCL, as it causes persistent skin and life-threatening systemic infections[39,42,46,47] seen in 44% to 76% of patients with advanced CTCL [40,45,48].

Staphylococcal enterotoxins (SEs), including the A type (SEA) are bacterial superantigens that circumvent normal antigen processing and recognition. SE binds directly to MHC class II molecules and crosslink T-cell receptors (TCR) by binding to their TCR-Vbeta chains (TCR-Vb) with very high affinity, which results in broad T-cell hyper-activation. Because SEs are only restricted by the TCR-Vb of the TCR complex, they can activate up to 20% of all naïve T cells[49]. The importance of SEs is emphasized by reports indicating that antibiotic therapy of staphylococcal infections in CTCL is associated with clinical improvement and, in some cases, remission of the lymphoma [40,45,50]. However, the mechanisms involved in disease aggravation and progression mediated by S. aureus and SE are poorly understood.

Here we report that SEA induces STAT3 activation and IL-17 expression in malignant T cells via engagement of non-malignant CD4 T cells. Our findings suggest that bacterial toxins play a central role in the activation of a key oncogenic pathway in CTCL.

Materials and Methods

Antibodies and reagents

ELISA kits and IL-2, IL-7 and IL-15 blocking antibodies were purchased from R&D Systems (McKinley Place NE, MN, USA). JAK3 and Erk1/2 antibodies (Ab) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while Stat3 Ab was purchased from Cell Signaling Technology (Beverly, MA, USA). Fluorochrome-conjugated CD3, CD4, CD25, CD26, MHC class II, pY(705)-Stat3 and the respective fluorochrome-conjugated isotype control Abs used for FACS were provided by R&D Systems, Biolegend (San Diego, CA, USA), BD Biosciences (San Jose, CA, USA) and Leinco (St. Louis, MO, USA), respectively. Other reagents were obtained as described below: TCR vβ kit from Ramcon (Bregnerød, Denmark), JAK3 inhibitor Tofacitinib (CP-690550) from Selleck Chemicals (Houston, TX, USA), siRNA against JAK3 and Stat3 from ThermoFisher Scientific (Waltham, MA, USA), SEs from Toxin Technology (Sarasota, FL, USA). SEA- mutants were generously provided by Active Biotech (Lund, Sweden).

Patients and isolation of SA bacteria

Malignant and non-malignant T cells were isolated from the blood of patients diagnosed with Sézary syndrome (SS) in accordance with the WHO-EORTC classification.[1] Malignant SS T cells typically lack the expression of cell surface markers CD26 and/or CD7 and often display reduced expression of CD4 when compared with non-malignant T cells[1,51,52]. Accordingly, T cells were identified as malignant ($CD4^{low/+}CD7^{-}$, $CD4^{low/+}CD26^{-}$) and non-malignant ($CD4^{+}CD7^{+}$, $CD4^{+}CD26^{+}$). Bacterial isolates were collected from CTCL patients using swabs wetted with 0.1% Triton X-100 in 0.075 M phosphate buffer, transferred to Stuart´s medium, and cultivated on blood agar overnight at 37ºC at 5% CO_2. In accordance with the Declaration of Helsinki, the samples were obtained with informed consent and after approval by the Committee on Health Research Ethics

Cell lines

The malignant T-cell line, SeAx, and the non-malignant T-cell line, MF1850, were established from patients diagnosed with CTCL[53] and cultured in media supplemented with 10% human serum (HS medium) and IL-2 as described elsewhere[54]. Prior to experimental setup, the CTCL cell lines were starved overnight in HS medium without IL-2.

ELISA

The concentrations of IL-17A in cell culture supernatants were measured using human IL-17A DuoSet ELISA development kit from R&D Systems (McKinley Place, NE, MN, USA) in accordance with the manufacturer's instructions.

Detection of Staphylococcal enterotoxins in bacterial isolate supernatants

The presence of Staphylococcal enterotoxins in bacterial cultures was examined using the RIDASCREEN SET A, B, C, D, E kit (R-Biopharm AG, Darmstadt, Germany) with a detection limit of 0.25 ng toxin/mL and in accordance with the manufacturer's instructions.

RNA purification, cDNA synthesis and QPCR

Total cellular RNA was purified and reverse transcribed into complementary DNA as previously described[55]. qPCR was performed using the TaqMan assay from ThermoFisher Scientific in accordance with the manufacturer's instructions and the samples were analyzed on an Mx3005P (Stratagene).

Cell isolation, Flow cytometry, and cell sorting

PBMCs were isolated from the blood of SS patients by Lymphoprep (Axis-shield, Oslo, Norway) density gradient centrifugation and used directly for flow cytometric analysis[56] or cultured in HS media with PBS or SEA or sorted by FACS using FACSAria (BD Bioscences) into populations of malignant and non-malignant T cells based on CD4 and CD26 surface expression and then mono- or co-cultured in HS media with PBS or SEA. Purity of the sorted malignant and non-malignant T cells was higher than 99% and 95% respectively. In experiments where co-cultured SeAx and MF1850 cells were sorted, the SeAx cells were stained prior culture with 1 µM CSFE as previously described.[24] The CSFE-positive (SeAx) and negative (MF1850) cells were sorted by FACSAria resulting in a purity of more than 98%. Data acquisition and flow cytometric analysis were done on Fortessa flow cytometers (from BD Biosciences) using FlowJo software (Tree Star, Ashland, OR).

Transient transfections

2 x10^6 cells per sample were transfected with small interfering RNA (siRNA) against JAK3, STAT3 or non-targeting control (ON-TARGETplus SMARTpool, Thermo Scientific, Lafayette, CO, USA). Pellets were resuspended in 100uL transfection solution (Ingenio Electroporation solution, Mirus Bio,

157 Madison, WI, USA) in the presence of 0,25 µM of the respective siRNAs and transfected with an
158 Amaxa Nucleofector (Amaxa GmbH, Cologne, Germany).

159 ## Statistics

160 For statistical analysis a two-tailed Student's t-test with a significance level of $p = 0.05$ was
161 used. A significant difference ($p < 0.05$) between a sample and control is indicated with an asterisk.

162

Results

SE- containing bacterial isolates from CTCL skin trigger expression of IL-17 by malignant cells.

It has been a matter of controversy whether or not malignant T cells express IL-17 in CTCL. Thus, some studies have reported on IL-17A and/or IL-17F expression by malignant T cells in lesional skin or blood[21,57-60] whereas others did not find IL-17 family cytokines despite the presence of IL-22 producing TH17 cells[61]. Since IL-17 is typically produced by CD4 T cells in response to bacteria such as *S. aureus* (reviewed in[62]) and SE-producing *S. aureus* often colonizes lesional skin, we hypothesized that SE can trigger IL-17 expression in CTCL. Accordingly, we tested whether bacterial isolates from lesional skin induced IL-17 production in co-cultures of malignant and non-malignant T cells. We analyzed for the presence of common enterotoxins in 46 bacterial isolates from CTCL skin (N= 6) and found that SEA was present in 21 out of 46 isolates, whereas SEB, SEC, SED and TSST-1 were not detected, therefore, confirming previous findings by others that lesional skin is often colonized by SEA-producing staphylococci[40]

Next, we performed co-cultures of malignant T and non-malignant T-cell lines stimulated with SEA positive and negative bacterial isolates from CTCL skin. As shown in Fig. 1A, SEA containing isolates stimulated vigorous production of IL-17A protein (average value 1515 pg/ml; range 485 – 3865 pg/ml, Fig. 1A, right panel), whereas SEA-negative bacterial isolates did not (average value 195 pg/ml; range 100-250 pg/ml; Fig. 1A, left panel). In order to address, whether malignant and/or non-malignant T cells produced IL-17, we stimulated co-cultures and separate cultures of malignant and non-malignant T cells in the presence or absence of SEA containing isolates prior to analysis of IL-17 protein in culture supernatants. As shown in Fig. 1B, SEA positive isolates induced a strong IL-17 response in co-cultures of malignant and non-malignant T-cell lines (Fig. 1B, right panel) whereas IL-17 production was not observed in separate cultures of malignant and non-malignant T cells, respectively (Fig. 1B, middle and left panels). The SEA-negative isolates induced only weak IL-17 response. Considering that SEA was by far the most prevalent SE in bacterial isolates from our patients, we tested whether recombinant SEA can also induce IL-17 production in co-cultures of malignant and non-malignant T-cells. Indeed, recombinant SEA produced almost identical results as presented in

Figure 1B. Notably, two non-stimulatory SEA-mutants (SEAAF47 and SEAD227/AF47[63] and SEB, SEC, SED, and TSST did not elicit significant IL-17 production (Fig. 1D) indicating that the IL-17 response was highly specific for intact SEA. The JAK3/STAT3 pathway drives IL-17 expression in malignant T cells[21], and as shown in Fig 1E, a clinical-grade JAK3 inhibitor Tofacitinib profoundly (> 70%) inhibited SEA-induced IL-17 production in co-cultures of malignant and non-malignant T cells.

SEA induces STAT3 activation in co-cultures.

As shown in Fig. 2, SEA induced a strong up-regulation and phosphorylation (pY705) of STAT3 in both malignant and non-malignant T cells following co-culture (Fig. 2, right panel) when compared to co-cultures stimulated with a vehicle control (Fig. 2, left panel). STAT3 phosphorylation was also increased in non-malignant T cells but not in malignant T cells following monoculture with SEA (Fig. 2, right panel) when compared to vehicle control (Fig. 2, left).

To address whether IL-17 in co-cultures originated from malignant cells, non-malignant cells, or both cell types, we separated the malignant and non-malignant T cells by Fluorescence-Activated Cell Sorting (FACS) after co-culture in the presence or absence of SEA as above and measured IL-17 As shown in Fig. 3A, SEA induced high expression of IL-17 mRNA in malignant T cells following co-culture with non-malignant T cells (Fig. 3A, right) when compared to vehicle control (Fig. 3A right). In contrast, SEA did not induce significant IL-17 mRNA expression in non-malignant T cells following co-culture with malignant T cells (Fig. 3A). Likewise, SEA did not induce IL-17 mRNA expression in monocultures of malignant and non-malignant T cells (Fig. 3A). As shown in Fig. 3B, siRNA-mediated depletion of STAT3 in malignant T cells profoundly inhibited IL-17 production in co-cultures of malignant and non-malignant T cells (Fig. 3B, third column) when compared to the effect of a non-targeting siRNA controls (Fig. 3B, first column). In contrast, STAT3 knockdown in non-malignant T cells had no effect on IL-17 production (Fig. 3B, second column) and STAT3 depletion in both malignant and non-malignant T cells had no additional effect when compared to siRNA-mediated depletion of STAT3 in malignant T cells alone (Fig. 3B, third versus fourth column). In parallel, malignant and non-malignant T cells were treated with JAK3 siRNA or a non-targeting control (NT) prior to co-culture in the presence or absence of SEA as above. JAK3 depletion in malignant T cells strongly inhibited IL-17 production in co-cultures (Fig. 3C) whereas JAK3 depletion in non-malignant T cells had no effect

indicating that SEA drives IL-17 expression through a JAK3/STAT3 dependent pathway in malignant T cells co-cultured with non-malignant T cells.

To address whether the cell cross-talk dependent induction of IL-17 requires cell-to-cell contact or was mediated through soluble factors, malignant and non-malignant T cells were co-cultured as above but separated by a cytokine-permeable membrane in Trans-Well plates. SEA induced high levels of IL-17 protein in supernatants isolated from malignant and non-malignant T cells, co-cultured in Trans-well plates (Fig. 3D). Likewise, SEA induced a significant increase in IL-17 mRNA expression in malignant T cells, but not in the non-malignant T cells following co-culture in Trans-well plates (Fig. 3E). Since IL-2 induces IL-17 expression in malignant T cells[21] and SEA induces IL-2 expression in non-malignant T cells[22], co-cultures were performed with and without SEA and IL-2 blocking and control antibody. As shown in Fig. 3F, inhibition of IL-2 almost completely blocked IL-17 production in co-cultures indicating the key role of IL-2 in SEA-mediated cross-talk between malignant and non-malignant T cell lines..

STAT3 activation and IL-17 expression in primary T cells from CTCL patients.

To address whether SEA also triggered STAT3 activation and IL-17 expression in primary T cells derived from CTCL patients, peripheral blood mononuclear cells (PBMC) were cultured in the presence or absence of SEA prior to FACS analysis of STAT3 activation in malignant ($CD4^+/CD26^-$) and non-malignant ($CD4^+/CD26^+$) T-cell populations. As observed from pY(705)-Stat3 staining, SEA induced a profound activation of STAT3 in both malignant ($CD4^+/CD26^-$, Fig. 4A upper panel) and non-malignant T cells ($CD4^+/CD26^+$, Fig. 4A, lower panel). Analysis of IL-17 expression showed induction of both mRNA (Fig. 4B) and protein (Fig. 4C) demonstrating significant IL-17A upregulation by SE in five out of six patients tested.

To further investigate SEA- mediated activation of primary malignant T cells, we performed TCR Vb staining of malignant ($CD4^+/CD26^-$) and non-malignant ($CD4^+/CD26^+$) T-cell compartments. As shown in a representative image in Fig. 5A, $CD4^+/CD26^-$ T cells expressed only the TCR-Vb17, whereas $CD4^+/CD26^+$ T cells displayed a typical Gaussian distribution of TCR-Vb indicating that the $CD4^+/CD26^-$

compartment consisted of only one malignant T cell clone whereas the $CD4^+/CD26^+$ compartment contained a non-malignant T-cell population with a normal TCR-Vb distribution (Fig. 5A).

Using FACS, we separated $CD4^+/CD26^-$ and $CD4^+/CD26^+$ T cells and performed mono- and co-cultures with or without SEA prior to analysis of STAT3 phosphorylation. As shown in Fig. 5B, both malignant and non-malignant T cells displayed a considerable baseline STAT3 phosphorylation in primary malignant and non-malignant T cells, which is in agreement with our previous findings[13]. Notably, SEA triggered a profound up-regulation of STAT3 phosphorylation in malignant T cells after co-culture with non-malignant T cells and in the presence of SEA (Fig. 5B), whereas SEA had little effect on STAT3 phosphorylation in monoculture of malignant T cells (Fig. 5B). In contrast, SEA induced a strong up-regulation of STAT3 phosphorylation in non-malignant T cells and this phosphorylation level was not further affected by addition of malignant T cells (Fig. 5B).

To address whether SEA triggered IL-17 expression in primary malignant T cells, PBMCs were cultured with and without SEA prior to qPCR analysis of IL-17A expression in $CD4^+/CD26^-$ malignant T cells (Fig. 6A, lower right) and $CD4^+/CD26^+$ non-malignant T cells (Fig. 6A, upper right). Notably, SEA induced IL-17A expression in both the large fraction (86%) of malignant T cells and the small fraction (5%) of non-malignant T cells (Fig. 6A, left versus right). Next, malignant T cells ($CD4^+/CD26^-$) were cultured in the presence and absence of SEA in monoculture and co-culture with non-malignant CD4 T cells. As shown in Fig.6B, SEA induced IL-17 production in primary malignant T cells only when co-cultured with non-malignant T cells, but not in monocultures of malignant T cells (Fig. 6B) showing that IL-17A expression in primary malignant T cells depended on SEA-driven cross-talk between malignant and non-malignant T cells. Next, co-cultures were treated with neutralizing antibodies against IL-2, IL-7, IL-15, (and a combination of the three antibodies), prior to stimulation with SEA. As shown in Fig. 6C, each individual antibody inhibited the IL-17A response by 15-20%, whereas the combination of antibodies inhibited the response by more than 40% (Fig. 6C) indicating the IL-17A response was at least partly driven by IL-2Rg cytokines.

Discussion

In this study we demonstrate for the first time that SEA induces STAT3 activation and IL-17 expression in immortalized and primary malignant T cells derived from CTCL patients. SEA-containing isolates of bacteria from CTCL skin, as well as recombinant SEA, triggered STAT3 activation and a robust IL-17 production in malignant T cells when co-cultured with non-malignant T cells but not with SEA alone. Activated STAT3 is oncogenic in animal models[10] and believed also to foster CTCL[12-17]. STAT3 provides survival signals through up-regulation of proto-oncogenes such as Bcl-2 and survivin[11,15], Interleukin-2 receptor (IL2R)[64] and pro-oncogenic miRNAs[65,66] and down-regulation of tumor suppressive miRNAs such as miR-22[67]. In addition, STAT3 drives expression of Suppressor of Cytokines Signaling (SOCS)[19], cytokines of the TH2 (IL-5 and IL-13)[68], TH17 (IL-17, IL-22)[21], regulatory T-cell (IL-10) phenotype[22], and other factors.

Our finding that SEA induced strong STAT3 activation in primary malignant T cells provides direct evidence linking bacterial toxins with activation of an oncogene in CTCL. Moreover, it suggests a mechanism whereby toxin-producing bacteria – via the activation of STAT3 - can augment an array of pathological processes in the lymphomagenesis. This is important because staphylococcal enterotoxins for decades have been suspected to play a tumor-promoting role in CTCL[39,40,45,50,69-71]. We now propose that SEA-mediated cross-talk between malignant and non-malignant T cells triggers oncogenic STAT3 activation *in vivo*. Our findings provide a plausible explanation for clinical observations indicating that SE-producing staphylococci promote tumor growth and aggravate the disease and, reversely, that antibiotic therapy may halt disease progression and even induce tumor regression in some CTCL patients[40,45,50].

Despite the well-established role of STAT3 in CTCL pathogenesis, it has not been clear what drives malignant STAT3 activation *in vivo*. Recently, activating mutations have been described in a subset (12,5%) of CTCL patients[28,29], but it remains unknown what drives aberrant STAT3 activation in the majority of patients. Early on it was discovered that malignant T cells under *ex vivo* conditions

rapidly lost expression of activated STAT3 indicating that *in vivo* signals and factors (such as IL-2Rg cytokines) present by the local environment play a key role in malignant STAT3 activation in CTCL patients[14]. In support, IL-2 and other IL-2Rg- binding cytokines like IL-7, IL-15, and IL-21 induce STAT3 activation in primary malignant T cells and immortalized T-cell lines[72-74] suggesting that these cytokines may also drive STAT3 activation *in vivo*. Although, both malignant and non-malignant T cells as well as stromal cells and keratinocytes may produce IL-2R-binding cytokines *in vivo*, the actual cells producing these factors and relative contribution by different sources remain unknown.

The present findings showing that SEA triggers STAT3 activation and IL-17 expression via an indirect mechanism involving non-malignant (i.e. infiltrating) T cells and soluble factors such as IL-2 and other IL-2Rg cytokines suggest that enterotoxins may also trigger IL-2Rg-mediated STAT3 activation *in vivo*. SE-producing *S. aureus* skin infection is more common in advanced disease when compared to less advanced CTCL. In fact, *S. aureus* was isolated from skin, blood, and other foci from the majority of CTCL patients with advanced disease and in half of these patients, the bacteria produced SEA, SEB, and/or TSST[40]. If the proposed mechanism is at play in these patients, higher loads of SE-producing bacteria in skin and blood in advanced disease would be predicted to translate into higher levels of activated STAT3 and may partially explain why malignant STAT3 activation is increased in advanced disease[13].

As mentioned above, staphylococcal enterotoxins have for long been suspected to drive chronic activation of malignant T cells[40,50,69-71,75]. Originally, it was thought that toxins triggered proliferation and expansion of malignant T cells through a direct binding and activation of malignant T-cell clones expressing the appropriate TCR-Vb but little data was available to support this hypothesis, while others contradicted it (reviewed in [38]). Our findings presented in this study have significant implications for our understanding of the interplay between bacterial toxins and malignant T cells. An indirect mode of action implies that toxin-mediated activation of malignant T cells does not rely on the expression of a single, toxin-specific TCR-Vb by these malignant T cells but on expression of multiple toxin-binding TCR-Vb expressed by non-malignant T infiltrating cells. Consistent with this

hypothesis, we observed that SEA induced STAT3 activation in a primary malignant T-cell clone expressing a SEA-non-responsive TCR-Vb (TCR-Vb17) only when co-cultured with non-malignant T cells expressing a full TCR-Vb repertoire including several SEA-binding TCR-Vb (such as TCR-Vb5).

In principle, this implies that not only a few patients harboring a single malignant T-cell clone expressing a SEA-responsive TCR-Vb, but all patients carrying non-malignant T cells with SEA-responsive TCR-Vb are susceptible to SEA- mediated STAT3 activation in malignant T cells. Thus, bacterial toxins might have a dramatic impact on malignant T cell activation in a much broader range of patients than previously thought. Moreover, our findings show that malignant T cells engage in a complex and delicate cross-talk with non-malignant T cells, which dramatically changes their response to signals and factors in the microenvironment. By inference, our data therefore indicate that conventional *in vitro* models using monocultures of purified malignant T cells have fundamental limitations, when it comes to mimicking the pathogenesis *in vivo*. Furthermore, it is likely that cytokines and factors other than IL-2Rg cytokines also influence toxin-mediated cross-talk between malignant and non-malignant T cells. Indeed, SEA triggers IL-10 expression in co-cultures of malignant and non-malignant T cells[76], IL-13 inhibits IL-17 but not IL-22 and IL-26 expression by TH17 cells[77], and prostaglandins such as PGE2 produced by malignant T cells are known to modulate differentiation and cytokine production by non-malignant T cells[76]. Accordingly, our data suggest that an inclusion of non-malignant T cells and possibly stromal cells and keratinocytes into cultures of malignant T cells would critically improve future *in vitro* models of CTCL to better mimic the dynamic interactions seen in CTCL patients.

It has been a matter of controversy whether or not IL-17 is expressed in CTCL. Some studies have reported IL-17 mRNA and/or protein expression *in situ* and *ex vivo*, whereas others reported its absence, despite the presence of IL-22 producing TH17-like cells[21,57-59,61]. The present findings offer a possible explanation for these opposite results. Specifically, that the differences in frequency and severity of skin colonization and infection by SE- producing bacteria between different cohorts of patients and even within a single cohort may explain why IL-17 expression differed between these studies and between patients within a single cohort[21,57,61]. The finding that SEA induces IL-17

353 expression in non-malignant primary T cells was not unexpected given that SEA mediates STAT3
354 activation in these cells[78], but important as it suggests that both malignant and non-malignant T cells
355 may contribute to IL-17 expression *in vivo*. As psoriasis is also associated with IL-17, de-regulated
356 STAT3 signaling, and skin colonization by superantigen- producing bacteria like staphylococcus aureus,
357 it is tempting to speculate that similar pathological mechanisms are involved in psoriasis and CTCL –
358 disorders, which have many histological and clinical features in common. Yet, it is an open question
359 whether IL-17 is involved in the antimicrobial defense and/or lymphomagenesis in CTCL patients
360 displaying skin colonization by enterotoxin producing staphylococcus aureus.
361
362 In conclusion, we show that SEA induces a cross-talk-dependent activation of STAT3 and
363 expression of IL-17 in malignant T cells suggesting a mechanism whereby SEA- producing bacteria
364 promote activation of an established oncogenic pathway (STAT3) previously implicated in the
365 pathogenesis of CTCL.

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372 ## Authorship

373 Contribution: A.W-O. performed the experiments; A.W-O. and N.O. analyzed and made the
374 figures; L.M.L., R.G., L.I. and M.K. provided essential materials and patient samples and A.W-O., T.K.,
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376 wrote the paper. All authors read, commented on and approved the manuscript.

377 ## Conflicts of interest

378 The authors declare no conflict of interest.

References

1. Kim YH, Liu HL, Mraz-Gernhard S, Varghese A, Hoppe RT. Long-term outcome of 525 patients with mycosis fungoides and Sezary syndrome: clinical prognostic factors and risk for disease progression. *Arch Dermatol*. 2003;139(7):857-866.
2. Wilcox RA. Cutaneous T-cell lymphoma: 2011 update on diagnosis, risk-stratification, and management. *Am J Hematol*. 2011;86(11):928-948.
3. Kaltoft K, Hansen BH, Thestrup-Pedersen K. Cytogenetic findings in cell lines from cutaneous T-cell lymphoma. *Dermatol Clin*. 1994;12(2):295-304.
4. Smoller BR, Santucci M, Wood GS, Whittaker SJ. Histopathology and genetics of cutaneous T-cell lymphoma. *Hematol Oncol Clin North Am*. 2003;17(6):1277-1311.
5. Vermeer MH, van Doorn R, Dijkman R, et al. Novel and highly recurrent chromosomal alterations in Sezary syndrome. *Cancer Res*. 2008;68(8):2689-2698.
6. Kopp KL, Kauczok CS, Lauenborg B, et al. COX-2-dependent PGE(2) acts as a growth factor in mycosis fungoides (MF). *Leukemia*. 2010;24(6):1179-1185.
7. Huang Y, Litvinov IV, Wang Y, et al. Thymocyte selection-associated high mobility group box gene (TOX) is aberrantly over-expressed in mycosis fungoides and correlates with poor prognosis. *Oncotarget*. 2014;5(12):4418-4425.
8. Krejsgaard T, Vetter-Kauczok CS, Woetmann A, et al. Ectopic expression of B-lymphoid kinase in cutaneous T-cell lymphoma. *Blood*. 2009;113(23):5896-5904.
9. Litvinov IV, Netchiporouk E, Cordeiro B, et al. Ectopic expression of embryonic stem cell and other developmental genes in cutaneous T-cell lymphoma. *Oncoimmunology*. 2014;3(11):e970025.
10. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. *Cell*. 1999;98(3):295-303.
11. Nielsen M, Kaestel CG, Eriksen KW, et al. Inhibition of constitutively activated Stat3 correlates with altered Bcl-2/Bax expression and induction of apoptosis in mycosis fungoides tumor cells. *Leukemia*. 1999;13(5):735-738.
12. Nielsen M, Kaltoft K, Nordahl M, et al. Constitutive activation of a slowly migrating isoform of Stat3 in mycosis fungoides: tyrphostin AG490 inhibits Stat3 activation and growth of mycosis fungoides tumor cell lines. *Proc Natl Acad Sci U S A*. 1997;94(13):6764-6769.
13. Sommer VH, Clemmensen OJ, Nielsen O, et al. In vivo activation of STAT3 in cutaneous T-cell lymphoma. Evidence for an antiapoptotic function of STAT3. *Leukemia*. 2004;18(7):1288-1295.
14. van Kester MS, Out-Luiting JJ, von dem Borne PA, Willemze R, Tensen CP, Vermeer MH. Cucurbitacin I inhibits Stat3 and induces apoptosis in Sezary cells. *J Invest Dermatol*. 2008;128(7):1691-1695.
15. Zhang C, Li B, Gaikwad AS, et al. Avicin D selectively induces apoptosis and downregulates p-STAT-3, bcl-2, and survivin in cutaneous T-cell lymphoma cells. *J Invest Dermatol*. 2008;128(11):2728-2735.
16. Zhang Q, Nowak I, Vonderheid EC, et al. Activation of Jak/STAT proteins involved in signal transduction pathway mediated by receptor for interleukin 2 in malignant T lymphocytes derived from cutaneous anaplastic large T-cell lymphoma and Sezary syndrome. *Proc Natl Acad Sci U S A*. 1996;93(17):9148-9153.
17. Verma NK, Davies AM, Long A, Kelleher D, Volkov Y. STAT3 knockdown by siRNA induces apoptosis in human cutaneous T-cell lymphoma line Hut78 via downregulation of Bcl-xL. *Cell Mol Biol Lett*. 2010;15(2):342-355.
18. Kasprzycka M, Zhang Q, Witkiewicz A, et al. Gamma c-signaling cytokines induce a regulatory T cell phenotype in malignant CD4+ T lymphocytes. *J Immunol*. 2008;181(4):2506-2512.
19. Brender C, Nielsen M, Kaltoft K, et al. STAT3-mediated constitutive expression of SOCS-3 in cutaneous T-cell lymphoma. *Blood*. 2001;97(4):1056-1062.

20. Brender C, Lovato P, Sommer VH, et al. Constitutive SOCS-3 expression protects T-cell lymphoma against growth inhibition by IFNalpha. *Leukemia*. 2005;19(2):209-213.
21. Krejsgaard T, Ralfkiaer U, Clasen-Linde E, et al. Malignant cutaneous T-cell lymphoma cells express IL-17 utilizing the Jak3/Stat3 signaling pathway. *J Invest Dermatol*. 2011;131(6):1331-1338.
22. Krejsgaard T, Willerslev-Olsen A, Lindahl LM, et al. Staphylococcal enterotoxins stimulate lymphoma-associated immune dysregulation. *Blood*. 2014;124(5):761-770.
23. Fogli LK, Sun A, Fanok M, Odum N, Hymes KB, Koralov SB. STAT3 Serine Phosphorylation and HDAC Inhibition In CTCL. Vol. 122; 2013.
24. Levidou G, Siakantaris M, Papadaki T, et al. A comprehensive immunohistochemical approach of AKT/mTOR pathway and p-STAT3 in mycosis fungoides. *J Am Acad Dermatol*. 2013;69(3):375-384.
25. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005;434(7037):1144-1148.
26. Walters DK, Mercher T, Gu TL, et al. Activating alleles of JAK3 in acute megakaryoblastic leukemia. *Cancer Cell*. 2006;10(1):65-75.
27. Flex E, Petrangeli V, Stella L, et al. Somatically acquired JAK1 mutations in adult acute lymphoblastic leukemia. *J Exp Med*. 2008;205(4):751-758.
28. McGirt LY, Jia P, Baerenwald DA, et al. Whole genome sequencing reveals oncogenic mutations in mycosis fungoides. *Blood*. 2015.
29. Choi J, Goh G, Walradt T, et al. Genomic landscape of cutaneous T cell lymphoma. *Nat Genet*. 2015;47(9):1011-1019.
30. Woetmann A, Nielsen M, Christensen ST, et al. Inhibition of protein phosphatase 2A induces serine/threonine phosphorylation, subcellular redistribution, and functional inhibition of STAT3. *Proc Natl Acad Sci U S A*. 1999;96(19):10620-10625.
31. Morales-Suarez-Varela MM, Olsen J, Johansen P, et al. Occupational risk factors for mycosis fungoides: a European multicenter case-control study. *J Occup Environ Med*. 2004;46(3):205-211.
32. Hodak E, Klein T, Gabay B, et al. Familial mycosis fungoides: report of 6 kindreds and a study of the HLA system. *J Am Acad Dermatol*. 2005;52(3 Pt 1):393-402.
33. Lynge E, Afonso N, Kaerlev L, et al. European multi-centre case-control study on risk factors for rare cancers of unknown aetiology. *Eur J Cancer*. 2005;41(4):601-612.
34. Jahan-Tigh RR, Huen AO, Lee GL, Pozadzides JV, Liu P, Duvic M. Hydrochlorothiazide and cutaneous T cell lymphoma: prospective analysis and case series. *Cancer*. 2013;119(4):825-831.
35. Aschebrook-Kilfoy B, Cocco P, La Vecchia C, et al. Medical history, lifestyle, family history, and occupational risk factors for mycosis fungoides and Sezary syndrome: the InterLymph Non-Hodgkin Lymphoma Subtypes Project. *J Natl Cancer Inst Monogr*. 2014;2014(48):98-105.
36. Litvinov IV, Tetzlaff MT, Rahme E, et al. Demographic patterns of cutaneous T-cell lymphoma incidence in Texas based on two different cancer registries. *Cancer Med*. 2015.
37. Moreau JF, Buchanich JM, Geskin JZ, Akilov OE, Geskin LJ. Non-random geographic distribution of patients with cutaneous T-cell lymphoma in the Greater Pittsburgh Area. *Dermatol Online J*. 2014;20(7).
38. Willerslev-Olsen A, Krejsgaard T, Lindahl LM, et al. Bacterial toxins fuel disease progression in cutaneous T-cell lymphoma. *Toxins (Basel)*. 2013;5(8):1402-1421.
39. Axelrod PI, Lorber B, Vonderheid EC. Infections complicating mycosis fungoides and Sezary syndrome. *JAMA*. 1992;267(10):1354-1358.
40. Jackow CM, Cather JC, Hearne V, Asano AT, Musser JM, Duvic M. Association of erythrodermic cutaneous T-cell lymphoma, superantigen-positive Staphylococcus aureus, and oligoclonal T-cell receptor V beta gene expansion. *Blood*. 1997;89(1):32-40.

41. Bonin S, Tothova SM, Barbazza R, Brunetti D, Stanta G, Trevisan G. Evidence of multiple infectious agents in mycosis fungoides lesions. *Exp Mol Pathol*. 2010;89(1):46-50.
42. Mirvish ED, Pomerantz RG, Geskin LJ. Infectious agents in cutaneous T-cell lymphoma. *J Am Acad Dermatol*. 2011;64(2):423-431.
43. Mirvish JJ, Pomerantz RG, Falo LD, Jr., Geskin LJ. Role of infectious agents in cutaneous T-cell lymphoma: facts and controversies. *Clin Dermatol*. 2013;31(4):423-431.
44. Dulmage BO, Feng H, Mirvish E, Geskin L. Black cat in a dark room: the absence of a directly oncogenic virus does not eliminate the role of an infectious agent in cutaneous T-cell lymphoma pathogenesis. *Br J Dermatol*. 2014.
45. Talpur R, Bassett R, Duvic M. Prevalence and treatment of Staphylococcus aureus colonization in patients with mycosis fungoides and Sezary syndrome. *Br J Dermatol*. 2008;159(1):105-112.
46. Posner LE, Fossieck BE, Jr., Eddy JL, Bunn PA, Jr. Septicemic complications of the cutaneous T-cell lymphomas. *Am J Med*. 1981;71(2):210-216.
47. Baser S, Onn A, Lin E, Morice RC, Duvic M. Pulmonary manifestations in patients with cutaneous T-cell lymphomas. *Cancer*. 2007;109(8):1550-1555.
48. Nguyen V, Huggins RH, Lertsburapa T, et al. Cutaneous T-cell lymphoma and Staphylococcus aureus colonization. *J Am Acad Dermatol*. 2008;59(6):949-952.
49. Pinchuk IV, Beswick EJ, Reyes VE. Staphylococcal enterotoxins. *Toxins (Basel)*. 2010;2(8):2177-2197.
50. Tokura Y, Yagi H, Ohshima A, et al. Cutaneous colonization with staphylococci influences the disease activity of Sezary syndrome: a potential role for bacterial superantigens. *Br J Dermatol*. 1995;133(1):6-12.
51. Girardi M, Heald PW, Wilson LD. The pathogenesis of mycosis fungoides. *N Engl J Med*. 2004;350(19):1978-1988.
52. Willemze R, Jaffe ES, Burg G, et al. WHO-EORTC classification for cutaneous lymphomas. *Blood*. 2005;105(10):3768-3785.
53. Kaltoft K, Bisballe S, Rasmussen HF, Thestrup-Pedersen K, Thomsen K, Sterry W. A continuous T-cell line from a patient with Sezary syndrome. *Arch Dermatol Res*. 1987;279(5):293-298.
54. Woetmann A, Lovato P, Eriksen KW, et al. Nonmalignant T cells stimulate growth of T-cell lymphoma cells in the presence of bacterial toxins. *Blood*. 2007;109(8):3325-3332.
55. Krejsgaard T, Litvinov IV, Wang Y, et al. Elucidating the role of interleukin-17F in cutaneous T-cell lymphoma. *Blood*. 2013;122(6):943-950.
56. Geisler C, Dietrich J, Nielsen BL, et al. Leucine-based receptor sorting motifs are dependent on the spacing relative to the plasma membrane. *J Biol Chem*. 1998;273(33):21316-21323.
57. Ciree A, Michel L, Camilleri-Broet S, et al. Expression and activity of IL-17 in cutaneous T-cell lymphomas (mycosis fungoides and Sezary syndrome). *Int J Cancer*. 2004;112(1):113-120.
58. Doherty SD, Ni X, Doherty CB, et al. Abnormal expression of interleukin-23 in mycosis fungoides/Sezary syndrome lesions. *Arch Dermatol Res*. 2006;298(7):353-356.
59. Chong BF, Wilson AJ, Gibson HM, et al. Immune function abnormalities in peripheral blood mononuclear cell cytokine expression differentiates stages of cutaneous T-cell lymphoma/mycosis fungoides. *Clin Cancer Res*. 2008;14(3):646-653.
60. Krejsgaard T, Litvinov IV, Wang Y, et al. Elucidating the role of interleukin-17F in cutaneous T-cell lymphoma. *Blood*. 2013.
61. Miyagaki T, Sugaya M, Suga H, et al. IL-22, but not IL-17, dominant environment in cutaneous T-cell lymphoma. *Clin Cancer Res*. 2011;17(24):7529-7538.
62. Lee Y, Collins M, Kuchroo VK. Unexpected targets and triggers of autoimmunity. *J Clin Immunol*. 2014;34 Suppl 1:S56-60.

63. Abrahmsen L, Dohlsten M, Segren S, Bjork P, Jonsson E, Kalland T. Characterization of two distinct MHC class II binding sites in the superantigen staphylococcal enterotoxin A. *EMBO J*. 1995;14(13):2978-2986.
64. Eriksen KW, Kaltoft K, Mikkelsen G, et al. Constitutive STAT3-activation in Sezary syndrome: tyrphostin AG490 inhibits STAT3-activation, interleukin-2 receptor expression and growth of leukemic Sezary cells. *Leukemia*. 2001;15(5):787-793.
65. van der Fits L, van Kester MS, Qin Y, et al. MicroRNA-21 expression in CD4+ T cells is regulated by STAT3 and is pathologically involved in Sezary syndrome. *J Invest Dermatol*. 2011;131(3):762-768.
66. van der Fits L, Out-Luiting JJ, Tensen CP, Zoutman WH, Vermeer MH. Exploring the IL-21-STAT3 axis as therapeutic target for Sezary syndrome. *J Invest Dermatol*. 2014;134(10):2639-2647.
67. Sibbesen N, Kopp KL, litvinov IV, et al. Jak3, STAT3, and STAT5 inhibit expression of mir-22, a novel tumor suppressor microRNA, in cutaneous T-cell lymphoma. Oncotarget; 2015.
68. Nielsen M, Nissen MH, Gerwien J, et al. Spontaneous interleukin-5 production in cutaneous T-cell lymphoma lines is mediated by constitutively activated Stat3. *Blood*. 2002;99(3):973-977.
69. Tokura Y, Heald PW, Yan SL, Edelson RL. Stimulation of cutaneous T-cell lymphoma cells with superantigenic staphylococcal toxins. *J Invest Dermatol*. 1992;98(1):33-37.
70. Linnemann T, Gellrich S, Lukowsky A, et al. Polyclonal expansion of T cells with the TCR V beta type of the tumour cell in lesions of cutaneous T-cell lymphoma: evidence for possible superantigen involvement. *Br J Dermatol*. 2004;150(5):1013-1017.
71. Vonderheid EC, Boselli CM, Conroy M, et al. Evidence for restricted Vbeta usage in the leukemic phase of cutaneous T cell lymphoma. *J Invest Dermatol*. 2005;124(3):651-661.
72. Marzec M, Liu X, Kasprzycka M, et al. IL-2- and IL-15-induced activation of the rapamycin-sensitive mTORC1 pathway in malignant CD4+ T lymphocytes. *Blood*. 2008;111(4):2181-2189.
73. van der Fits L, Out-Luiting JJ, van Leeuwen MA, et al. Autocrine IL-21 stimulation is involved in the maintenance of constitutive STAT3 activation in Sezary syndrome. *J Invest Dermatol*. 2012;132(2):440-447.
74. Qin JZ, Kamarashev J, Zhang CL, Dummer R, Burg G, Dobbeling U. Constitutive and interleukin-7- and interleukin-15-stimulated DNA binding of STAT and novel factors in cutaneous T cell lymphoma cells. *J Invest Dermatol*. 2001;117(3):583-589.
75. van der Fits L, Sandberg Y, Darzentas N, et al. A restricted clonal T-cell receptor alphabeta repertoire in Sezary syndrome is indicative of superantigenic stimulation. *Br J Dermatol*. 2011;165(1):78-84.
76. Boniface K, Bak-Jensen KS, Li Y, et al. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med*. 2009;206(3):535-548.
77. Wolk K, Mitsui H, Witte K, et al. Deficient cutaneous antibacterial competence in cutaneous T-cell lymphomas: role of Th2-mediated biased Th17 function. *Clin Cancer Res*. 2014;20(21):5507-5516.
78. Muranski P, Restifo NP. Essentials of Th17 cell commitment and plasticity. *Blood*. 2013;121(13):2402-2414.

Figure legends

Figure 1.

Bacterial isolates from CTCL patients contain staphylococcal enterotoxins. (A) Mixed bacterial isolates from patients were tested for SE expression (SEA, SEB, SEC, SED + TSST-1) and categorized accordingly as either positive or negative. Co-cultures with Malignant T cells (SeAx) and Non-malignant T cells (MF1850) were then stimulated with SE-positive or SE-negative isolates and incubated for 24 hours. IL-17 concentration in the supernatants was determined by ELISA. (B) Malignant (SeAx) - and Non-malignant (MF1850) T cell lines were mono- and co-cultured in the absence (Media) or presence (Isolate) of a mixed bacterial isolate from a CTCL patient. IL-17A protein was measured in the supernatant after 24 hours of incubation with ELISA. (C) Malignant (SeAx) - and Non-malignant (MF1850) T cell lines were mono- and co-cultured with either vehicle (PBS) or (C) recombinant SEA (50 ng/mL) and (D) SEAwt or SEAD227A, SEAF47A/D227A, SEB, SEC2, SED, TSST-1 toxins (50 ng/mL). IL-17A protein was measured in the supernatant after 24 hours of incubation with ELISA. (E) Malignant (SeAx) and non-malignant (MF1850) T cell lines were mono – and co-cultured with SEA (50 ng/ml) and Tofacitinib (0.3 µM) or vehicle (DMSO) for 24 hours. After incubation IL-17A protein concentration was determined by ELISA. Error bars represent SEM of three independent experiments.

Figure 2.

Staphylococcal enterotoxins activate and phosphorylate STAT3 in both malignant and non-malignant T cells. (A) Representative flow cytometric analysis of CFSE stained Malignant - (SeAx) and Non-malignant T cell lines (MF1850) mono- and co-cultured with either vehicle (PBS) or recombinant SEA (50 ng/mL) for 24 hours. All samples were stained for pY(705)-Stat3. “PBS + Malignant” signifies gated Non-malignant T cells co-cultured with Malignant T cells and vice versa for “SEA + Non-malignant”.

Figure 3.

Enterotoxin induces IL-17 production in co-cultured malignant T cells.

(A) Malignant (SeAx) and non-malignant (MF1850) T cell lines were either mono-cultured or co-cultured with vehicle (PBS) or SEA (50 ng/ml) for 16 hours. The co-cultured malignant and non-

malignant T cells were sorted by FACS and the relative level of IL-17A and GAPDH mRNA were determined in all samples by qPCR. In each sample the level of IL-17A mRNA was normalized to that of GAPDH mRNA and it is depicted as fold change compared to mono-cultured malignant T cells with PBS. "Malign (Cocultured)" signifies IL-17A expression in malignant T cells co-cultured with non-malignant T cells and vice versa for "Non-malign (Cocultured)". (B) Malignant (SeAx) and non-malignant (MF1850) T cells were transiently transfected with NT or Stat3-specific siRNA (B) or JAK3 specific siRNA (C) and monocultured for 24 hours. Then, the transfected cells were washed and cocultured in the presence of SEA (50 ng/mL) for another 24 hours before the concentrations of IL-17A in cell culture supernatants was determined by ELISA. Presented as percent of IL-17A secretion relative to cocultures of malignant and non-malignant T cells transfected with NT siRNA. (D) Malignant (SeAx) and non-malignant (MF1850) T cell lines were co-cultured separated by transwells with vehicle (PBS) or SEA (50 ng/ml) for 24h. IL-17 concentrations in the supernatants were determined by ELISA. (E) Malignant (SeAx) and non-malignant (MF1850) T cell lines were either mono-cultured with transwells or co-cultured separated by transwells for 24h. The relative level of IL-17A and GAPDH mRNA were determined in all samples by qPCR. In each sample the level of IL-17A mRNA was normalized to that of GAPDH mRNA and it is depicted as fold change compared to mono-cultured malignant T cells with PBS. "Malign Transwell" signifies IL-17A expression in malignant T cells co-cultured with non-malignant T cells separated by a transwell and vice versa for "Non-malign. Transwell". (F) Malignant - (SeAx) and Non-malignant T cell lines (MF1850) were mono- and co-cultured with either vehicle (PBS), SEA, SEA and IgG isotype control or SEA and neutralizing IL-2 antibody. IL-17 concentrations in the supernatants were determined by ELISA. Error bars represent SEM of three independent experiments.

Figure 4

Staphylococcal enterotoxins treatment leads to STAT3 phosphorylation and subsequent IL-17 secretion in primary T cells from CTCL patients. (A) Representative flow cytometric analysis of peripheral blood mononuclear cells freshly purified from a CTCL patient and cultured for 24 hours with SEA (200 ng/mL) or vehicle (PBS). After incubation cells were stained for py-Stat3 and CD3, CD4 and CD26. Non-malignant T cells stain $CD3^+$, $CD4^+$, $CD26^+$ and malignant T cells stain $CD3^+$, $CD4^+$, $CD26^-$

. (B) PBMCs from CTCL patients were stimulated with a cocktail of SEA, SEB, SEC2 SEE, SEI, TSST-1 (200 ng/mL) (SE) or vehicle (PBS) for 24 hours. After incubation IL17A expression and GAPDH expression was determined by qPCR. In each sample IL17A expression is normalized to GAPDH. (C) Pooled data of PBMCs from CTCL patients stimulated for 24 hours with a cocktail of SEA, SEB, SEC2 SEE, SEI, TSST-1 (200 ng/mL) (SE) or vehicle (PBS). IL-17A concentrations were determined by ELISA and normalized to 10^6 cells. * represents statistical significance of $p<0.05$. "ND", No IL17A gene expression detected.

Figure 5

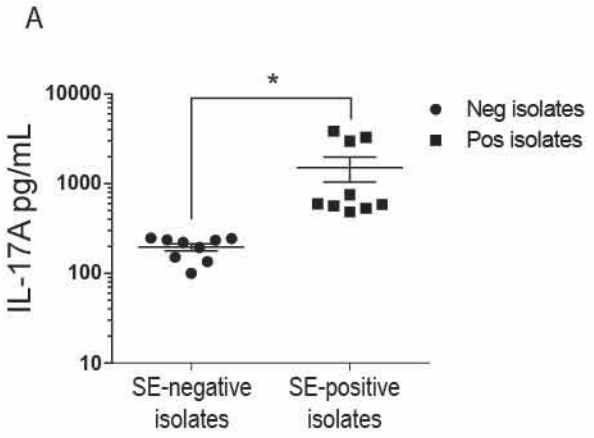
Staphylococcal enterotoxins induce Stat3 phosphorylation in primary malignant T cells cultured with non-malignant T cells. (A) Representative flow cytometric analysis of freshly purified PBMCs from a CTCL patient stained with CD3, CD4, CD26 and a TCR-Vbeta panel. Bar plot demonstrates TCR-Vbeta repertoire of the malignant ($CD3^+$, $CD4^+$, $CD26^-$) T cell compartment and the non-malignant ($CD3^+$, $CD4^+$, $CD26^+$) compartment. (B) $CD4^+$, $CD26^-$ (malignant T cells) and $CD4^+$, $CD26^+$ (normal T cells) were separated by FACS from freshly purified PBMCs from a CTCL patient. $CD4^+$, $CD26^-$ and $CD4^+$, $CD26^+$ T cells were mono - and co-cultured with either vehicle (PBS) or SEA (200 ng/mL) for 24 hours. After incubation cells were stained for pY-Stat3. Intensity of pY-Stat3 staining is shown in contour plot. "PBS + Non-malignant" signifies gated malignant T cells co-cultured with non-malignant T cells and stimulated with vehicle and vice versa for "SEA + Malignant"

Figure 6

Staphylococcal enterotoxins induce IL-17 production from cocultures of primary malignant T cells and non-malignant CD4 T cells. (A) PBMCs from a CTCL patient were stimulated with either vehicle (PBS) or SEA (200 ng/mL) for 24 hours and then sorted by CD4, CD26. IL17A gene expression from malignant and non-malignant cells were determined by qPCR and normalized to GAPDH expression. (B) Primary malignant T cells from a CTCL patient and non-malignant CD4 T cells were mono- and co-cultured with either vehicle (PBS) or SEA (200 ng/mL). IL-17A protein was measured in the supernatant after 24 hours of incubation with ELISA. (C) Primary malignant T cells from a CTCL patient and non-malignant CD4 T cells were co-cultured with SEA and blocking antibodies against either IL-2, IL-7 or IL-15 or a combination of IL-2, IL-7 and IL-15 for 24 hours. IL-17A concentrations were

632 determined by ELISA and normalized to 10^6 cells and shown in absolute concentrations and in percent
633 inhibition of IC control. “ND”, No IL17A gene expression detected.

Figure 1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Pos isolates
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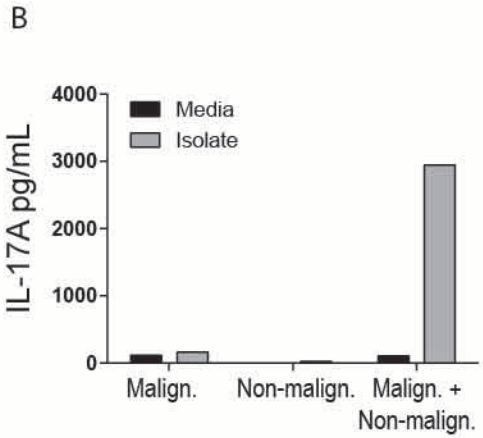
SE-negative isolates
SE-positive isolates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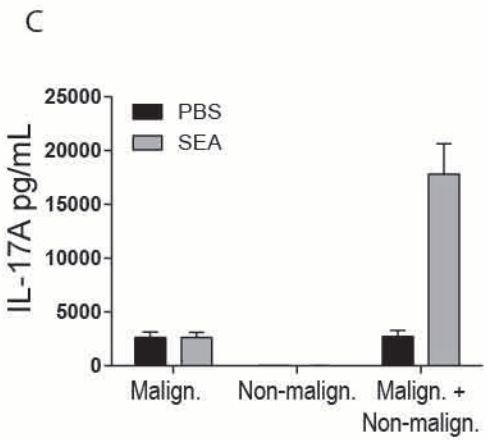
1000
0
Malign.
Non-malign.
Malign. + Non-malign.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Malign.
Non-malign.
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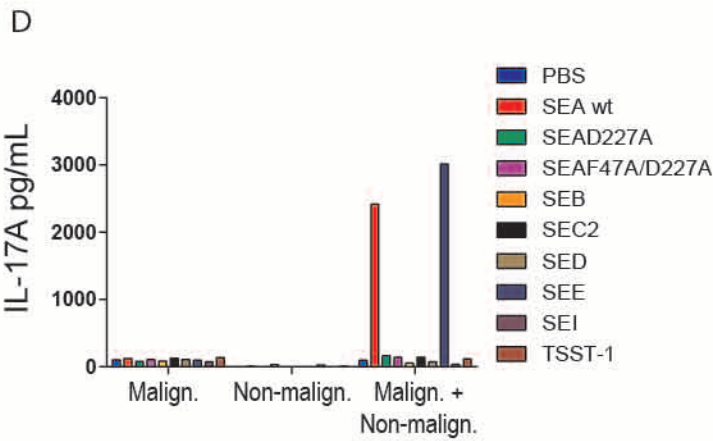
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SEA wt
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SEC2
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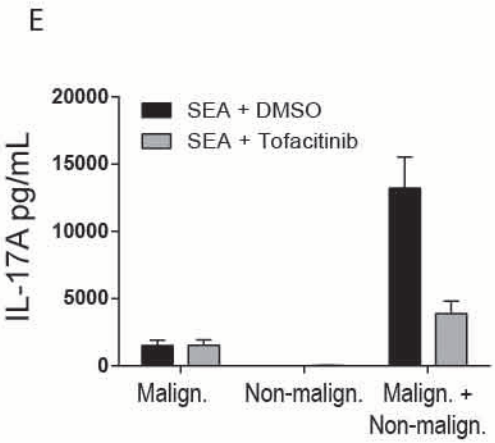
Malign.
Non-malign.
Malign. + Non-malign.


E
SEA + DMSO
SEA + Tofacitinib
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Malign.
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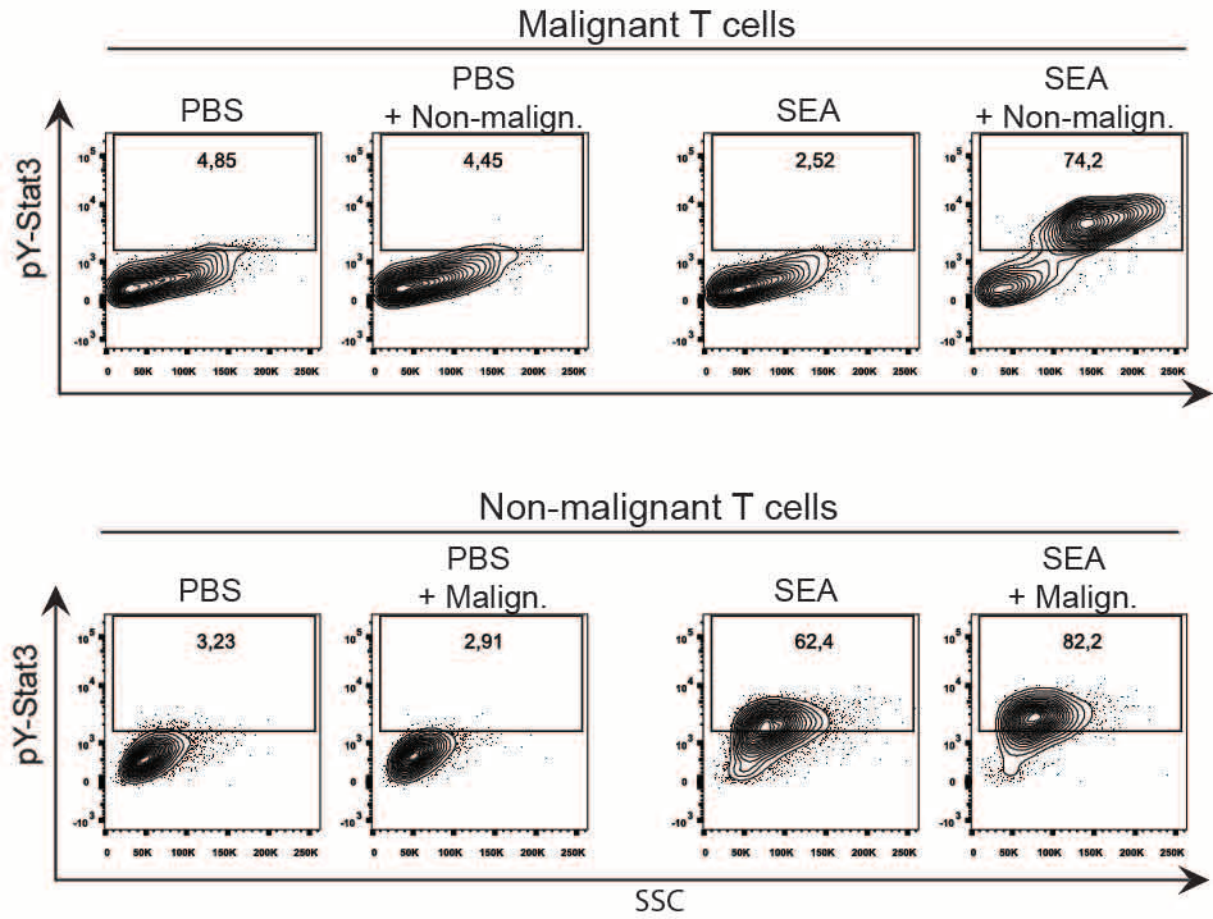
Malign. + Non-malign.

Figure 2


Malignant T cells
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+ Non-malign.
SEA
SEA
+ Non-malign.
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pY-Stat3
Non-malignant T cells
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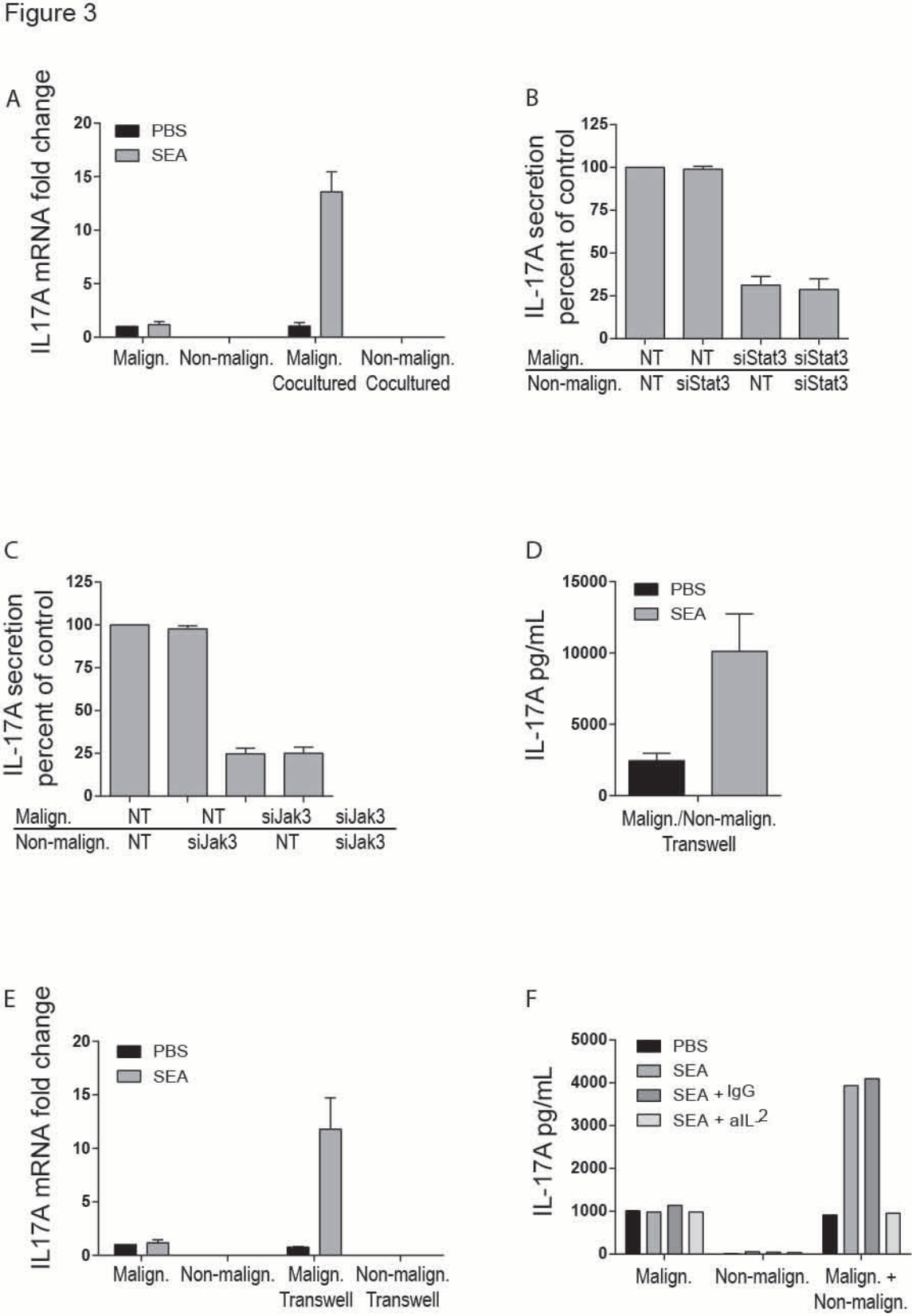
pY-Stat3
SSC

Figure 3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SEA
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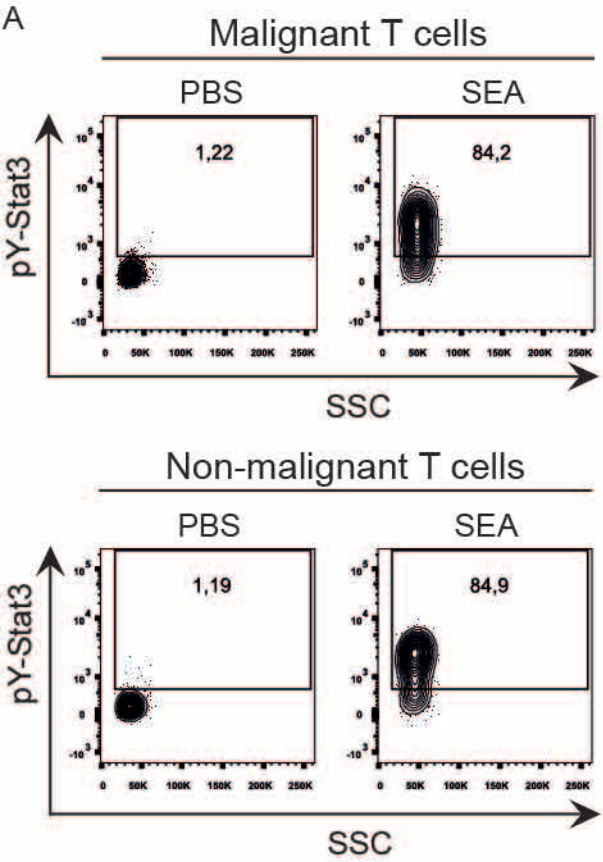
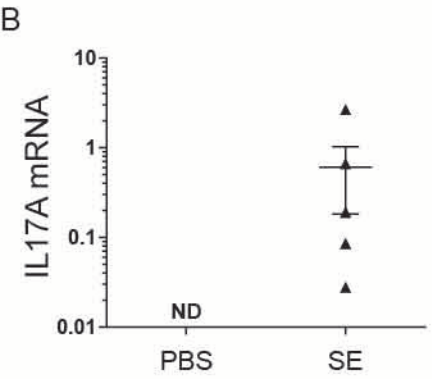
Malign. Cocultured
Non-malign. Cocultured
B
IL-17A secretion percent of control
Malign.
Non-malign.
NT
siStat3
C
IL-17A secretion percent of control
Malign.
Non-malign.
NT
siJak3
D
IL-17A pg/mL
PBS
SEA
Malign./Non-malign. Transwell
E
IL17A mRNA fold change
PBS
SEA
Malign.
Non-malign.
Malign. Transwell
Non-malign. Transwell
F
IL-17A pg/mL
PBS
SEA
SEA + IgG
SEA + aIL-2
Malign.
Non-malign.
Malign. + Non-malign.

Figure 4


A
Malignant T cells
PBS
SEA
1,22
84,2
pY-Stat3
SSC
Non-malignant T cells
PBS
SEA
1,19
84,9
pY-Stat3
SSC


B
IL17A mRNA
ND
PBS
SE

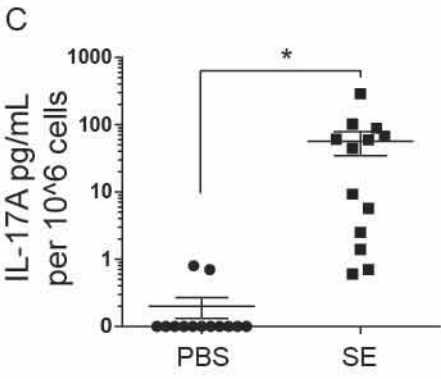
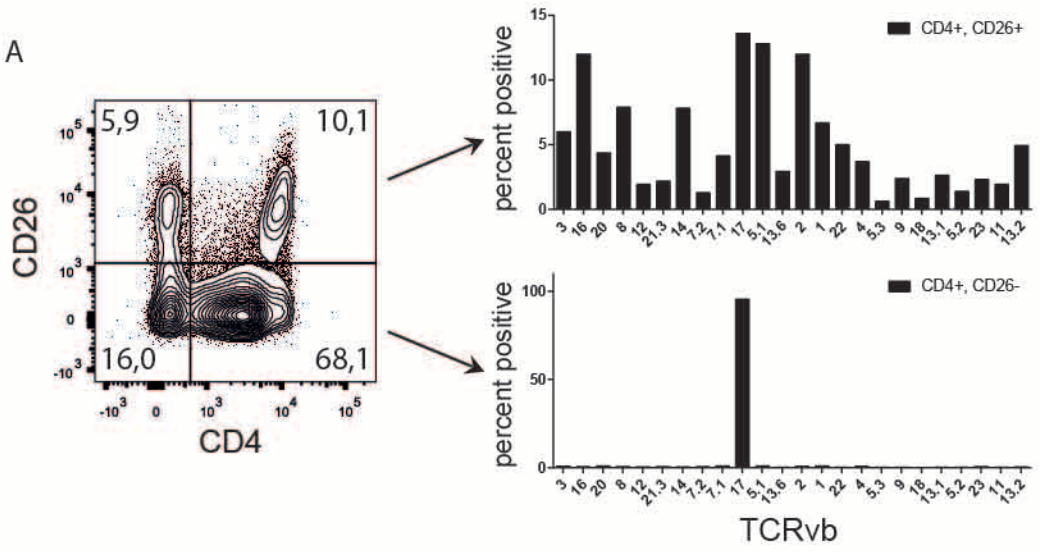

C
IL-17A pg/mL per 10^6 cells
*
PBS
SE

Figure 5


A
5,9
10,1
16,0
68,1
CD26
CD4
percent positive
CD4+, CD26+
CD4+, CD26-
TCRvb

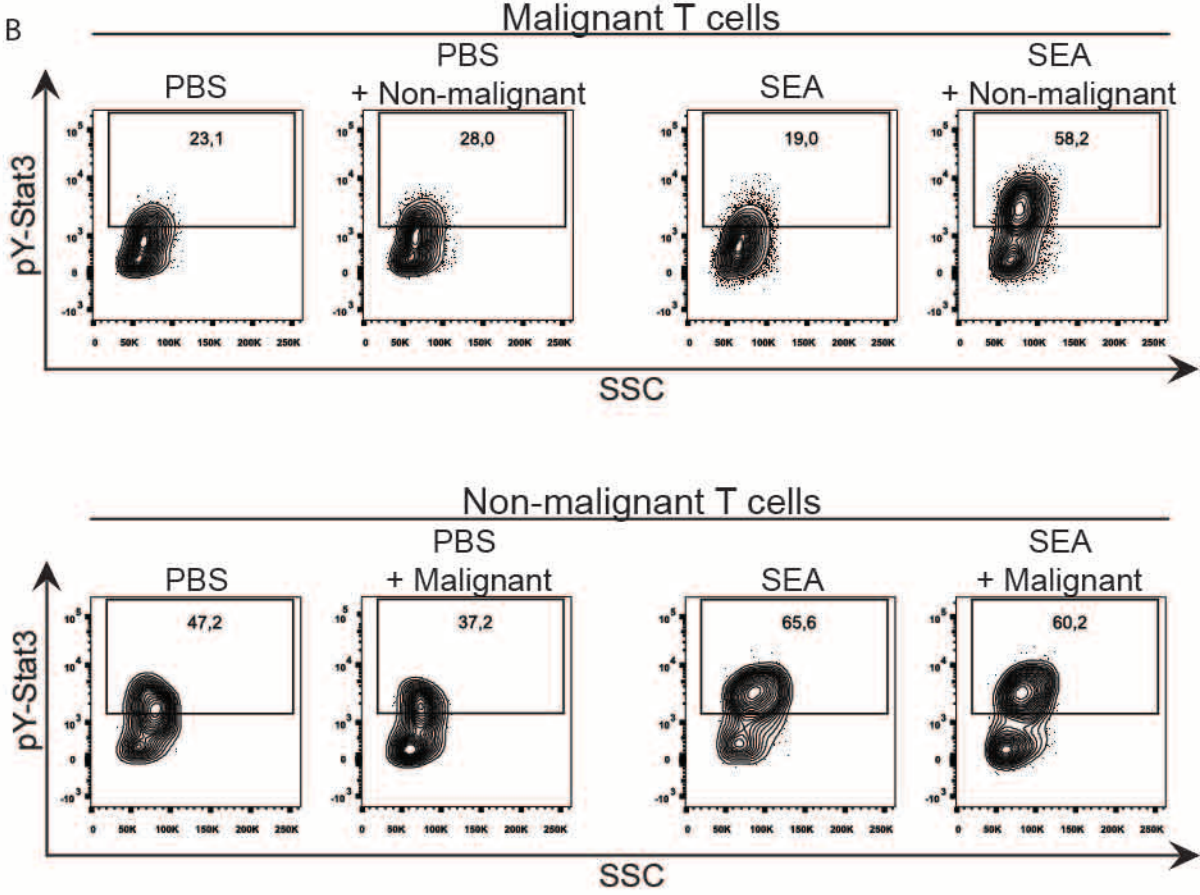
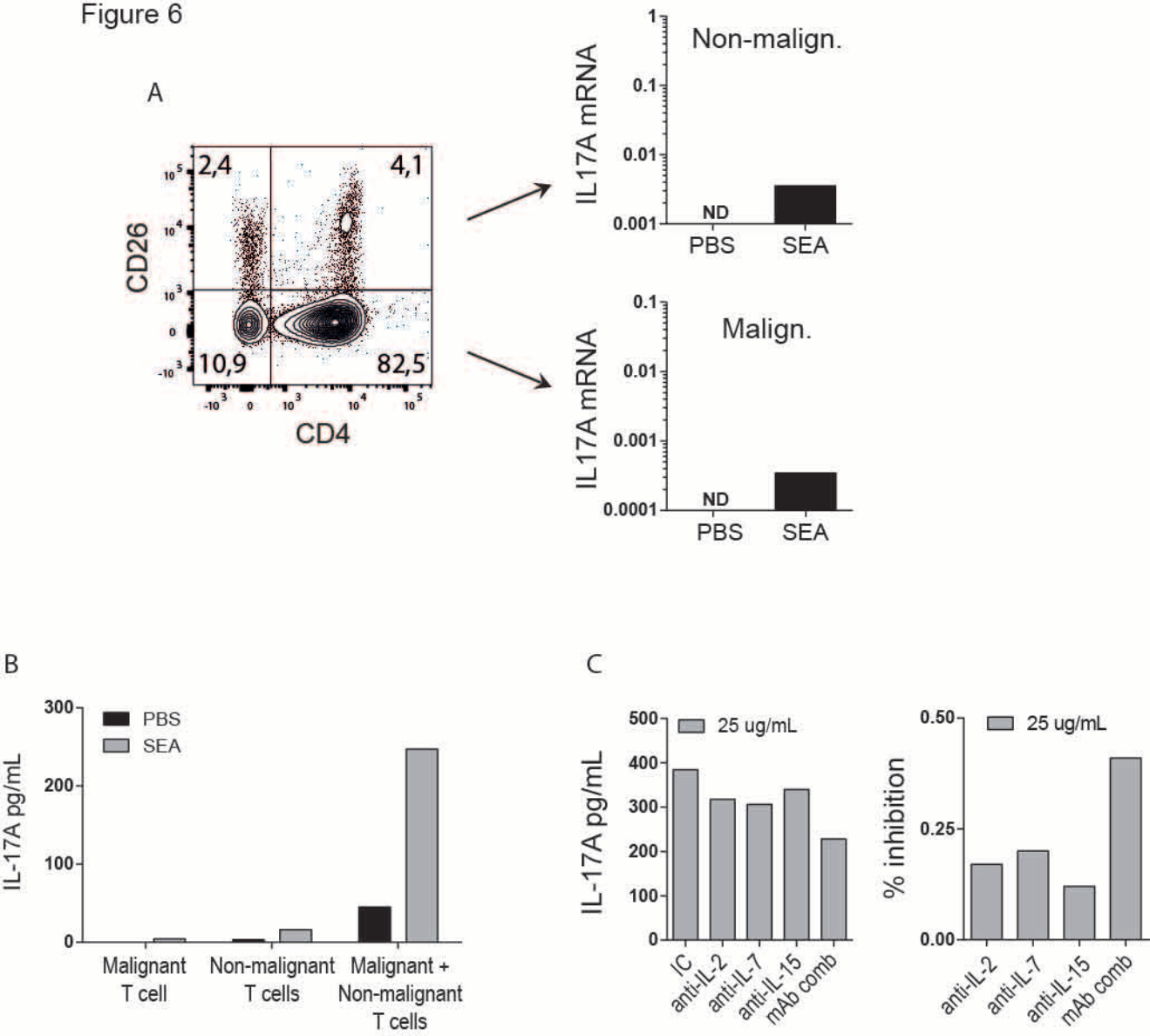

B
Malignant T cells
PBS
PBS + Non-malignant
SEA
SEA + Non-malignant
23,1
28,0
19,0
58,2
pY-Stat3
SSC
Non-malignant T cells
PBS
PBS + Malignant
SEA
SEA + Malignant
47,2
37,2
65,6
60,2
pY-Stat3
SSC

Figure 6


A
2,4
4,1
10,9
82,5
CD26
CD4
IL17A mRNA
Non-malign.
ND
PBS
SEA
Malign.
B
PBS
SEA
IL-17A pg/mL
Malignant T cell
Non-malignant T cells
Malignant + Non-malignant T cells
C
25 ug/mL
IL-17A pg/mL
IC
anti-IL-2
anti-IL-7
anti-IL-15
mAb comb
% inhibition

blood

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Staphylococcus aureus enterotoxin A (SEA) stimulates STAT3 activation and IL-17 expression in cutaneous T-cell lymphoma

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