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Leiter: Prof. Dr.Dr.h.c. Th. Ruzicka

Visualization and functional characterization of T-T cell interactions *in vivo*

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Alexander Christian Schipf

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2. Berichterstatter:	Prof.Dr. D.J. Schendel
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	Dr. rer.nat. R. Roeder
Dekan:	Prof. Dr. D. Reinhardt
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INHALTSVERZEICHNIS:

1. KURZZUSAMMENFASSUNG

1.2. Ziel

Ziel der Arbeit ist die Darstellung und funktionelle Charakterisierung von T-T Zell Interaktionen während der Differenzierung von naïven $CD4^+$ T Helfer Zellen *in vivo*. Dabei sollen die Interaktionen von naïven $CD4^+$ T Helfer Zellen mit differenzierten T Helfer Zellen Typ 2 (T_H2) mittels Messung spezifischer T Helfer (T_H) Zytokine dargestellt werden.

1.3. Methoden

T Zell-homing naïver $CD4^+$ und T_H2 T Zellen – spezifisch für Influenza-Hämagglutinin(HA)- oder Ovalbumin(OVA)-Peptid - in drainierende Lymphknoten wurde nach Immunisierung und subkutanem Peptidstimulus bei naïven BALB/c Empfänger-Mäusen *in vivo* untersucht.
Zur Darstellung der ’homing’ Kapazität wurden Methoden der Durchflußzytometrie (FACS) und Immunfluoreszenz verwendet. Die Messung der einzelnen T-Zell Zytokine wurde unter Verwendung von Enzyme-Linked-Immunosorbent-Assays (ELISA) und Enzyme-Linked-Immunospot(ELISPOT) Analysemethoden zur Messung von Interferon(IFN)-γ sowie CT.4S-Assay und ELISPOT zur IL-4 Analyse durchgeführt.

1.4. Ergebnisse

Es konnte gezeigt werden, dass sich nach *in vivo* Stimulation mit dem jeweiligen Peptid und Complete Freund’s Adjuvant (CFA) eine annährend gleiche Anzahl naïver T Zellen und T_H2 Zellen in den drainierenden Lymphknoten wiederfanden und sich in unmittelbarer Nachbarschaft in der parakortikalen T-Zell Zone darstellten.
Mit dieser Versuchsanordnung konnten T_H2 Zellen die CFA vermittelte T_H1 Induktion bei naïven T-Zellen verhindern und eine Differenzierung in T_H2 Zellen bewirken.

Dabei vermittelten die T_H2 Zellen die T_H2-Induktion bei naïven T Zellen nur, wenn beide –naïve T Zellen und T_H2 Zellen– zeitgleich und im gleichen Lymphknoten mit dem jeweiligen Peptid aktiviert wurden. Hingegen konnten T_H2 Zellen von IL-4-negativen Mäusen eine CFA-vermittelte T_H1 Induktion nicht verhindern. Somit zeigte sich, dass eine T_H2-Induktion bei naïven T-Zellen stets IL-4 von T_H2 Zellen unter gleichzeitiger Aktivierung beider T Zell Subpopulationen bedarf.

1.4. Zusammenfassung

Bei der Differenzierung naïver T Zellen in einen T_H2 Phänotyp spielen zahlreiche Signale eine wichtige Rolle. IL-4 ist dabei einer der wichtigsten Faktoren für die Differenzierung von naïven T Zellen in einen T_H2 Phänotyp. Es konnte gezeigt werden, dass direkte, IL-4-vermittelte, T-T-Zell Interaktionen stattfinden und dass unter bestimmten Bedingungen IL-4 von differenzierten T_H2 Zellen eine wichtige Rolle in der T_H Zell Differenzierung *in vivo* einnimmt.

2. ABSTRACT

2.1. Purpose

To define the T-T cell interactions during differentiation of naïve $CD4^+$ T helper cells *in vivo* displaying the interactions of naïve $CD4^+$ and differentiated type 2 (T_H2) T helper cells by measuring specific T helper (T_H) cytokines.

2.2. Methods

The homing capacity of naïve T cells bearing a T cell receptor (TCR) specific for influenza hemagglutinin (HA) or T_H2 cells bearing a TCR specific for ovalbumin (OVA) was analyzed in draining lymph nodes of naïve murine BALB/c recipients after immunization and peptide challenge *in vivo*.
T cell homing was visualized by flow cytometry (FACS) and fluorescence microscopy. For measuring different T cell specific cytokines we used established systems as Enzyme-Linked-Immunosorbent Assay (ELISA) and Enzyme-Linked-Immunospot-(ELISPOT) Assay for interferon(IFN)-γ and CT.4S assay and ELISPOT for IL-4 analysis.

2.3. Results

We showed that naïve and T_H2 cells migrate with similar dynamics into draining lymph nodes and were found in close vicinity in T cell areas, when driven by antigen in complete Freunds' adjuvant (CFA). Under these conditions, T_H2 cells prevented CFA-mediated T_H1-induction and deviated the differentiation of naïve T_H cells toward a T_H2 phenotype. OVA- T_H2 cells induced T_H2-differentiation in naïve T cells exclusively, if naïve and T_H2 memory cells were both activated simultaneously in the same lymph node.

'T_H2'-cells from IL-$4^{-/-}$ mice were unable to affect CFA-mediated T_H1-induction showing that T_H2-derived IL-4 was strictly required to deviate naïve HA T_H cells toward a T_H2 phenotype.

2.4. Conclusions

Among the various signals capable of promoting T_H2 differentiation IL-4 is one of the most important factor for differentiation of naïve T cells into T_H2 phenotype. Direct, IL-4-mediated, T-T cell interactions could be measured and demonstrated that T_H2 derived IL-4 under restricted conditions plays a major role in T_H cell differentiation *in vivo*.

3. INTRODUCTION

After engagement of the T-cell receptor (TCR) by the appropriate peptide MHC complex, which triggers clonal expansion, helper T (T_H) cells rapidly undergo programmed differentiation. This differentiation process can result in highly polarized immune responses in the case of chronic infections, such as parasitic infections. A more heterogenous response is often shown by allergies or acute immune responses (Abbas and Sher 1996). This heterogeneity of immune responses was presented with strikingly different and polarized clinical and pathological features.

In general, the regulation of immune response upon antigenic stimulus is dependent on a variety of different cells and accessory signals. The different pathways of immune responses are determined by the antigen itself and its way of presentation to different cell types. To eliminate antigens specifically, antigen-presenting cells (APC) offer these proteins after phagocytosis and antigen-processing either to $CD8^+$ cytotoxic T cells (CTL) or regulatory $CD4^+$ T helper cells. Multiple accessory signals, such as soluble mediators, hormons and cytokines, are additionally required in regulation of cellular immunity.

Thereby, $CD4^+$ T helper cells play a critical role in immune regulation steering immune responses in different pathways. Naïve $CD4^+$ T cells differentiate during initial priming by dendritic antigen presenting cells (DC) into distinct phenotypes that differ by the spectrum of cytokines they produce upon re-stimulation. The two most discernible populations of $CD4^+$ T lymphocytes are T helper cells type 1 (T_H1) and T helper cells type 2 (T_H2), where T_H1 clones produce interferon- γ (IFN-γ), interleukin(IL)-2 and tumor-necrosis factor(TNF)-β, and T_H2 clones IL-4, IL-5, IL-6 and IL-13 (**Fig.1**).

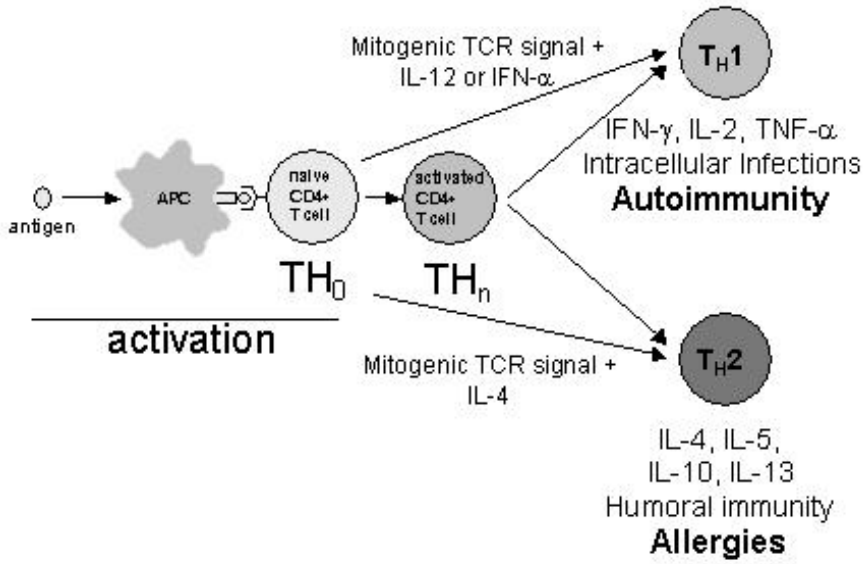


Fig. 1 **CD4+ T cell differentiation after antigen stimulus.**
T_H1 and T_H2 T cell differentiation, secreted cytokine profiles and inflammatory responses (modified to Mosmann et al., 1986).

Studies of autoimmune diseases and infectious diseases demonstrated that the cytokine pattern of $CD4^+$ memory T cells ultimately determines their biological function. Interferon γ (IFN-γ) producing T_H1 cells are responsible for cell-mediated immunity, whereas interleukin 4 (IL-4) T_H2 cells are responsible for extracellular immunity. T_H1 cells establish delayed type hypersensitivity responses (DTHR) that provide solid protection against intracellular pathogens, such as *Leishmania* species and *Toxoplasma gondii*, but may become harmful when directed against autoantigens expressed by solid organs (Abbas and Sher 1996, Paul and Seder 1994, Rocken and Shevach 1996). On the other side IL-4 producing T_H2 cells promote production of non-complement binding immunoglobulin such as IgE protecting against extracellular pathogens and promoting T_H2 responses to multiple aero-allergens, which are involved in allergic asthma (Urban and Schreiber 1994, Kay 2001).

The process by which an uncommitted T_H cell develops into a mature T_H1 or T_H2 cell is highly plastic (Murphy and Reiner 2002). During the process of T cell stimulation (Rocken and Hauser 1992) T cells are influenced by a large spectrum of different signals, such as TCR-ligand interaction (Tsitoura and Lamb 1996, Constant and Bottomly 1997), the functional phenotype of the stimulating DC (Moser and Murphy 2000, Rissoan and Liu 1999, Langenkamp and Sallusto 2000, Biedermann and Rocken 2001), co-stimulatory signals (Rodriguez-Palmero and Hunig 1999, Bennett and Heath 1998, Ridge and Matzinger 1998), various soluble mediators such as prostaglandins (Snijdewint and Kapsenberg 1993) or corticosteroids (Ramirez and Mason 1996), and by cytokines (Paul and Seder 1994, Diehl and Rincon 2002, Trinchieri 1995).

The cytokines IL-12 and IL-4, acting through signal transducer and activator of transcription 4 (STAT4) and STAT6, respectively, are key determinants of the outcome. It has been proposed also that antigen dose, co-stimulators, genetic modifiers and other non-cytokine factors have crucial roles in determining the dominance of a T_H cell response. Many data indicate that certain crucial transcription factors play causal roles in the gene-expression programmes of T_H1 and T_H2 cells. For example the zinc-finger transcritpion factor GATA3 seems to be crucial for inducing some important attributes, as genes encoding for IL-4, IL-13 and IL-5 (Szabo 2000, Ouyang 1998+2000, Zheng and Flavell 1997).

Although all these factors may influence the determination of lymphokine-producing phenotype, the effects appear to be secondary to the dominant role of the lymphokines and cytokines during early stimulus. The two strongest signals on T_H cell differentiation are IL-4, which induces T_H2 cells, and IL-12 mainly produced by activated macrophages and dendritic cells, which induces T_H1 differentiation (Abbas and Sher 1996, Rocken and Shevach 1996, O'Garra 1998).

Some of these signals, such as IL-6 or IL-12, can be directly be provided by the stimulating DC (Paul and Seder 1994, Langenkamp and Sallusto 2000, Diehl and Rincon 2002, Trinchieri 1995). IL-2, a major cytokine for T_H cell activation, itself appears to be required for naïve cells to develop into T_H1- or T_H2-like cells but is not deterministic of their differentiation fate.

As the T_H1 and T_H2 subpopulations interact and cross-regulate each other, the development of IFN-γ producing cells is strikingly inhibited by IL-4. INF-γ producing T_H1 cells amplify T_H1 development and inhibits proliferation of T_H2 cells, whereas

IL-10 produced by T_H2 cells blocks activation of T_H1 cells. Understanding these regulatory pathways, a conceptual problem raised. IL-4 is required for T_H2 differentiation, but is only produced by T cells after their differentiation into T_H2 effector cells (Racke and Rocken 1994). Therefore IL-4 must be provided by third party cells. Mast cells, basophils (Yoshimoto and Paul 1994) and especially NK T cells (Bendelac and Roak 1997, Lanouis and Louis 1997) are important *in vivo*-sources of IL-4. Some studies provided strong evidence that early IL-4 producing NK T cells play a central role in the instruction of T_H2 responses and may be involved in the protection against T_H1-mediated autoimmune diseases (Bendelac and Roak 1997, Lanouis and Louis 1997). In sharp contrast, the role of IL-4-producing T_H2 cells on the differentiation of freshly primed $CD4^+$ T cells remains enigmatic. Studies on infectious diseases such as *leishmania major* (*L. major*) in BALB/c mice showed that early IL-4 producing $CD4^+$ T cells are essential for the T_H2 differentiation of co-stimulated 'by-stander' T cells (Schmitz and Radbruch 1994). However, these findings did not show whether this differentiation relies on T-T cell interaction or on early effects of the developing T_H2 cells on DC modifying DC functions.

Substantial evidence suggests the central role of T cell-derived IL-4 on surrounding T cells can modulate the T cell differentiation of a naïve population, especially if their respective antigens are presented on the same DC (Schuhbauer and Mueller2000) and T cell differentiation relies primarily on differentiation status of antigen presenting cells (Rissoan and Liu 1999, Stetson and Locksley 2002, Racke and Rocken 1994).

In this study we analyzed the interaction between naïve $CD4^+$ T cells expressing a T cell receptor (TCR) specific for influenza hemagglutinin (HA) and primed T_H2 cells, bearing a TCR specific for ovalbumin (OVA) after stimulation in the presence of complete Freunds' adjuvant, which induces T_H1 differentiation. When re-stimulated *in vivo* by the specific peptide, naïve $CD4^+$ T cells and T_H2 cells enriched inside T cell areas of draining lymph nodes with identical dynamics and bound to the same DC. Under these conditions, activated T_H2 cells overcome the T_H1-inducing capacity of CFA and deviated the differentiation of the freshly activated $CD4^+$ T cells from a T_H1 toward a T_H2 phenotype. This deviation strictly required activation of the T_H2 cells by specific peptide, close temporal and physical vicinity of naïve and T_H2 memory cells and T_H2 cell-derived IL-4, which directly shows that T_H2 cell-derived

IL-4 can instruct surrounding T_H cells to develop a T_H2 phenotype. Such contagious T_H2-induction may be of central relevance for the consecutive spreading of T_H2 responses to multiple protein-antigens in allergic asthma or allergic rhinitis (Biedermann and Rocken 2001).

4. METHODS

4.1. Reagents and antibodies

Highly purified murine recombinant (r) IL-4 was purchased from Strathmann (Hamburg, Germany), 1 μg corresponding to 2 x 10^6 U and IL-2 of Chiron Therapeutics (Ratingen, Germany). Anti-CD4 (L3T4), anti-CD8 (Ly-2) and anti-B220 (CD45R) microbeads were from Miltenyi Biotech (Bergisch-Gladbach, Germany) and soluble CD4 mAb (Gk1.5) and CD8 mAb (5367.2) were hybridoma supernatants (ATCC, Manassas, VA). HA-peptide (H-SFERFEIFPK-OH) and OVA-peptide (H-ISQAVHAAHAEINEAGR-OH) were from Biotrend (Cologne, Germany). Mouse cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany) supplemented with 5% heat inactivated fetal calf serum, L-glutamine (216 mg/ml), penicillin, streptomycin (all from Life Technologies, Karlsruhe, Germany), 10 mM HEPES, and 5 x 10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany).

For FACS analysis we used biotin-labelled mAb recognizing the idiotype for the HA-TCR (mAb 6.5, Lanoue and Sarukhan 1997), FITC-labelled mAb KJ1.26 recognizing OVA-TCR cells, was purchased from Caltag (Burlingame, CA). SA-Cy5 blue fluorescent avidin was from Caltag, rat anti-mouse CD4 mAb (GK1.5), PE- or FITC-labelled, and mAbs for immunohistology, ELISA and ELISPOT analysis were from PharMingen (Hamburg, Germany). PKH-26 red-fluorescent and PKH-67 green fluorescent dyes were from Sigma-Aldrich (Munich, Germany).

4.2. Mice

BALB/c mice were purchased from either Harlan-Winkelmann (Borchen, Germany) or Harlan Olac Ltd. (Bicester, UK). DO11.10 mice transgenic for the OVA-peptide specific TCR and BALB/cxIL-$4^{-/-}$ mice (Kopf and Solbach 1996) were originally from Max Plank Institute Freiburg (Germany), transgenic BALB/c mice carrying an I-E^d-restricted TCR specific for the HA 110-119 peptide (Prescott and Sly 1999) from Basel Institute of Immunology (Basel, Switzerland). Mice were maintained under pathogen-free conditions in isolator cages in the animal facility of the Department of Experimental Surgery, Ludwig-Maximilians-University.

4.3. Cell culture

$CD4^+$ T cells were isolated from lymph nodes harvested and sorted using MACS magnetic beads (Miltenyi Biotec) according to the manufacture's protocol and 90-98% $CD4^+$ T cells were stimulated for three days in 96 well flat bottom tissue culture plates with APC (T-depleted spleen cells, prepared by incubating total spleen cells first with CD4 and CD8 mAb and subsequently with complement, Dade Behring, Marburg, Germany), 1,000 U ml^{-1} rIL-4 and 0.3 µM OVA-peptide and further expanded with IL-2 (50 IU ml^{-1}). Cells were re-stimulated once weekly. In all experiments, APC were derived from BALB/cxIL-$4^{-/-}$ mice. 'IL-$4^{-/-}$ – T_H2 cells' were generated by priming BALB/cxIL-$4^{-/-}$ mice with OVA dissolved in incomplete Freunds' adjuvant and OVA-peptide. Subsequently, $CD4^+$ T cells were purified and expanded as above. For *ex vivo* analysis, $CD4^+$ T cells were isolated from draining lymph nodes by MACS-sorting and 10^5 sorted cells were stimulated for 48 hours with either medium alone, HA-peptide (7.5 µM) or OVA-peptide (0.3 µM) and 10^6 IL-$4^{-/-}$-APC in 200 µl medium.

4.4. *In vivo* experiments

Polarized OVA-TCR T_H2 cells ($5x10^6$) and $5x10^6$ freshly isolated, naïve HA-TCR cells were intraperitoneally transferred into naïve, 6-8 weeks old BALB/c mice on day -4. On day 0, mice were challenged subcutaneously into the right and left flanks with HA- or OVA-peptide (100µg ml^{-1}) dissolved in CFA (DIFCO, Kansas City, MO) on day 4 after adoptive transfer. T cells from draining nodes and control nodes were analyzed at the time indicated.

4.5. FACS analysis and immunhistology

At the indicated time, draining lymph nodes were harvested, cells counted and T cells were quantified by FACS analysis using a FACScan cytometer (Becton Dickinson, Heidelberg, Germany). For immunohistology T cells were stained prior to injection with PKH-red (OVA-TCR) or PKH-green (HA-TCR) and lymph nodes were isolated at the indicated time after priming. Cryo-sections of draining lymph nodes were stained and analyzed using immunfluorescent microscopy (Leitz, Wetzlar, Germany).

4.6. Proliferation and cytokine assays

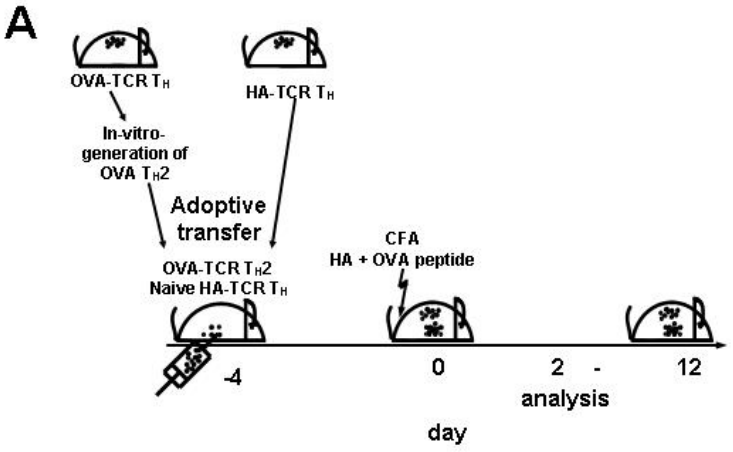
For proliferation analysis T cells ($0.1x10^6$ $well^{-1}$) were cultured in round-bottomed Maxi Sorp surface microplates (Nunc-Gibco, Wiesbaden, Germany). After 48 hours [^{3}H] thymidine (1 μCi $well^{-1}$) was added and plates were harvested 8 hours later. For cytokine assays T cells ($1.0x10^6$ $well^{-1}$) were cultured in 96 flat-bottomed well plates and supernatants were harvested for cytokine analysis after 48 hours. IFN-γ was assayed by ELISA (PharMingen, Hamburg, Germany) and IL-4 using the CT.4S cell line (Hu-Li and Paul 1989), with a detection limit at 10 U ml^{-1}.

For ELISPOT analysis we coated 96 round-bottomed plates (Multiscreen-Millipore, Schwalbach, Germany) with mAb rat anti-mouse IFN-γ (4 μg ml^{-1}) or 11B11 IL-4 mAb (5 μg ml^{-1}). T cells ($0.5x10^6$ $well^{-1}$) and irradiated APC ($0,5x10^6$ $well^{-1}$) were cultured with 0.3 μM OVA- or 7.5 μM HA-peptide and 5 U ml^{-1} IL-2 for 48 hours. Cytokine producing T cells were stained with secondary mAb and the number of cells was analysed by ELISPOT-Reader (Biosys, Karben, Germany).

5. RESULTS

5.1. Migration-dynamics and localization of naïve CD4$^+$ T cells and T_H2 memory cells in draining lymph nodes

To investigate potential interactions between naïve CD4$^+$ T cells and T_H2 cells we installed different experimental groups and studied migration dynamics of these two distinct populations in draining lymph nodes. CD4$^+$ T cells specific for either influenza hemagglutinin antigen (HA) or ovalbumin antigen (OVA) peptide were derived from naïve TCR-transgenic BALB/c mice (**Fig.2**).



B

Experimental groups

group	T cells transferred	challenge
I	HA-TCR + OVA-TCR Th2	HA
II	HA-TCR + OVA-TCR Th2	HA + OVA
III	HA-TCR	HA + OVA
IV	OVA-TCR Th2	HA + OVA

Fig.2. **Experimental protocol and groups to test T–T cell interactions.**
(**A**) *In vitro* generation of OVA-TCR transgenic T_H2 cells and chronologic treatment of BALB/c recipients. Cells were injected intra-peritoneally (day -4) and mice were challenged on day 0 with either HA-, OVA-peptide or both. Analysis of draining LN after 2-12 d by FACS, immunhistology and cytokine assays.
(**B**) Installing experimental groups to indicate T-T cell interactions. Naïve group received only PBS injections, group III and IV were controls for activity of injected T_H cells.

Primarily we analyzed the migration dynamics of these two distinct populations in draining lymph nodes.

We primed OVA-TCR cells for one week *in vitro* with antigen presenting cells (APC), OVA peptide and IL-4, and re-stimulated cells 3-4 times in the presence of IL-4 in order to establish stable T_H2 cell lines (Seder and Fazekas de St Groth 1992, Swain 1994).

After *in vitro* T cell peptide priming under polarizing conditions, about 98% of the OVA-specific T_H2 cells expressed the transgenic TCR and the cells acquired regular cytokine profiles, whereas naïve cells showed very low amounts of cytokines. Upon re-stimulation with peptide and APC, $4x10^5$ cells T cells proliferated and released more than 6000 U/ml IL-4 and no detectable IFN-γ (<30 ngml-1, **Fig.3**).

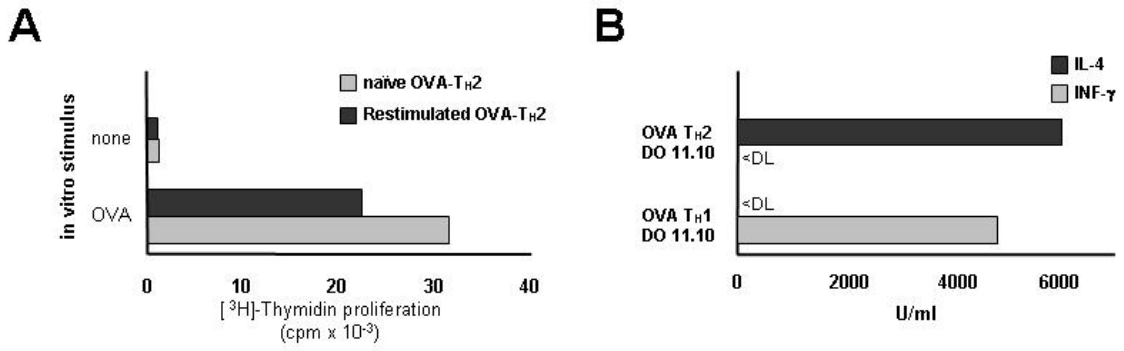


***Fig.3. In vitro* proliferation and cytokine release of OVA-T_H2 cells.**
(**A**) After *in vitro* generation of OVA-TCR T_H1 and T_H2 cells proliferation of either DO11.10 OVA-TCR transgenic naïve T_H cells (black histograms) or restimulated OVA-TCR transgenic T_H2 cells (gray histograms) is shown.
(**B**) IL-4 (black histograms) or IFN-γ (gray histograms) secretion by the referring cell lines. Data showing one of 6 experiments, negative controls (unstimulated cells) showed either cytokine secretion under detection limit. Background values are subtracted. cpm-counts per minute; DL, detection limit.

One critical condition in the experimental model was that naïve and *in vitro*-polarized T cell populations should home in sufficient numbers into draining lymph nodes when reintroduced *in vivo* and that they retain their characteristic cytokine pattern when re-stimulated.

Therefore we installed different experimental groups, varying in injected T cell types and *in vivo* peptide stimulus. The first two groups received naïve HA-transgenic T cells (group I) with one or both peptides simultaneously (HA- and OVA-peptide, group II). Control groups III and IV were immunized with one of the transgenic T cells and challenged with both peptides. Naïve group received only PBS injections (**Fig. 2B**).

To illustrate whether polarized OVA T_H2 and naïve HA-transgenic T cells home into draining lymph nodes with similar dynamics and in sufficient numbers, we injected $5x10^6$ OVA-TCR T_H2 cells together with $5x10^6$ freshly isolated naïve HA-TCR transgenic T cells from 6.5 HA-transgenic mice intra-peritoneally to naïve BALB/c recipients (day -4, **Fig. 2A**).
Four days later (day 0), we primed the mice with either OVA peptide, HA peptide or both peptides (100 µg ml^{-1}) emulsified in CFA subcutaneously into the right and left flank (**Fig. 2**). Investigating the migration of each of the transgenic T cell populations into draining or distant lymph nodes we daily analyzed axillar or inguinal lymph nodes by FACS.

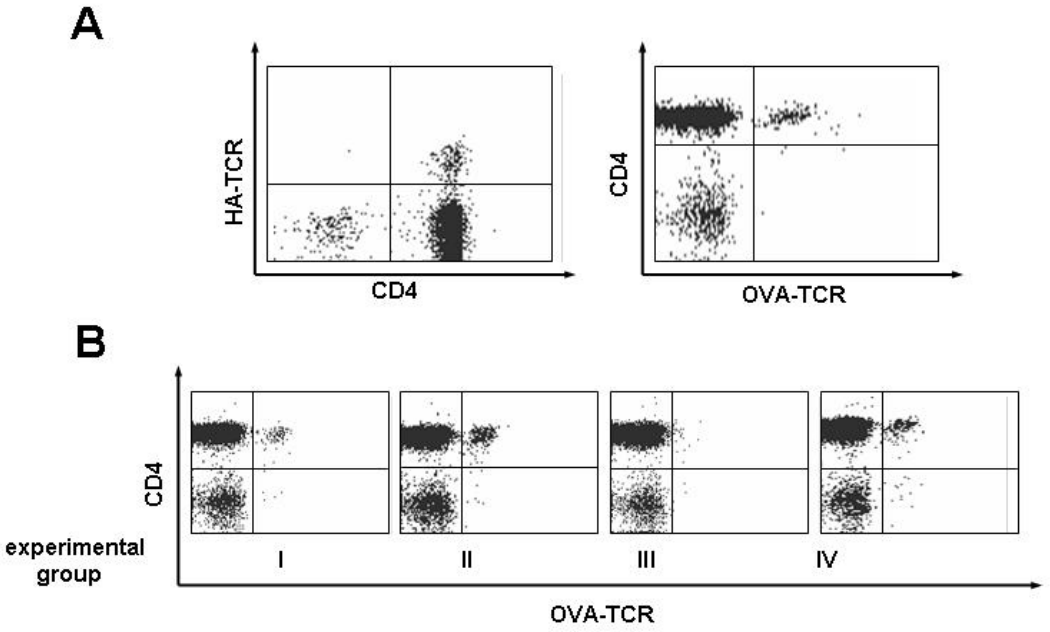


Fig. 4. **Naïve HA-TCR cells and OVA-TCR T_H2 cells migrate with similar dynamics into draining lymph nodes.**
Between day 3 and 5 injected OVA- and HA-TCR T_H cells accumulated in draining lymph nodes. FACS analysis in group II after cell harvesting, staining either with 6.5-Biotin-Streptavidin-PE and CD4-FITC for HA-TCR transgenic or with CD4-PE and KJ1.26-FITC for OVA-TCR transgenic T_H cells. In multiple experiments frequency of OVA-TCR and HA-TCR cells varied between 2 and 4% of total T cells in draining lymph nodes. Both transgenic T_H cells were found in similar amounts in draining lymph nodes.
(**A**) Relative HA-TCR T_H cells and (**B**) OVA-TCR T_H2 cell frequency in groups I-IV; memory T_H2 cells migrated only when activated by referring peptide in draining lymph node (group I and II).

Measuring the quantity of transgenic T cells, naïve HA-TCR cells constituted about 0,4% of all lymph node $CD4^+$ T cells on day of priming. Following injection of complete Freunds' adjuvant (CFA), HA-TCR cells neither accumulated in draining lymph nodes. After priming with CFA and specific HA-peptide their frequency increased up to 10-fold in draining nodes, peaking between days 3 and 5 at 2-6% of total $CD4^+$ T cells (**Fig. 4A**). Similar migration we observed in injected OVA-TCR T_H2 cells. They migrated into axillar lymph nodes and, after *in vivo*-priming, became enriched to the same extend as naïve HA-TCR T_H cells (**Fig. 4B)**. In multiple experiments the frequency of either OVA-TCR cells or HA-TCR cells varied between 2% and 4% of total CD4+ T cells. Importantly, only when specific peptide was emulsified in CFA OVA-specific $CD4^+KJ6.5^+$ were attracted into the

lymph nodes (**Fig. 4**), underlining that both naïve and memory cells migrate and proliferate only when specific peptide is present. The finding that naïve HA-TCR cells and OVA-TCR T_H2 cells migrated with similar dynamics into draining nodes when activated by peptide in CFA also implies that the two cell populations did not compete for space.

Further we investigated the morphology of draining lymph nodes on analysis day using cryo-sections of axillary lymph nodes and analyzing by immunfluorescence microscopy. Prior to injection OVA-TCR T_H2 stained with PKH26-red and HA-TCR T_H cells with PKH67-green fluorescent antibody before adoptive transfer. About 85-94% of transgenic T cells were stained and transferred intraperitoneally into naïve BALB/c recipients. *In vivo* we stimulated both populations with peptide in CFA referring experimental protocol group II. Draining lymph node cells were analyzed between day 4-6 after peptide challenge. Thus, naïve HA-TCR cells and OVA-TCR T_H2 cells accumulated in the same paracortical T cell areas and both cell types were observed in close vicinity at day 4-6 after *in vivo* challenge (**Fig. 5**). In the absence of specific peptide neither naïve nor T_H2 cells enriched inside paracortical T cell areas. These observations suggest that inside the paracortical T cell area the two T cell populations were activated in close vicinity and communicate either direct or indirectly using a common dendritic cell (DC).

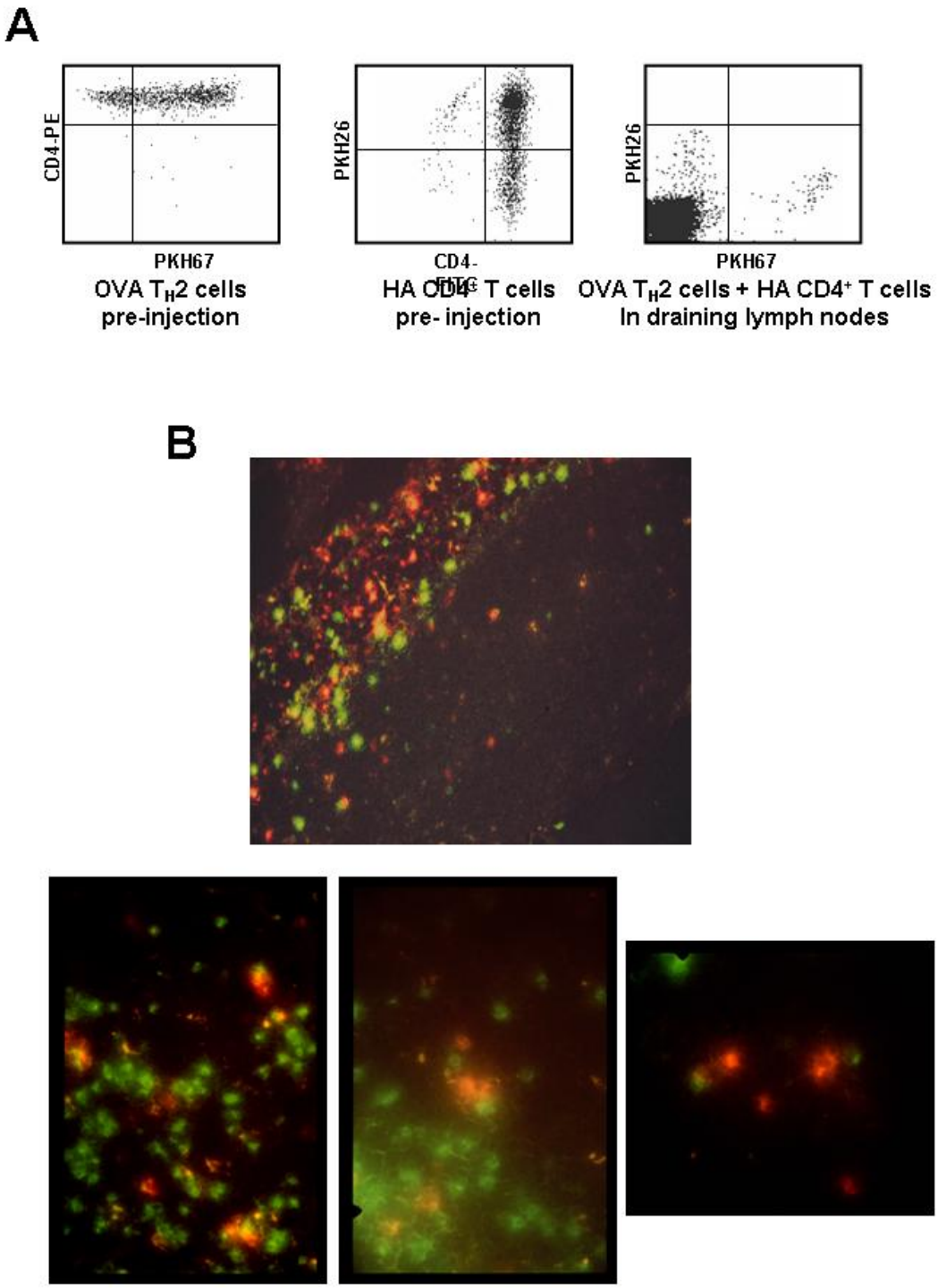


Fig.5. **Localization of $CD4^+$ HA T cells (green and OVA T_H2 cells (red) in draining lymph nodes.** Prior to injection OVA-TCR T_H2 cells were stained with PKH26 red-fluorescent antibody and HA-TCR T cells with PKH67 green-fluorescent antibody before adoptive transfer. (**A**) Cells were analyzed by FACS before and after intraperitoneal injection and *in vivo* stimulation with referring peptides in CFA. (**B**) Cryo-sections of draining lymph nodes were analysed by fluorescence microscopy. Transferred T cells were found in close vicinity in paracortical T cell area.

The following analysis step was to investigate the homing dynamics of the two transgenic T cells. Therefore the number of HA-$CD4^+$ and OVA-TCR T_H2 cells in draining lymph nodes was quantified during the first week of T cell stimulation with peptide in CFA. Both transgenic T cells peaked between day 4-6 after peptide challenge and were present in sufficient numbers (**Fig. 6 A, B**). In former experiments others have shown that in the absence of additional *in vivo* stimulation, T_H2 and naïve $CD4^+$ T cells home differently into surrounding lymph nodes. In sharp contrast, when stimulated by peptide in CFA, naïve HA-TCR cells and OVA-TCR T_H2 cells homed in our experiments with same dynamics and expanded to similar extent in draining lymph nodes.

Besides the morphological vicinity of injected T cells these findings underlined that naïve HA-TCR cells and OVA-TCR T_H2 cells migrated with similar dynamics into draining lymph nodes using potential T cell attractants.

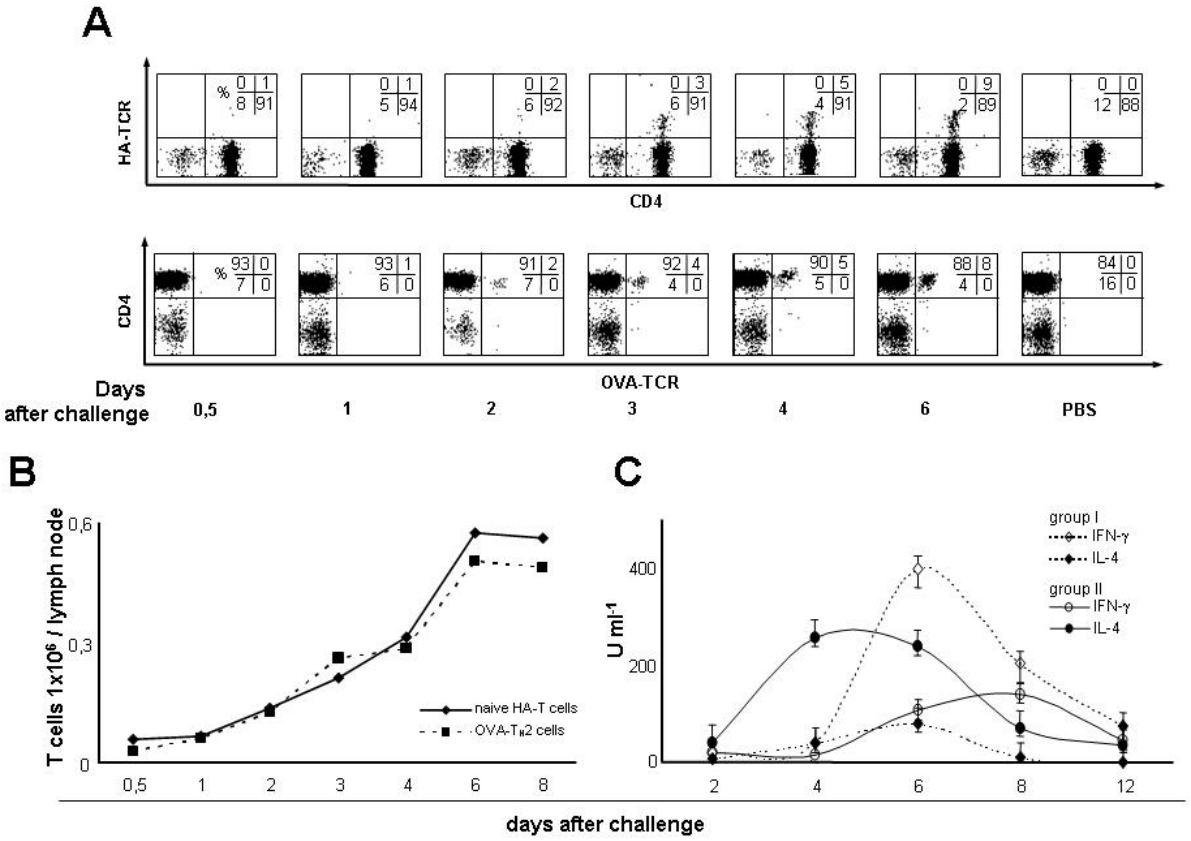


Fig. 6. **Time course analysis and quantification of HA-CD4+ cells and OVA-T_H2 cells in draining lymph nodes** during the first week of T cell stimulation with peptide in CFA, control groups received PBS only. (**A**) Relative and (**B**) absolute numbers of T cells detected in draining lymph node using FACS analysis. (**C**) Time course analysis of cytokines (IL-4 and IFN-γ) secreted by HA TCR transgenic T cells primed *in vivo* either in presence (solid line, group II) or absence (dotted line, group I) of OVA-TCR T_H2 cells. Stimulation protocol see methods, each data point reflects mean ± standard deviation from 2-4 independent experiments. Negative controls (unstimulated cells) were either cytokine negative or, if not, background values were subtracted.

5.2. Functional *in vivo* interaction between naïve CD4$^+$ T cells and T_H2 memory cells

As OVA-TCR T_H2 cells and naïve HA-TCR CD4$^+$ T cells migrated with similar dynamics into draining lymph nodes and co-localized in close vicinity in the paracortical lymph node area, we asked whether IL-4 secreted by activated T_H2 cells could potentially influence the differentiation of naïve CD4$^+$ T cells during initial priming. Therefore we first activated naïve HA-TCR CD4$^+$ T cells with HA peptide in CFA and isolated these T cells after priming at different times to determine their cytokine phenotype (**Fig. 2**). Between days 5 and 6, CD4$^+$ T cells were isolated from draining lymph nodes and stimulated for cytokine release. We incubated for *ex vivo* experiments 1 x 10^6 negatively selected CD4$^+$ T cells *in vitro* with 7.5 µM HA- or 0,3 µM OVA-peptide and 1 x 10^6 BALB/c x IL-4$^{-/-}$ APC. Each data point reflects mean ± standard deviations of cytokine secretion from 2-4 independent experiments (**Fig. 6 B, C**).

As expected, priming HA-TCR CD4$^+$ T cells with HA-peptide dissolved in CFA induced within 4 days HA-specific T_H1 cells that produced large amounts of IFN-γ but no or only very little IL-4 upon *in vitro* stimulation. Cytokine producing capacity peaked between days 5 and 7 and rapidly declined thereafter (**Fig. 6 C**, dotted line). T cells produced no cytokine in the absence of peptide and underlined hereby the specifity of peptide activation.

Importantly, during priming with HA peptide in CFA, HA-TCR cells differentiated into T_H1 cells whether resting OVA-T_H2 cells were present or not (**Fig. 7 A**, group I and III). Even though OVA-TCR T_H2 cells were present in draining lymph nodes (**Fig. 3 B**, group I), resting T_H2 cells did not affect CFA-driven T_H1 development in HA-TCR cells (**Fig. 7 A**, group I versus group III). But when we dissolved HA and OVA peptide in CFA and activated simultaneously memory OVA-TCR T_H2 cells and naïve HA-TCR in the same lymph node, HA-TCR developed a strongly polarized T_H2 phenotype, even in the presence of CFA, which normally drives CD4$^+$ T cells into T_H1 phenotype direction (**Fig. 7 A**, group II). This effect was specific for OVA-TCR T_H2 cells, because naïve OVA-specific CD4$^+$ T cells did not affect CFA-mediated T_H2 induction in HA-TCR cells (**Fig. 7 A,** group III). Cytokine production was specific, as T cells produced no detectable cytokine in the absence of peptide (**fig.7 A,** naïve

group).

To underline the role of activated T_H2 cells for naïve T cell differentiation we tested secreted cytokines in all experimental groups. Therefore BALB/c mice received intraperitoneally naïve HA-TCR T_H cells together with or without OVA-TCR T_H2 cells and groups I–IV were primed (**Fig. 1b**). Between days 5 and 6, $CD4^+$ T cells were isolated from draining lymph nodes and stimulated for cytokine release. We incubated for *ex vivo* stimulation $1{,}0x10^6$ T_H cells + $1{,}0x10^6$ BALB/cxIL-$4^{-/-}$ APC and 7,5 µM HA- or 0,3 µM OVA-peptide. In CT.4S assay serial dilutions of supernatants were compared to those elicted by known amounts of murine rIL-4 as standards ($5x10^3$ CT.4S cells/well; incubation for 48h and thymidin staining). For ELISPOT analysis, incubation of $0{,}4x10^6$ T_H cells with $0{,}8x10^6$ APC from IL-4 deficient mice, IL-2 and referring peptides for 48h and visualization by ELISPOT-reader.

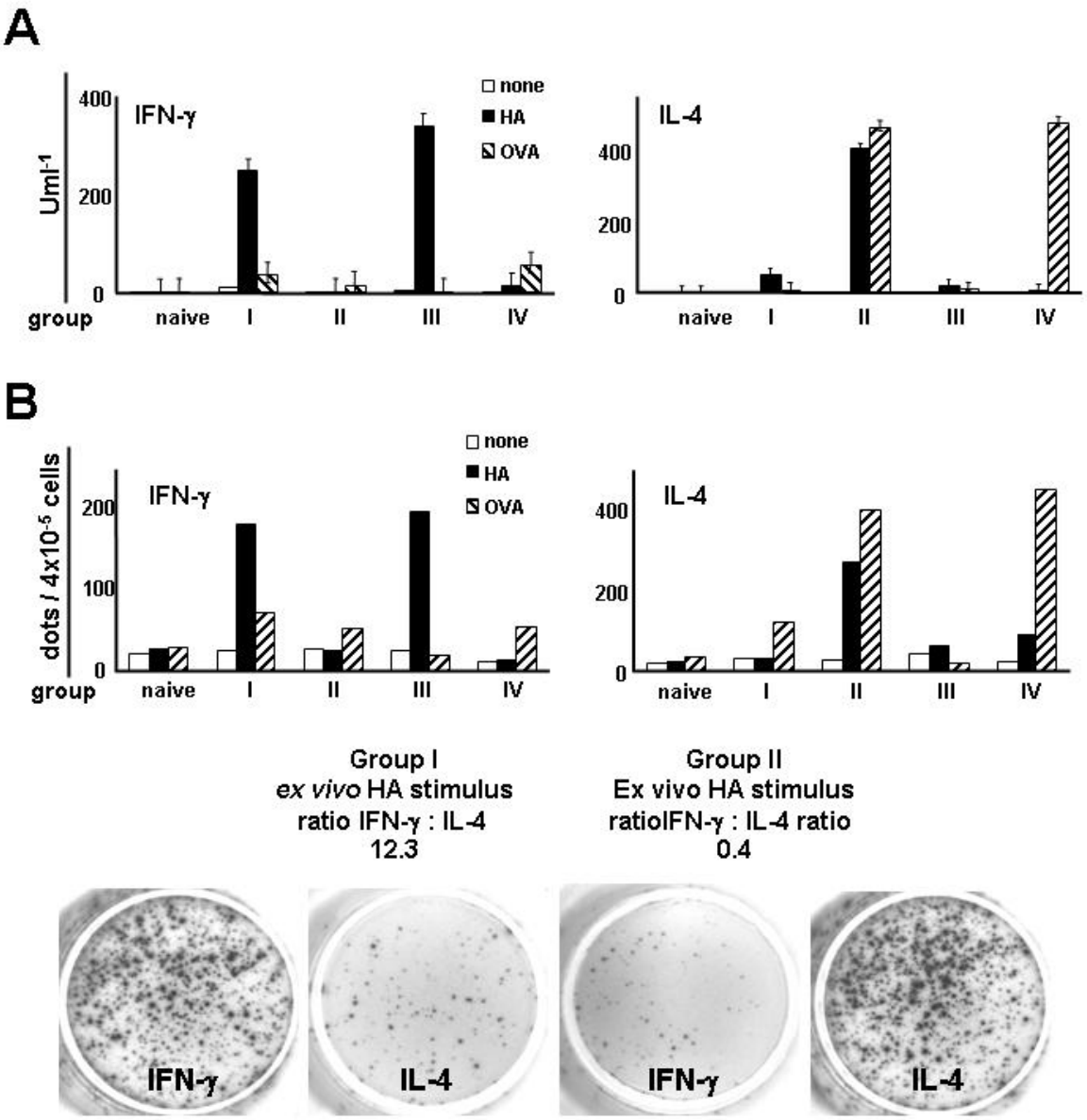


Fig. 7. **T_H2 cell-derived IL-4 deviates CFA-driven T_H1 differentiation towards a T_H2 phenotype.** BALB/c mice received intraperitoneally naïve HA-TCR T_H cells together with or without OVA-TCR T_H2 cells and groups I-IV were primed according to the protocol outlined in Fig.2B. (**A**) Analysis of secreted cytokines by ELISA for IFN-γ and CT.4S bioassay for IL-4 production in supernatants. Cells were harvested between day 5 and 6 and re-stimulated for 48 h. In CT.4S assay serial dilutions of supernatants were compared to those elicted by known amounts of murine rIL-4 as standards ($5x10^3$ CT.4S cells/well; incubated for 48h and thymidin staining). (**B**) For ELISPOT analysis, incubation of $0,4x10^6$ T_H cells with $0,8x10^6$ APC from IL-4 deficient mice (IL-$4^{-/-}$), IL-2 and referring peptides for 48h and visualized by ELISPOT reader. Data show one representative out of five experiments. HA-reactive cells (black histograms), OVA-reactive cells (dashed histograms). Ratio of IFN-γ or IL-4 producing cells were 12,3 in group I and 0,4 in group II. Wells were photographed by ELISPOT reader™. Dot-forming unit=cytokine producing HA-TCR T cells.

Whether we determined cytokine-release in the supernatant upon re-stimulation (**Fig.7A**, groups I and II) or the frequency of cytokine producing T cells using ELISPOT analysis (**Fig. 7 B**, groups I and II), simultaneous stimulation of OVA-TCR T_H2 cells with the naïve HA-TCR strongly reduced the IFN-γ-production and increased the amount of IL-4 by HA-TCR cells. In the supernatant, IFN-γ-release decreased about 10-30 fold while IL-4-release increased 10-30 fold, diminishing the IFN-γ : IL-4-ratio 100-1,000-fold. ELISPOT analysis provided equivalent results, as the ratio of IFN-γ^+ : IL-4^+ cells switched by a factor of 30 (12.3 in group I and 0.4 in group II, **Fig. 7 A**). Thus, simultaneous activation of naïve HA-TCR $CD4^+$ T cells together with OVA-TCR T_H2 cells entirely overruled the T_H1-inducing signals provided by CFA and deviated the freshly activated $CD4^+$ T cells towards a T_H2 phenotype (**Fig. 6, 7 A, B**).

Time-course analysis revealed that OVA-TCR T_H2 cells affected not only HA-specific T cell differentiation but also accelerated the appearance of HA-specific T cell responses (**Fig. 6**), with a stable reversal of the IFN-γ : IL-4-ratio over the entire period when transgenic T cells were enriched in the nodes (**Fig. 6**).

5.3. T_H2 cell-derived IL-4 induces a T_H 2 phenotype in naïve $CD4^+$ T cells

The data above showed that OVA-TCR T_H2 cells prevented T_H1 differentiation of CFA-stimulated, naïve HA-TCR T cells and deviated their development toward a T_H2 phenotype. As IL-4 is the strongest single factor capable of deviating T cell differentiation toward a T_H2 phenotype, it was likely that the IL-4 derived from OVA-TCR T_H2 cells directly induced differentiation of the HA-TCR T_H2 phenotype. Alternatively, OVA-TCR T_H2 cells might have influenced the surrounding environment, especially by causing the DC to acquire a T_H2-inducing phenotype (Moser and Murphy 2000, Rissoan and Liu 1999, Langenkamp and Sallusto 2000, Liu and Gilliet 2001). Even though this is unlikely, as IL-4 itself primes DC (Biedermann and Rocken 2001, Hochrein and Shortman 2000, Kalinski and Kapsenberg 1999) directly, we could not exclude the possibility that other unknown T_H2 cell-associated factors induced maturation of a T_H2-differentiating DC2-phenotype.

To address the role of T_H2-derived IL-4 in the deviation of HA-TCR cells from a T_H1-

toward a T_H2-phenotype, we generated OVA-specific 'T_H2' cell lines from IL-$4^{-/-}$ mice. We developed these lines by immunizing IL-4- deficient BALB/c mice (IL-$4^{-/-}$) with OVA in incomplete Freunds' adjuvant (IFA). Subsequently we stimulated and expanded $CD4^+$ T cells from draining lymph nodes with APC, OVA-peptide, IL-2 and IL-4 *in vitro* (Seder and Fazekas de St Groth 1992).

These T cell lines proliferated normally in response to OVA peptide but produced neither IL-4 nor IFN-γ (**Fig. 8 A, B**). Functional analysis of $CD4^+$ T cells revealed that these OVA-specific T cells migrated with similar dynamics to draining lymph nodes as OVA-TCR T_H2 cells, since $CD4^+$ T cells from draining lymph nodes proliferated vigorously in response to OVA peptide. No difference was seen, whether peptide-specific T cells originated from a transgenic animal or whether from T cell lines established by repetitive *in vitro* stimulation (**Fig. 8 C**).

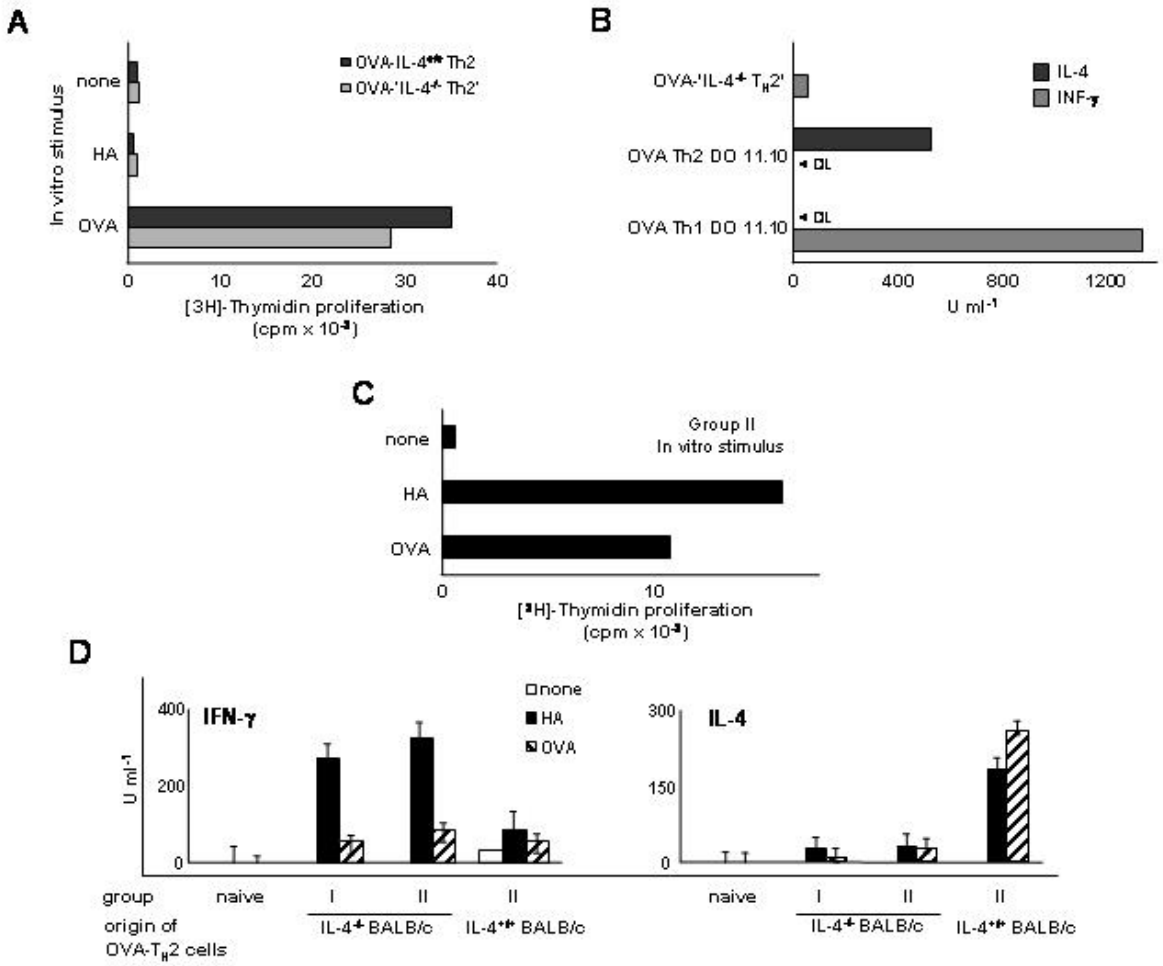


Fig. 8. OVA- T_H2 cells providing IL-4 in a paracrine fashion deviate surrounding naïve T_H cells towards a T_H2 phenotype. *In vitro* generation of IL-4 deficient 'OVA-TCR T_H2' cells from BALB/c x IL4$^{-/-}$ mice. (**A**) After repetitive stimulation, proliferation of IL-4-deficient and non-deficient T cell cultures by thymidin assay and (**B**) cytokine secretion of IL-4$^{-/-}$, DO11.10 IL-4$^{+/+}$ and T_H1 *in vitro* cultures was measured by ELISA and CT.4S assays. IL-4 deficient OVA-TCR T_H2 cells and naïve HA-TCR T_H cells were injected, 4 days later challenged and harvested on day 3-5 after peptide challenge. (**C**) *Ex vivo* proliferation and (**D**) cytokine secretion were tested by ELISA and ELISPOT assays. HA-reactive cells (black histograms), OVA-reactive cells (dashed histograms). Untreated mice or mice primed with HA peptide in CFA alone were positive and negative controls. One out of two similar experiments is shown; cpm-counts per minute, DL-detection limit.

Subsequently we injected OVA-'IL-4$^{-/-}$ T_H2 cells' together with naïve HA-TCR cells intraperitoneally and primed mice to the protocol of group II (**Fig. 2 B**). On day 6 we isolated CD4$^+$ T cells from draining lymph nodes and stimulated cells with either medium, HA- or OVA peptide either for proliferation or cytokine production into the supernatant. While IL-4-producing OVA-TCR T_H2 cells from IL-4-producing BALB/c (IL-4$^{+/+}$) strongly promoted T_H2-differentiation of HA-TCR cells (**Fig. 8 D**), IL-4-deficient 'OVA- T_H2' cells were not capable of affecting CFA-driven T_H1 development of HA-specific CD4$^+$ T cells (**Fig. 8 D**). As OVA- T_H2 cells from IL-4$^{-/-}$ mice did not

differ from OVA-TCR T_H2 cells, except from their capacity to produce IL-4, the data underline the central role of T_H2 cell-derived IL-4 in deviating the surrounding HA-TCR cells from a CFA-driven T_H1 development toward a T_H2 phenotype.

5.4. Deviation of the CFA-driven T_H1-development toward a T_H2-phenotype requires co-localization of OVA-T_H2 and naïve $CD4^+$ T cells

As mentioned above T_H2 cells can deviate CFA-driven T_H1-development of naïve $CD4^+$ T cells toward a T_H2-phenotype, but only if T_H2 cells were able to release IL-4 and if both T cell populations were activated simultaneously. As *in vivo* activation of naïve and T_H2 cells occurred in close vicinity in the paracortical region in draining lymph nodes (**Fig. 5**), it was likely that the IL-4 secreted by activated OVA-TCR T_H2 cells deviated the expanding HA-TCR T cells toward a T_H2-phenotype. To test the requirement for physical co-localization, we first injected both naïve and T_H2 cell lines intraperitoneally. During the subsequent immunization we then separated these two T cell populations *in vivo* by injecting the OVA-CFA suspension (50 µg ml^{-1} OVA-peptide) into the right flank and the HA-CFA suspension (50 µg ml^{-1} HA-peptide) into the left flank. On day 6 we isolated $CD4^+$ T cells separately from the right and the left axillary lymph nodes. FACS analysis confirmed that OVA-TCR T_H2 cells migrated preferentially into draining lymph nodes of the right axilla, while HA-TCR cells enriched primarily in draining nodes of the left axilla and functional analysis revealed these results (**Fig. 9**).

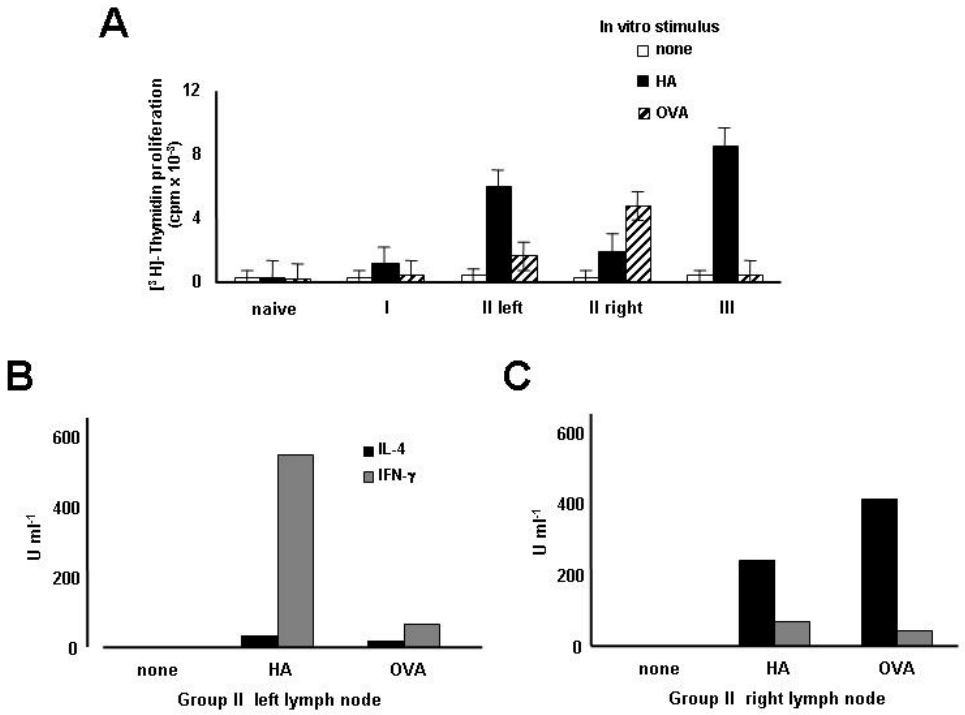


Fig. 9. **Deviation toward a T_H2 phenotype requires co-localization of OVA-T_H2 and naïve HA-TCR cells.** Challenge with HA- and OVA-peptide in separated flanks of BALB/c mice (according to group II in experimental protocol) 4 days after intraperitoneal injection of OVA-TCR T_H2 and HA-TCR T_H cells. (**A**) *In vitro*-proliferation in response to each peptide separately for the right and the left axillary lymph nodes, IL-4 and IFN-γ production in response to medium, HA or OVA peptide separately for $CD4^+$ T cells from left (**B**) or right (**C**) axillary lymph nodes. Controls were $CD4^+$ T cells from unprimed mice or mice treated according to protocol group I or III (Fig. 2 B). Negative controls of unstimulated cells were either cytokine-negative or, if not, background values were subtracted, cpm-counts per minute.

T cells from the right axillar node proliferated strongly in response to OVA-peptide (**Fig. 9a**) and expressed a typically T_H2 pattern (**Fig. 9b**). Importantly, the few HA-TCR cells that migrated to this right axillar node (**Fig. 9a**) also developed a T_H2-phenotype (**Fig. 9c**).

Thus, T_H2 cells can indeed instruct the surrounding naïve T cells to differentiate toward a T_H2 phenotype exclusively if both populations, the naïve $CD4^+$ T cells and the T_H2 cells are activated simultaneously and inside the same lymph node.

6. DISCUSSION

T cell differentiation, a crucial step in immune responses, is the result of complex cellular and molecular regulation. After activation, $CD4^+$ helper T cells differentiate into T_H1 or T_H2 effector cells, which specialize in producing distinct cytokines to mediate different types of immune responses (Paul and Seder 1994). T_H cell activation and differentiation is an essential step of T-dependent immune responses. Prior investigations have revealed a complex regulation of the T_H differentiation program, which includes T cell receptor (TCR) and costimulatory receptor signals, polarizing cytokines, lineage-specific transcriptional factors and signal transduction pathways (Contant and Bottomly 1997, Glimcher and Murphy 2000). For example, STAT6 induced expression of T_H2 specific lineage factors and enhances T_H2 differentiation (Takeda and Akira 1996, Kaplan and Grusby 1996 and Shimoda and Ihle 1996).

In this study we introduced an *in vivo* model of interactions between $CD4^+$ T cells of different specificity polarized towards T_H1 or T_H2 type and demonstrated that naïve and T_H2 memory T cells can co-migrate with indistinguishable dynamics into draining lymph nodes, home to the paracortical T cell area and are located in close vicinity when activated by specific peptide antigen. Although previous studies have emphasized that effector memory T cells are hindered in their ability to home to lymph nodes (Iezzi and Lanzavecchia 2001). Because naïve T_H cells and primed T_H2 cells express different sets of chemokine receptors, their migration dynamics might be different. Thus, others have shown that in the absence of additional *in vivo* stimulation, T_H2 memory cells or naïve $CD4^+$ T cells home differently into the surrounding lymph nodes, we established that polarized T cells did reach lymph nodes in adequate numbers after transfer intraperitoneally.

The finding that naïve HA-TCR cells and OVA-TCR T_H2 cells migrated with similar dynamics into draining nodes when activated by peptide in CFA also implies that the two cell populations did not compete for space. In sharp contrast, when stimulated by peptide in CFA, naïve HA-TCR cells and OVA-TCR T_H2 cells arrived with the same dynamics and expanded to the same extent in the draining node. T_H2 cell-derived IL-4 was obligatory to turn over CFA-driven T_H1 development and deviated the differentiation of naïve T cells from a T_H1- toward a T_H2-phenotype. Moreover these results argue that under certain sets of conditions, naïve $CD4^+$ T cells can be

stimulated down the T_H2 pathway through autocrine IL-4. Campbell *et al* (Campbell and Kelso 2001) have reported similar experiments in which they examined the capacity of $CD4^+$ T cells with a naïve phenotype to give rise to IL-4-producing clones in the presence or absence of added IL-4 when stimulated. Others argue that IL-4 induces a new program of gene activation that results in the T_H2 phenotype and a molecular basis is discussed for an IL-4-mediated selective growth advantage on T_H2 cells (Noben-Trauth and Paul 2002).

Under these conditions, T_H2 cell-derived IL-4 directly abrogated CFA-driven T_H1 development and deviated the differentiation of naïve T cells from a T_H1- toward a T_H2-phenotype. When primed under the influence of T_H2 cell-derived IL-4, the ratio of IFN-γ- : IL-4-producing cells changed 30-fold within HA-specific T cell population, showing that this concomitant stimulation of naïve and T_H2 cells affected not only the ratio of cytokine production (100- to 1,000-fold), but also the number of cytokine-releasing cells. The critical dependence on T_H2 cell-derived IL-4 and on the close physical association of naïve and T_H2 cells strongly argues that the effect was directly mediated by T-T cell interactions and not due to an indirect modification of the DC phenotype (Chitnis and Khoury 2004).

In addition, multiple “three-cell type” clusters of T cell interaction have been postulated (Kalinski and Kapsenberg 1999, Tucker and Bretscher 1982, Mitchison and O’Malley 1987). Strong evidence for such interactions came from *in vitro* and *in vivo* data suggesting that paracrine production of IL-2 by ‘T helper’ cells might be required for efficient generation of cytotoxic T cells, especially conditions where TCR-ligand interactions are low (Kurts and Miller 1997). More recent data questioned the concept of direct T-T cell interactions as it could be shown that T cell help for the induction of cytotoxic T cell responses relies on the priming of DC through CD40-CD40L-mediated signal exchange between DC and T_H1 cells rather than on paracrine IL-2 production by T helper cells (Schoenberger and Melief 1998, Bennett and Heath 1998, Ridge and Matzinger 1998). Others mentioned that antigen dose, type of antigen-presenting cell and time of differentiation can contribute to T cell differentiation (Rothoeft and Schauer 2003).

Further investigations demonstrated that in allergic individuals an irregular proportion of T regulatory 1-like cells mediating multiple suppressive mechanisms in healthy individuals and allergy-inducing T_H2 cells may be decisive in the development of allergy (Akdis 2004). Alternatively, polarized effector T cells might modulate successive immune responses via modulation of DC function. An elegant study by

Alpan (2004) demonstrated that antigen-specific $CD4^+$ T effector cells could influence the $CD4^+$ T cell differentiation directed against an unrelated antigen by modulating DC function, but only if antigens were presented by the same DCs. This DC-modulating capacity of activated $CD4^+$ T cells is IL-4 and IL-10 dependent, possibly explaining why - in a T_H2-adoptive transfer model of asthma - T_H2 priming to an unrelated novel inhaled antigen is dependent on IL-4 produced by the transferred T_H2 cells (Alpan and Matzinger 2004, De Heer and Lambrecht 2004). Dendritic cells have functions not only in the induction of allergic T_H2 responses but also during the effector response as it occurs in clinical asthma. From a therapeutic perspective, the capacity of DCs to stimulate the expansion and function of antigen-specific regulatory and type 2 helper T cells might be exploited to treat allergic disease and could lead to the design of better prevention or therapeutic strategies for asthma (Lambrecht and Pauwels 1998).

Similarly, two competing concepts are established for the development of T_H1 or T_H2 responses in humans and rodents. As IL-4 delivery during T cell stimulation allows to deviate the development of T_H1-responses in an antigen-specific mode (Kurts and Miller 1997, Rocken and Shevach 1994) and seems to be the strongest single signal capable of inducing IL-4-producing T cells also *in vivo* (Rocken and Shevach 1996, O'Garra 1998). It is likely that 'paracrine' delivery of IL-4 is also important for the development of IL-4-producing T_H2 cells. Despite this strong evidence for a critical role of IL-4 in the development of T_H2 cells, the physiological source and the significance of IL-4 during the development of T_H2-responses remaines enigmatic. Mast cells, basophils and, especially, NK T cells are potential sources of IL-4 that might promote T_H2 differentiation of naïve T cells (Voehringer and Locksley 2004).

Elegant studies provided strong evidence that early IL-4-producing NK T cells play a central role in the instruction of T_H2 responses and may be involved in the protection against T_H1-mediated autoimmune diseases. The role of 'paracrine' delivered IL-4 was especially investigated in NK T cells, as these cells produce IL-4 very rapidly during early T cell priming (Yoshimoto and Paul 1994). The absence of IL-4-producing NK T cells has been associated with aberrant development of T_H1-responses even favor the development of T_H1-mediated autoimmune disease (Bendelac and Roak 1997). These data, suggesting a central role for NK T cell-derived IL-4 in the development of T_H2 responses, are in conflict with showing that highly purified naïve $CD4^+$ T cells can normally acquire a T_H2 phenotype, even in the

environment of IL-4$^{-/-}$ mice (Schmitz and Radbruch 1994) and others which unraveled the normal development of T_H2-responses in *L. major*-infected BALB/c mice, even in the absence of NK T cells (Launois and Louis 1995). Even though spreading of T_H2 responses during early phases of *L. major*-infection is well established (Launois and Louis 1997, Stetson and Locksley 2002, Julia and Glaichenhaus 1996), the trigger leading to T_H2-differentiation remains unclear. In addition, an important body of evidence suggests that *in vivo* T_H2 responses primarily rely on the functional phenotype of the DC priming naïve T_H cells (Moser and Murphy 2000, Langenkamp and Sallusto 2000, Stetson and Locksley 2002, Liu and Gilliet 2001, Kalinski and Kapsenberg 1999). In terms of DCs orchestrate the optimal balance in the development of the different types of T_H cells (De Heusch and Moser 2004, De Jong and Kapsenberg 2005). These data now provide experimental prove to the concept that T cell differentiation is not only directed by DC and IL-4 producing NK T cells, but also directly by T-T-cell interactions. Even though unprimed T_H2 memory cells have a defect to home spontaneously into lymph nodes (Iezzi and Lanzavecchia 2001), T_H2 cells enrich in paracortical lymph node areas with the same dynamics as naïve T cells, if attracted by specific peptide dissolved in CFA (Stoll and Germain 2002, Miller and Cahalan 2002).

Previous data showing that IL-4 paradoxically primes DC to mature toward a T_H1-inducing phenotype of Dendritic cells (DC1) and that IL-4 induces T_H2 maturation exclusively if present during the period of T cell priming (Biedermann and Rocken 2001). Thus, it is likely that, in the system described here, T_H2 cell-derived IL-4 did not induce DC2 but induced T_H2-differentiation in HA-TCR cells through T-T-cell interactions. In agreement with this interpretation we found that deviation of CFA-stimulated HA-TCR cells was strictly dependent on T_H2 cell-derived IL-4, the strict co-localization of naïve and T_H2 cells and the requirement on the simultaneous activation of naïve and T_H2 memory cells. Thus, the simple presence of T_H2 memory cells did not affect CFA-driven T_H1 development of HA-TCR cells. This is fundamentally different from DC-priming through CD40-CD40L-interactions, where activated T_H cells modify the functional phenotype of DC in a way that they prime cytotoxic T cell responses more efficiently during subsequent interactions with naïve $CD8^+$ T cells.

The data reported here thus provide a solid explanation that T_H1 and T_H2 responses can normally co-exist even in individuals with multiple T_H2 responses, such as in

patients with allergic asthma or allergic rhinitis. These individuals develop T_H2-responses to environmental aero-allergens that tend to spread to multiple epitopes (Prescott 1999, Kulig 1999), while they raise normal T_H1-resposes against viruses, as long as these viruses do not infect the airways. Even though it is generally believed that T_H2-development to multiple aero-allergens is genetically determined and T_H2-responses to aero-allergens start with only a single or a small spectrum of T cell epitopes. As these individuals are concomitantly exposed to multiple aero-allergens, T_H2 cell-derived IL-4 may induce spreading of T_H2 responses in lung-draining lymph nodes. This may also explain that silencing of T_H2-responses to as little as 2-3 leading aero-allergens significantly improves allergic rhinitis and why these individuals may raise normal T_H1-responses if antigens are presented in lymph nodes draining regions distant from lung (Biedermann and Rocken 1999).

The differentiation of naïve T cells either into T_H1 or T_H2 effector cells *in vivo* has also essential implications in specific tumor therapy. In cancer immunotherapy many vaccine strategies aim to stimulate the T_H response specific for a tumor antigen. Furthermore early clinical trials have shown that modulating the immune system with tumor-specific T_H1 and T_H2 cells can result in significant levels of antigen-specific T_H cells and cytotoxic T cells generating long lasting tumor immunity (Nishimura and Otha 1999, Hung and Levitsky 1998, Disis and Knutson 2004). Recent studies underlined that *in vivo* allosensitation during immunotherapy generates T_H1 or T_H2 populations with differential *in vivo* capacity for expansion to alloantigen, resulting in differential graft-versus-tumor effects and graft-versus-host diseases (Jung and Fowler 2003).

Thus, treatment with IL-4 is capable of inducing T_H2 responses and to treat inflammatory autoimmune disease or contact-hypersensitivity in rodents (Racke and Rocken 1994, Biedermann and Rocken 2001) and psoriasis, a T_H1-associated autoimmune disease in humans (Ghoreschi and Rocken 2003) using a cytokine-based therapy that is currently based on systemic application of IL-4. This indicates that, in the future, a T cell cytokine such as IL-4 should be vectored either into clusters by *in vitro*-primed T cells (Gross and Kosmatopoulos 2004) or by means of gene transfer into Dendritic cells (Yamazaki and Steinman 2003) or into specific T cells (Biedermann and Carballido 2004).

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- **Lebenslauf**

Alexander, Christian Schipf
19.07.1975 in München
Karneidplatz 25
81547 München
Tel. 089/64956597
mail: alexander.schipf@web.de

Schulausbildung:

1982-1986	Grundschule Vaterstetten
1986-1995	Neusprachliches Gymnasium Vaterstetten

Zivildienst:

08/95-08/96	Zivildienst, Maltester Hilfsdienst

Studium:

11/96-04/03	Studium der Humanmedizin an der LMU München, Alicante, Spanien Barcelona, Spanien
05/03	3.Staatsexamen, Teilapprobation

Arzt im Praktikum:

05/03-09/04	Pathologisches Institut der LMU München
10/04	Approbation als Arzt

Assistenzarzt:

Seit 10/04	Wissenschaftlicher Mitarbeiter am Pathologischen Institut der LMU München

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