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CD30 Discriminates Heat Shock Protein 60-Induced FOXP3+ CD4+ T Cells with a Regulatory Phenotype

Ismé de Kleer,* Yvonne Vercoulen,* Mark Klein,* Jenny Meerding,* Salvatore Albani,† Ruurd van der Zee,‡ Birgit Sawitzki,§ Alf Hamann,§ Wietsce Kuis,* and Berent Prakken*

In many animal models, the manifestations of inflammatory diseases can be prevented by the adoptive transfer of CD4+FOXP3+ regulatory T cells (Tregs). CD4+FOXP3+ Tregs can be obtained by isolation and expansion of polyclonal naturally occurring Tregs or by Ag-specific activation of CD4+CD25+ FOXP3+ T cells. Two major obstacles are hampering the translation of this latter protocol into therapeutic application. First, there is a lack of knowledge on relevant autoantigens. Second, the resulting population is contaminated with activated CD4+ T cells that transiently express Forkhead box P3 but gain no regulatory function. Therefore, these cells may not be safe for clinical application. In this study, we demonstrate that highly suppressive FOXP3+ Tregs can be induced in vitro by the activation of CD4+CD25+ T cells with the self-Ag human 60-kDa heat shock protein (HSP60). The activation induced suppressive FOXP3+ Tregs can be distinguished by surface expression of CD30 from nonsuppressive FOXP3+ effector cells. We confirm that the induced CD30+FOXP3+ Tregs recognize HSP60 epitopes and that the induction of Tregs by HSP60 is enhanced by signaling via TLR4 on APCs. These findings have implications for the generation and isolation of pure populations of Ag-specific Tregs, with the potential to prevent and treat human inflammatory diseases. The Journal of Immunology, 2010, 185: 000–000.

As a result of intense research during the past decade, CD4+ FOXP3+ regulatory T cells (Tregs) have emerged as a central T cell population for preserving peripheral tolerance. Reduced frequencies or impaired function of this Treg population leads to a wide range of autoimmune disorders in animals and putatively also in humans (1–7). This indicates that an imbalance between autoreactive T cells and Tregs may contribute to the pathogenesis of such diseases. Thus, learning how to manipulate this balance, by either expanding the pool of CD4+FOXP3+ Tregs and/or enhancing their suppressive activity has obvious therapeutic relevance.

In many animal models, the onset or progression of inflammatory (auto)immune diseases can be prevented by an adoptive transfer of Tregs. This approach has been successfully applied in the treatment of experimental autoimmune encephalitis (8), experimental type I diabetes, arthritis (9), and colitis (10). Of specific interest are studies suggesting that the success of Treg-based immunotherapy depends on the Ag specificity of the Tregs (11, 12). For example, studies using nonlymphopenic mouse models of autoimmune diabetes showed that organ-specific Tregs are superior in disease protection compared with polyclonal Tregs (11, 13). The use of Ag-specific Tregs may also avoid dampening of immune responses to tumors and infectious agents, which are possible side effects of transferring large numbers of polyclonal Tregs. Because transferred Ag-specific Tregs can suppress T cells through bystander suppression, therapeutic Tregs do not require specificity for the Ag initiating the disease. However, the Tregs need to be specific for tissue Ags capable of activating Tregs. Here lays one of the most critical issues with respect to translating the successful results of Treg-based immunotherapy in transgenic mice to human autoimmune diseases; in most human autoimmune diseases, relevant autoantigens have not been identified yet, despite intensive research. In addition, the establishment of Treg lines to predefined Ags is technically challenging. Ag-specific FOXP3+ Tregs can be generated in vitro by activating CD4+CD25FOXP3+ T cells in the presence of TGF-β. However, Forkhead box P3 (FOXP3) can also be transiently expressed in effector T cells without gain of regulatory function (14–16). The resulting population therefore contains a mixture of FOXP3+ Tregs and FOXP3+ effector cells and may not be safe for clinical application. It was recently shown that the two FOXP3+ cell types can be distinguished by the methylation pattern at the FOXP3 locus (17). However, a surface marker that enables selective sorting of activation-induced FOXP3+ Tregs is still lacking.

Earlier studies suggested that heat shock proteins (HSPs) are good targets for Ag-specific Treg-based immunotherapy in human inflammatory disease (18). HSPs are evolutionary strongly conserved proteins present in all eukaryotic and prokaryotic cellular organisms. They are expressed both constitutively and under stressful conditions, such as UV radiation, infections, and malignancies...
(19–21). Besides being potent activators of the innate immune system (22, 23), HSPs have important immune regulatory effects. The human 60-kDa HSP (HSP60) molecule can downregulate T cell proliferation (24) and inhibit the secretion of proinflammatory cytokines by activated T cells (25). This anti-inflammatory effect may be mediated by a direct TLR2-mediated effect of HSP60 on intrinsic Tregs, resulting in enhancement of regulatory T cell function (26). Furthermore, extensive studies in animal models, as well as juvenile idiopathic arthritis (JIA) patients (27–29), have provided evidence that specific T cell responses against HSPs, especially HSP60, are associated with anti-inflammatory regulation (30). In almost all models of experimental arthritis, including adjuvant arthritis, preimmunization with HSP65 protects animals from arthritis (31–33). This protection is mediated by the activation of self-HSP-specific T cells with regulatory characteristics and capable of downregulating inflammation (31, 34). Also in arthritis patients, specific T cell responses against HSP60 are found. These T cell responses against endogenously produced HSP60, which is abundantly expressed in the synovial lining cells of the patients (35), are associated with a good prognosis (28, 36). Thus, HSPs fulfill two nonredundant criteria for Ag-specific Treg-based immunotherapy, namely selective expression at sites of inflammation and immunogenicity.

In the present report, we explored the potential of human HSP60 as a target for Treg-based immunotherapy. We found that suppressive HSP60-specific FOXP3+ Tregs can be generated in vitro and distinguished from FOXP3− cells and from FOXP3+ effector cells without regulatory function by the surface expression of CD30. We therefore conclude that CD30 represents a marker that separates contaminating HSP60-induced FOXP3+ effector T cells from immune-suppressive FOXP3+ Tregs. The HSP60-mediated differentiation to a Treg phenotype was enhanced by TLR4 signaling on APCs. These findings may allow the generation and isolation of regulatory CD4+CD25+ T cells with regulatory characteristics and capable of downregulating inflammation for the development of Treg-based immunotherapy for human inflammatory diseases.

Materials and Methods

Participants

Buffy coats were taken from healthy volunteers (n = 15) and 20 ml blood samples from patients with JIA (n = 6) or rheumatoid arthritis (RA; n = 1). The JIA patients were followed up at the Department of Pediatric Immunology and Rheumatology and the RA patient at the Department of Rheumatology of the University Medical Center (Utrecht, The Netherlands). The study was approved by the local Institutional Review Board, and oral consent was obtained from all healthy volunteers and patients or their parents.

Cells, medium, and reagents

PBMCs were isolated using Ficoll Isopaque density gradient centrifugation (Amer sham Biosciences, Piscataway, NJ). Very low endotoxin RPMI 1640 (Seromed, Berlin, Germany) containing 10 mM HEPES (Seromed), 2 mM L-glutamine (Seromed), 20 μg/ml streptomycin, 60 mg/ml penicillin, and 10% human AB-serum was used as a culture medium. Low endotoxin HSP60 (<0.3 μg/ml protein) was obtained as a gift from Dr. R. van der Zee, from the Faculty of Veterinary Medicine (Utrecht, The Netherlands), and from StressGen Biotechnologies (Victoria, British Columbia, Canada). Measurement of the endotoxin levels in the used HSP60 samples was contracted out to Cambrex (Rapid Endo-test; Cambrex, Verviers, Belgium). In some experiments, TLR4 blocking Ab (clone HTA152; Serotec, Oxford, U.K.) or TLR2 blocking Ab (clone TL2.1; BioLegend, San Diego, CA) was used. For these experiments, APCs or CD4+CD25+ T cells were incubated (45 min, 37°C) with 20 μg/ml TLR4 blocking Ab before addition to the cultures (37). The survival of the cells was measured at day 7 by staining the cells with 7-aminoactinomycin D (BD Biosciences, San Jose, CA).

Flow cytometry

PBMCs were washed twice in FACS buffer (PBS containing 2% FCS) and adjusted to 0.5–1 × 106 cells/ml in staining buffer (FACS buffer containing 0.1% sodium azide) and blocked with mouse serum (30 min at 4°C). Subsequently, the cells were incubated in 50 μl FACS buffer containing three or four appropriately diluted PE-, FITC-, Cy5-, or APC-labeled mAbs against human CD4 (clone RPA-T4), CD25 (clone 2A3), CD30 (clone Ber-H3), CD127 (clone hIL-7R-m21), CCR4 (clone 1G1), glucocorticoid-induced TNFR related protein (GITR) (clone 110416), and TLR-4 (HTA125). For intracellular staining of CTLA-4 (clone BNI3), the cells were first surface stained, then fixed in Cytofix/Cytoperm solution (20 min, 4°C), and washed twice in perm/wash solution (Cytofix/perm kit; BD Biosciences), followed by incubation with anti–CTLA-4, anti–IL-10, or anti–IFN-γ mAb. For FOXP3 staining (clone PCH101) the cells were first surface stained and subsequently treated with a FOXP3 staining kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions. GITR mAb was obtained from R&D Systems (Wiesbaden, Germany). TLR4 mAb was obtained from Serotec and FOXP3 mAb from eBioscience. All other mAbs were obtained from BD Biosciences. Stained mononuclear cells were diluted in sheath fluid and run on a FACS Calibur (BD Biosciences). CellQuest software (BD Biosciences) was used for analysis.

Induction of regulatory T cells

CD4+CD25+ T cells from healthy donors were obtained by a first step of CD4 T cell enrichment and a second step of CD25+ selection using CD4 T cell isolation kit, CD25 microbeads, and VarioMACS selection columns (Miltenyi Biotec, Bisley, Surrey, U.K.). Contamination of the CD4+CD25+ T cell fraction was <3%. Irradiated (3500 rad) CD4+ T cell-depleted PBMCs were used as APCs. CD4+CD25+ T cells and APCs (1:3) were cultured in the presence of human HSP60 (10 μg/ml) for 7 d. After 7 d, the cells were evaluated on the expression of the above-described markers. Furthermore, CD4+CD25+ and induced CD4+CD25+ T cells were sorted by FACS and analyzed on the expression of mRNA FOXP3. The purity of the sorted populations, determined by FACS reanalysis of an aliquot of sorted cells, was 97% on average.

Suppression and proliferation assays

The 3% CD4+CD25− T cells with the brightest expression of CD25 were tested in coculture experiments on suppressive function and compared with CD25+CD30+ and CD25−CD30− CD4+ T cells. Fresh CD4+CD25+ effector T cells (3–5 × 103) from the same donor and induced CD25+CD30+, CD25+CD30−, and CD25−CD30−. CD4+ T cells were directly sorted by FACS (FACS Vantage, BD Biosciences) into plate-bound anti-CD3-coated wells (OKT-3, 1.5 μg/ml), in different ratios. T cell-depleted, irradiated PBMCs (3500 rad) from the same donor were used as APCs (1:3). The cells were incubated at 37°C for 6 d, the last 18 h in the presence of [3H]thymidine (1 μCi/well). The suppressive activity was determined by calculating the relative difference in proliferative response (mean [3H] thymidine incorporation [cpm] of triplicate wells) between CD4+CD25− T cells cultured alone and CD4+ CD25+ T cells cultured in the presence of induced regulatory T cells. For CFSE assays, CD4+CD25+ effector T cells (Teffs) isolated by magnetic selection, labeled with 2 μM CFSE, plated 25,000/well, and irradiated T cell-depleted PBMCs were used as APCs, plated 25,000/well. Alternatively, 10,000 CFSE-labeled PBMCs were used as effector cells and plated per well. Induced Tregs were isolated by FACS sorting and added in different ratios to the cultures. Cells were stimulated with anti-CD3 (clone OKT3). On day 4 of culture, CD4+ T effector proliferation was analyzed by FACS Canto (BD Biosciences). The percentage of suppression was calculated by comparing the percentage of proliferated CD4+CD25+ T cells in wells with CD4+CD25+ T cells cultured alone and CD4+CD25− T cells cultured in the presence of HSP60-induced regulatory T cells.

Stability of FOXP3 expression and cytokine production

To determine FOXP3 stability and cytokine secretion by CD30-expressing cells and CD30− cells, CD25+CD30+ and CD25−CD30− CD4+ T cells were FACS sorted and cultured separately in presence of 1.5 μg/ml plate-bound anti-CD3. For FOXP3 analysis, cells were stained on day 7. Supernatants were taken on days 3, 5, and 7 for cytokine analysis by multiplex immunoassay (see below).

Ag-specific suppression assay

To assess specificity of Treg suppression, CD4+CD25+ effector T cells were isolated by magnetic selection, labeled with 2 μM CFSE, and plated 25,000/well, and irradiated T cell-depleted PBMCs were used as APCs, plated at 25,000 cells per well. Irradiated PBMCs induced Tregs were isolated by FACS sorting and added in different ratios to the cultures. Cultures were either stimulated with CD3 (clone OKT3). On day 4 of culture, CD4+ T effector proliferation was analyzed by FACS Canto (BD Biosciences).
Biosciences). Percentage of suppression was calculated by comparing the percentage of proliferated CD4+CD25− T cells in wells with CD4+CD25+ T cells cultured alone and CD4+CD25+ T cells cultured in the presence of HSP60-induced regulatory T cells.

**Methylation of FOXP3 Treg-specific demethylated region**

To determine the methylation of the FOXP3 Treg-specific demethylated region (TSDR), only healthy male donors were included. DNA was isolated from sorted CD25+CD30+ and CD25−CD30− CD4+ T cells, induced by HSP60, using QiaAmp DNA Mini Kit (Qiagen, Valencia, CA). Methylation of the FOXP3 TSDR was determined according to the previously published methods (38).

**mRNA analysis by quantitative PCR**

Total RNA from FACS-sorted cells was isolated using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 500 ng/ml. First-strand cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) with 1 μg/μl oligo(dT) and 10 mM 2′-deoxynucleoside 5′-triphosphates (both Amersham Biosciences, Uppsala, Sweden). The reaction mixture was incubated at 40°C for 90 min, followed by incubation at 70°C for 15 min. To ensure the fidelity of mRNA extraction and reverse transcription to first-strand cDNA, all samples were subjected to real-time PCR amplification with primers specific for the constitutively expressed gene β2-microglobulin (β2m). For FOXP3 and β2m transcripts, real-time quantitative PCR was performed with a LightCycler (Roche Diagnostics) based on specific primers and general fluorescence detection with SYBR Green. The following primer combinations were used: IL-10, 5′-TGGAGACACGTGCACCATCT-3′ (forward) and 5′-GCTGGAACAGCTGGCAGAT-3′ (reverse); IFN-γ, 5′-GGAAAGCCAAATTTG-TCTCCCT-3′ (forward) and 5′-ATGCCTCTGCCACCTGGAAAC-3′ (reverse); FOXP3, 5′-TCAAGAGCCTGCCGAGGCG-3′ (forward) and 5′-CACGAGCC-CTTGTGGAG-3′ (reverse); and β2m, 5′-CCAGCAGAACTGGAGGAAAGTC-3′ (forward) and 5′-GATGCTGCTTCATGCTGCG-3′ (reverse). All PCRs were performed using LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics). A pool of cDNA from tetanus toxoid-stimulated human PBMCs was used as a standard, and normalization to β2m was performed for each sample. Semiquantitative levels of FOXP3 are expressed as percentage of the FOXP3 expression of the cDNA pool.

**Analysis of cytokine production by multiplexed particle-based flow cytometric assay**

Cell culture supernatants were collected, stored at −80°C, and processed within 1 mo. Cytokine concentrations were measured by the Bio-Plex system in combination with the Bio-Plex Manager software, version 4.0 (Bio-Rad, Hercules, CA), which uses the Luminex XMAP technology as described previously (39). The following cytokines were measured: IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, TNF-α, and IFN-γ.

**T cell capture and artificial APcs**

This technique is extensively described earlier (40). Compared with the previously described protocol a few improvements were made. In short, PBMCs of DR4-positive arthritis patients were cultured with or without human HSP60. After 7 d, the cells were prestained with anti-CD19 or anti-CD30, washed twice, and resuspended in FACS buffer. CD4-CY and CTB-FITC high double-positive cells were sorted by FACS (FACS Vantage; BD Biosciences) and analyzed on the expression of mRNA FOXP3, IL-10, and IFN-γ.

**Results**

**Activation of CD4+CD25− T cells with human HSP60 induces a clear population of CD4+FOXP3+ T cells**

Stimulation of human CD4+CD25− T cells can result in considerable FOXP3 expression and development of suppressor activity (41–43). We examined the potential of human HSP60 to generate FOXP3+ Treg by stimulating CD4+CD25− T cells obtained from healthy adults and JA patients. In both groups, similar results were seen. Stimulation of CD4+CD25− T cells with HSP60 resulted in the expression of CD25 in intermediate and bright levels, suggestive of two populations (Fig. 1A). Flow cytometric staining for FOXP3 showed that all CD4+CD25bright T cells and most CD4+CD25int T cells (82.2 ± 2.9%, mean ± SEM) induced after activation with HSP60 were FOXP3+ whereas only a minority of the CD4+CD25− T cells exhibited FOXP3 expression (Fig. 1A). These findings were confirmed by the measurement of FOXP3 mRNA levels in sorted CD4+ T cell populations (Fig. 1B). Thus, HSP60 can induce expression of CD25 and FOXP3 in CD4+ T cells. Next, we tested the suppressive capacity of the HSP60-induced CD4+CD25bright T cells as described before (2). Clear suppression was found in only three of the six donors, even though in all six donors 100% of the CD4+ CD25bright T cells expressed FOXP3 (Fig. 1C).

**Surface expression of CD30 discriminates activation-induced FOXP3+ Tregs**

Despite the high prevalence of FOXP3+ cells, HSP60-induced CD4+CD25bright T cells lacked suppressive capacity in half of the donors. Because FOXP3 can be transiently expressed in activated cells without acquiring regulatory function, this finding may indicate that, in addition to contamination with FOXP3− cells, the induced CD25bright T cell population contains FOXP3+ effector T cells without regulatory function. Whether the HSP60-induced CD25bright T cells are suppressive depends on the proportion of functional Treg within this population. Thus, to rely on CD25bright expression alone is not sufficient in identifying activation-induced Tregs for use in suppressor function tests or for the isolation of therapeutic Tregs, even though all of these cells express FOXP3. We therefore sought to find a surface marker that correlates with suppressive function rather than induced FOXP3 expression.

CD30 is a member of the TNFR superfamily and originally described as a marker of Reed-Sternberg cells in Hodgkin’s disease (44, 45). Although in peripheral blood the expression of CD30 is extremely low to zero, it is expressed upon activation on T and B lymphocytes. Although engagement of CD30 by its ligand has extremely low to zero, it is expressed upon activation on T and B lymphocytes. Although engagement of CD30 by its ligand has shown to provide costimulatory signals to activated T cells and to enhance cytokine production and secondary proliferative responses (46), the exact function of CD30 in mature T cells is still unknown. Of interest are reports suggesting that CD30 is required for or contributes to immune regulation (36, 47–49). In a previous study, we again at room temperature and washed three times in PBS. As negative controls, empty liposomes (blanco), liposomes with anti-CD19 or anti-CD28 were used. Finally, the aAPCs were incubated with the stained cells, washed twice, and resuspended in FACS buffer. CD4-CY and CTB-FITC high double-positive cells were sorted by FACS (FACS Vantage; BD Biosciences) and analyzed on the expression of mRNA FOXP3, IL-10, and IFN-γ.
in mice, CD30 signaling on CD4+CD25+ Tregs has shown to be important in the prevention of allograft rejection and acute graft-versus-host disease (50, 51). These studies suggest a role for CD30 expression, and CD25+CD127low cells had a lower expression of FOXP3 (Supplemental Fig. 2). We therefore concluded that cell surface expression of CD30, but not CD127 low, CD27, CTLA4, or GITR, allows a distinction of activation-induced FOXP3 + T cells. Other markers tested were CD127, CD27, GITR, and CTLA4, all described to play a role in Treg function (54–56). CD127 held our interest particularly, because it is a marker used for the identification of naturally occurring CD4+FOXP3+ Tregs (54). However, none of these markers, including CD127low, showed a similar pure positive correlation with FOXP3 expression in activation-induced Tregs (Fig. 3). Furthermore, CD127low expression did not correlate with CD30 expression, and CD25+CD127low cells had a lower expression of FOXP3 (Supplemental Fig. 2). We therefore concluded that cell surface expression of CD30, but not CD127low, CD27, CTLA4, or GITR, allows a distinction of activation-induced FOXP3+ T cells.

**The induction of CD4+ Foxp3+ Treg through activation is enhanced by TLR4 triggering on APCs**

Besides being an Ag for T cells, HSP60 can also function as an innate ligand for TLR2 (24) and TLR4 (57, 58). Both TLR2 and TLR4 are the receptors for bacterial lipopolysaccharide (LPS), which can stimulate macrophages and monocytes, whereas TLR4 is also involved in innate immune responses to viral infections.

showed that CD30+ T cells present in the synovial fluid of JIA patients correlate with a favorable disease course (36). Furthermore, in mice, CD30 signaling on CD4+CD25+ Tregs has been shown to be important in the prevention of allograft rejection and acute graft-versus-host disease (50, 51). These studies suggest a role for CD30 in activation-induced regulatory cells and prompted us to study the expression of CD30 in our system. First, we analyzed the relationship between the expression of CD30 and FOXP3 by FACS and RT-PCR in CD4+CD25+, CD4+CD25int, and CD4+CD25bright T cells obtained after 7-d cultures of HSP60-stimulated CD4+CD25+ T cells. Single-cell analysis by FACS showed that CD30 expression is restricted to FOXP3-expressing cells and that 56% (range, 53–62%) of the FOXP3-expressing T cells coexpressed CD30 (Fig. 2A). Furthermore, the expression of FOXP3 per cell was higher in CD30-expressing cells, compared with the CD30− FOXP3-expressing cells (Fig. 2C). These data were confirmed by RT-PCR. Although no significance was reached (one-way ANOVA with Bonferroni correction), RT-PCR showed a clear trend toward higher mRNA FOXP3 levels in T cell subsets coexpressing CD30 (Fig. 2B).

The restricted expression of CD30 to FOXP3+ cells enabled us to compare the suppressive capacity of activation-induced CD25bright, CD25−CD30−, and CD25+CD30+ T cells. Interestingly, only CD4+ T cells coexpressing CD30 manifested regulatory function in vitro, whereas CD4+CD30− T cells did not, and for CD25bright, only half of the donors (three of six) showed suppression, whereas two donors showed high proliferation of Tregs, causing an average negative level of suppression (Fig. 2D, for CD25bright; see also Fig. 1C). In addition, we showed a similar pattern in suppression assays using CFSE dilution as readout, discriminating CD4+ effector T cells proliferation from Treg proliferation. The difference in vitro-suppressive capacity between CD30+ and CD30− CD4+ T cells was less pronounced in this system (Fig. 2E). This fits with other reports that, especially for induced Tregs, in vitro suppression assays are not as consistent as for freshly isolated Tregs (52).

Furthermore, we tested stability of FOXP3 expression within the CD30+ cells. Indeed, after 7 d of separate culture of sorted CD30+ cells, the cells that maintained CD30 on their surface were all FOXP3+ (Supplemental Fig. 1). A demethylated state of the TSDR within the FOXP3 locus relates to Treg function and stability of FOXP3 expression. However, TSDR demethylation was similarly low in both HSP60-induced CD25+CD30+ T cells and CD25+CD30− T cells (data not shown).

Next, we tested cytokine production by both CD30+ and CD30− T cells after stimulation with anti-CD3, similar to stimulation in suppression assays. Although not significant, CD30-expressing cells seem to excrete less IL-2 (p = 0.1), probably due to high FOXP3 expression. Both CD30+ and CD30− cells excreted high levels of IL-17, which was a little higher in the CD30+ cells. For IL−4, IL−5, IL−10, IL−13, IFN−γ, and TNF−α excretion, no significant difference between CD30+ and CD30− cells was observed (Fig. 2F).

Cytokine excretion profiles of both populations are very different from freshly isolated CD4+CD25+CD127low T cells (Tregs), which produce only low levels of cytokine, but are comparable to Teffs, except for a higher IL−17 and lower TNF−α production (see Supplemental Table I and our previously published data [53]).

Other markers tested were CD127, CD27, GITR, and CTLA4, all described to play a role in Treg function (54–56). CD127 held our interest particularly, because it is a marker used for the identification of naturally occurring CD4+FOXP3+ Tregs (54). However, none of these markers, including CD127low, showed a similar pure positive correlation with FOXP3 expression in activation-induced Tregs (Fig. 3). Furthermore, CD127low expression did not correlate with CD30 expression, and CD25+CD127low cells had a lower expression of FOXP3 (Supplemental Fig. 2). We therefore concluded that cell surface expression of CD30, but not CD127low, CD27, CTLA4, or GITR, allows a distinction of activation-induced FOXP3+ T cells.
FIGURE 2. FOXP3 mRNA expression and suppressive function of HSP60-induced CD4+CD25+CD30+ T cells. A, Flow cytometric staining of CD25, CD30, and FOXP3 on CD4+CD25- T cells activated with HSP60 in the presence of APCs. B, Semiquantitative RT-PCR analysis of FOXP3 mRNA in sorted CD4+ T cells derived after 6-d cultures of CD4+CD25- T cells in the presence of human HSP60. Shown is the mean ± SD of six experiments with blood of healthy controls. The sort gates for the six populations analyzed are given in A. C, FOXP3 MFI of gated CD4+CD25+CD30+FOX3+ T cells compared with CD4+CD25-CD30-FOX3+ T cells derived after 7-d cultures of CD4+CD25- T cells in the presence of human HSP60 for six healthy donors. D and E, Comparison of suppressive potential of CD4+CD25+CD30+, CD4+CD25+CD30-, and CD4+CD25+CD25+bright T cells derived after 7 d cultures of CD4+CD25- T cells in the presence of human HSP60. The dot plot shows the used sort gates. D, Four separate experiments with blood of three healthy donors are shown for CD25+CD30+ and CD25+CD30- and six experiments with blood of six donors for CD25+bright. Level of suppression of effector T cell (Teff) proliferation, calculated for several ratios of Teff + Treg, compared with culture of Teff alone (suppression = 0%). E, Effector T cells were labeled with CFSE. Percentage of Teff proliferation, and Teff + Teff, compared with culture of Teff with Tregs. Histograms from 1 of 10 donors are shown. F, CD4+CD25+CD30+ T cells and CD4+CD25+CD30- T cells were sorted on day 6 and cultured separately for 5 d in the presence of anti-CD3. Supernatants were collected out of triplicates of cultured wells and measured by multiplexed particle-based flow cytometric assay. Mean cytokine levels in supernatants are depicted of three healthy controls. Shown are means ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001. MFI, mean fluorescence intensity.
TLR4 are abundantly expressed on cells of the innate immune system, such as dendritic cells (DCs) and macrophages. In our experiments, HSP60-induced FOXP3 expression required the addition of APCs (Fig. 4A). To test whether TLR2 or TLR4 ligation on APCs plays a role in the induction of FOXP3, we pretreated APCs with neutralizing anti-TLR2 or anti-TLR4 mAb before addition to the cultures. Interestingly, blocking TLR4 on APCs resulted in a significant reduction of CD25 and FOXP3 mRNA expression, whereas blocking TLR2 on APCs had no such effect (Fig. 4B). Although several batches of recombinant human HSP60 were shown to contain minimal residual LPS and LPS lipoproteins (59), it is unlikely that TLR4 ligation with contaminating LPS played a role in our assays. The highly purified recombinant human HSP60 used in this study contained undetectable amounts of bacterial endotoxin (i.e., <0.003 EU/µl or <0.3 pg/µg protein). We therefore conclude that HSP60 can enhance activation-induced FOXP3+ Treg induction via TLR4 ligation on APCs.

The activation-induced CD4+CD30+ Tregs are Ag specific

Because HSP60 functions both as TCR and TLR4 ligand, we wanted to exclude a pure innate effect and evaluated whether we could induce CD4+CD30+FOXP3+ T cells by activating CD4+CD25− T cells with peptides from HSP60. Healthy controls (n = 2) were selected on their responsiveness to previously identified pan-DR binding peptide epitopes from human HSP60 (60, 61). In both donors, activation of CD4+CD25− T cells with a peptide of HSP60 resulted in a similar CD30 and FOXP3 expression pattern as activation with the whole protein (Fig. 5A). This shows that CD4+CD30+FOXP3+ Treg development is the result of activation
through the TCR and that TLR signaling enhances but is redundant for Treg development.

In addition, the Ag specificity of the HSP60-induced CD4+CD30+ FOXP3+ Tregs was evaluated using the so-called T cell capture technique. This technique uses aAPCs consisting of liposomes with a high relative density of MHC–peptide complexes enabling the capture of low-avidity class II-restricted Ag-specific T cells (40). CD4+CD25+ T cells derived from the peripheral blood of three healthy volunteers (Fig. 5B), two DR4+ JIA patients and one DR4+ RA patient, were stimulated with HSP60, in the presence of APCs. We next used CTB-labeled aAPCs containing DR4 molecules loaded with a peptide of HSP60 to isolate T cells able to recognize HSP60 epitopes from the cultures. Cells were stained with fluorescence-labeled aAPCs, CD4 and CD30. The aAPC-positive cells were analyzed on the expression of CD30 (Fig. 5B, Supplemental Fig. 3) and after sorting on the expression of IL-10, IFN-γ, and FOXP3 mRNA by RT-PCR (Fig. 5D). A comparison in mRNA content was made with ex vivo-isolated CD4+CD25+ T cells and CD4+CD25highCD127low natural Tregs. In each experiment, HSP60 peptide-specific T cells expressing CD30 and FOXP3 mRNA could be identified, producing high levels of IL-10 and no IFN-γ, which is compatible with intracellular staining of total HSP60-induced Tregs, showing that CD30 expression correlates with high IL-10 and low IFN-γ (Supplemental Fig. 4). This indicates that at least part of the HSP60-induced CD4+CD30+FOXP3+ T cells recognize HSP60 epitopes and have a regulatory cytokine production profile. To test specific suppressive capacity, we undertook suppression assays in which stimulation of the effector T cells with specific Ag (HSP60) or a specific Ag (tetanus toxoid) was used. Because these Ags activate only few effector cells, Teff proliferation is similarly low for both Ags, compared with anti-CD3 stimulation. Therefore, optimal suppression is already reached at a coculture ratio of 10:1. HSP60 responses were suppressed similar compared with tetanus toxoid responses (Fig. 5E). This suggests that HSP60-induced CD4+CD25+CD30+ T cells are able to perform bystander suppression as well as specific suppression.

**Discussion**

Although Ag- and/or site-specific suppression of inflammation seems the ultimate goal of immunotherapy for specific inflammatory
diseases, knowledge on relevant Ags is still lacking in most cases. In addition, thus far it has not been possible to identify and isolate pure activation-induced Treg populations as a result of the lack of an appropriate surface marker that can distinguish FOXP3+ Tregs.

In the current study, we show that the activation of CD4+CD25− T cells with human HSP60 results in de novo generation of a subset of FOXP3-expressing T cells. In addition, we show that within this population the surface expression of CD30 marks a very homogenous population of activation-induced FOXP3+ T cells with a stable expression of high levels of FOXP3, leading to suppressive function in vitro.

The exact function of CD30 on mature T cells is unknown. Previous in vitro studies have shown that CD30-CD30 ligand interaction has effect on both costimulation and cytokine production (62, 63) and recent ex vivo studies implicated a role for CD30+ T cells in the regulation of autoimmunity and tumor rejection (36, 47–49). In mice, CD30 signaling plays a role in the suppression of allograft rejection and acute graft-versus-host disease (50, 51). In line with these studies, our data now strongly point at an immunoregulatory role for CD30 on activation-induced Tregs.

The specificity of the HSP60-induced FOXP3+ Tregs was confirmed by identifying HSP60 peptide-specific T cells among the induced CD4+CD30+ T cell population using the T cell capture technique (40, 64). To find Ag specificity was of special interest because HSP60 can have a TCR-mediated effect but also functions as a ligand for TLRs. TLR4 triggering on APCs indeed had an enhancing effect on the induction of CD4+CD30+FOXP3+ Tregs. This observation adds to previous reports showing that ligation of TLRs on APCs modulates immune regulation by Tregs. For example, TLR4-triggered DCs have shown to induce Treg proliferation by a cooperative action of IL-1 and IL-6 (65). And TLR9-induced IL-6 production by DCs releases effector T cells from Treg-mediated suppression (66). Thus, depending on the local milieu, TLR signaling on APCs confers signals that either augment or attenuate T cell-mediated immunoregulation.

In addition to this indirect, APC-mediated effect, TLRs have shown to control regulatory function via a direct effect on Tregs. Zanin-Zhorov et al. (26) previously showed that direct TLR2 triggering of Tregs enhances their suppressive capacity. As with ex vivo-isolated Tregs, HSP60-induced Tregs express high levels of CD30 and no TLR4 (data not shown). Neither HSP60 nor LPS exerts any increased proliferation or survival of human CD4+CD25+ Tregs (I. de Kleer and Y. Vercoulen, unpublished data). It therefore seems unlikely that an additional innate effect of HSP60 on the proliferation and/or survival of de novo-generated HSP60-specific CD4+CD25+ Tregs plays a role in our assays.

Furthermore, both total CD30+ and the induced HSP60 peptide-specific Tregs produce high levels of IL-10 and low IFN-γ and are not only able to suppress HSP60-specific responses but also responses to tetanus toxoid. This may be because we did not select HSP60 peptide-specific Tregs for these assays or because the highly activated state of the Tregs in the HSP60 cultures enables them to suppress T eff responses to different Ags. This suggests that these HSP60-induced Tregs are suitable to suppress independent of the Ag that is causing inflammation. However, given the uncertainty in interpreting human in vitro Treg phenotype and functional assays (52), we realize that our findings cannot be directly translated into predicting suppressive function in vivo.

Altogether, these data suggest a model whereby de novo-generated Tregs that are the result of prolonged or repeated innate and/or specific T cell activation by HSPs at sites of tissue damage are involved in controlling the spread of an inflammatory response. This may explain the high numbers of Tregs isolated from sites of inflammation in various human inflammatory diseases (2).

The ability of HSP60 to drive de novo induction of Treg in vitro and the identification of CD30 as a surface marker for activation-induced Tregs described in this paper may prove useful in new therapeutic approaches for human inflammatory diseases.

Disclosures
The authors have no financial conflicts of interest.

References


